Data Article

Experimental data-sets on the histopathological and immunohistological assessment of the Interrenal gland (adrenal homolog) of Japanese medaka (Oryzias latipes) fish exposed to graphene oxide

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ARTICLE INFO

Article history:
Received 7 September 2022
Revised 18 October 2022
Accepted 19 October 2022
Available online 25 October 2022

Keywords:
Graphene oxide
Endocrine disruption
Japanese medaka
Adrenal homolog
Interrenal gland
Immersion
Intraperitoneal injection
Cell sorting

ABSTRACT

The datasets of this article present the experimental parameters resulting from the assessment of adrenal gland as a potential biomarker of endocrine disruption mediated by graphene oxide (GO), a nanocarbon, using Japanese medaka fish as the model. These data sets support the article “Histopathological evaluation of the interrenal gland (adrenal homolog) of Japanese medaka (Oryzias latipes) exposed to graphene oxide”. The experiments were conducted on reproductively active adult fish maintained as a breeding pair (one male and one female) in 500 mL balanced salt solution (BSS) either by immersion in GO (20 mg/L in BSS) continuously for 96 h with refreshing of media once in every 24 h or by a single intraperitoneal (IP) injection of GO (100 μg/g) to both male and female fish. The experimental fish were allowed breeding and assessed after 21 days post-treatment. Moreover, one day-post hatch (dph) Japanese medaka fries (orange-red variety) were exposed to different concentrations of GO (2.5–20 mg/L) by immersion in embryo-rearing medium (ERM) for 96 h (1–5 dph) with refreshing of media every 24h. Food was given to the adults, however, the lar-
vae remained fasting during the GO-exposure (0–5 dph) period. Control adults and larvae were identically maintained either in BSS (adults) or ERM (larvae), with no GO. After treatment, both adults and the larvae were maintained in BSS with feeding in a GO-free environment. After 21 days post-treatment, adults, and after six weeks post-treatment, larvae, were anaesthetized in MS-222, and the trunk region was preserved in 4% paraformaldehyde in PBS (20 mM) containing 0.05% Tween 20. Evaluation of interrenal gland (IRG) in kidneys were made in 5 μm thick sections stained on haematoxylin-eosin (HE). The phenotypic sex of adults was assessed by secondary sex characters (fin features) and gonad (testis and ovary) histology; in larvae, phenotypic sex was determined by gonad histology and the genotypic sex by genotyping dmy gene. The location of IRG in the kidney were determined by immunohistochemical technique using rabbit polyclonal antityrosine hydroxylase antibody as primary antibody. The digital images of sections were captured using an Olympus CKX53 inverted microscope with DP22 camera and CellSens software. Using imagej software, a minimum of 3 images of kidney consisting IRG were assessed for cell (separated as dark and pale stained nucleus after HE staining) sorting (cells/ mm²) and also measured the nuclear area (μm²). Counting of IRG cells, lined between the cardinal vein and the interstitial cells in the kidneys, were limited to maximum three layers in a given area. Numerical data, presented as means ± SEM, were analysed by one-way ANOVA followed by post-hoc Tukey’s multiple comparison test or unpaired parametric ‘t’ test including Welch’s correction, if distributed normally; or by Kruskal-Wallis test followed by Mann-Whitney’s test as post hoc test, if the data did not meet the criteria of using a parametric test. Statistically significant difference were considered for p ≤ 0.05. The collected data on IRG of Japanese medaka fish will be used for the assessment of GO as an EDC disposed in the environment.

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Specifications Table

| Subject | Biological Sciences |
|---------|---------------------|
| Specific subject area | Zoology |
| Type of data | Table |
| How data were acquired | Image |
| | Graph |
| | Figure |

Microtome (Olympus cut 4055)
Transmission Electron microscope (TEM) (JEM 1011) (manufacturer JEOL ltd)
Barnstead Nanopure, model D11901, Thermo Fisher Scientific, Waltham, MA, USA
MS-222 (Ethyl 3-aminobenzoate methanesulfonate,Sigma-Aldrich, St. Louis, MO, USA). Product number E10521
Haematoxylin Eosin Staining (HE) (Sigma-Aldrich, St. Louis, MO, USA)

(continued on next page)
Masson’s Trichrome Staining (MT) (Sigma-Aldrich, St. Louis, MO, USA)
Periodic acid-Schiff staining (PAS) (Sigma-Aldrich, St. Louis, MO, USA)
Immunohistochemistry using polyclonal rabbit antityrosine hydroxylase antibody as primary antibody (Catalog # AB152; Sigma-Aldrich, St. Louis, MO, USA)
ABC kit (Vector lab, Burlingame, CA, USA)
Vector Dab (Vector lab, Burlingame, CA, USA)
Blockall (Vector lab, Burlingame, CA, USA)
Olympus CKX53 inverted microscope with DP22 camera (Olympus U-TV0.5X C-3; serial number 0E53865) and CellSens software (Hunt optics & Imaging, Pittsburgh, PA, USA)
Thermal cycler (Technie, Cole Parmer, Chicago, IL, USA)
UV illuminator and vertical gel electrophoresis apparatus (Thermo Fisher Scientific, St. Louis, MO)
Smartphone digital camera
GraphPad Prism 7 (San Diego, CA, USA)
imagej (imagej.nih.gov)

Data format
Raw
Analyzed

Parameters for data collection
GO, used in the experiments, are obtained either commercially or synthesized in the laboratory, sonicated in BSS (adults) or ERM (larva), and characterized by TEM [1–6]. Adults were exposed to GO (20 mg/L) either by continuous immersion for 96 h or by single IP injection (100 μg/g) and assed the interrenal gland after 21 days post-treatment. Larvae were exposed to different concentration of GO (2.5, 5.0, 10.0, and 20.0 mg/L) continuously for 96 hours (1–5 dph) and assessed the interrenal gland 6 weeks post treatment (47 dph). Phenotypic sex were evaluated histologically. Genotypic sex by genotyping male sex determining gene dmy [3–6]. The location of interrenal gland cells in the kidney of adult fish were determined by immunohistochemical technique using rabbit polyclonal antityrosine hydroxylase antibody as primary antibody. Distribution of cells (number/ mm²) and nuclear area (μm²) in the adrenal homolog were analyzed by imagej software. The cells were sorted as dark stained nuclei (DSN) and pale-stained nuclei (PSN) depending on the HE staining intensity. The nuclear area of both DSN and PSN cells were determined by imagej software and expressed as μm².

Description of data collection
GO obtained from Sigma (St. Louis MO), or synthesized in the laboratory are sonicated either in ERM and BSS [1–6]. Adult fish with food were exposed to GO either by continuous immersion for 96 h (20 mg/L) or by single IP injection (100 μg/g) and assed the IR gland after 21 days post-treatment. The larvae without food were exposed to different concentration of GO (2.5, 5.0, 10.0, and 20.0 mg/L) continuously for 96 hours (1–5 dph) and assessed the interrenal gland 6 weeks post treatment (47 dph). The phenotypic sex and the effects of GO on adrenal homolog were evaluated histologically in 5μm thick HE stained sections and were analyzed by imagej software. Genotypic sex of the larvae was determined by genotyping dmy gene. The location of interrenal gland cells in the kidney of adult fish were determined by immunohistochemical technique using rabbit polyclonal antityrosine hydroxylase antibody as primary antibody. The digital images of sections were captured using an Olympus CKX53 inverted microscope with DP22 camera and CellSens software. The counted interrenal gland cells were further grouped as dark stained nucleus (DSN) and pale-stained nucleus (PSN) depending on the HE staining intensity and expressed as number of cells/ mm². The nuclear area of DSN and PSN cells were expressed as μm².

Data source location
RCMI Centre for Environmental Health, Jackson State University, Jackson, MS 39217, USA.

Data accessibility
Repository name: Figshare.com
Data identification number: not available
Direct URL to data: https://doi.org/10.6084/m9.figshare.20660412.v1

Related research article
A.K. Dasmahapatra, P.B. Tchounwou. Histopathological evaluation of the interrenal gland (adrenal homolog) of Japanese medaka (Oryzias latipes) exposed to graphene oxide, Environ.Toxicol. 37 (2022) 2460–2482. https://doi.org/10.1002/tox.23610.
Value of the Data

- Adrenal gland in medaka, like other teleost fish, has an interrenal distribution around the cardinal vein passing through the kidneys and not as a compact organ encapsulated as cortex and medulla as in mammals that imposed a potential problem of using fish IRG as a target organ of EDCs. Our innovative data indicate that GO-induced impairments of IR glands in Japanese medaka are nonspecific, and be affected by factors such as exposure route, sex, and age of the fish.
- The data will be useful to the organizations worldwide, who are developing strategies and tests for screening EDCs disposed in the environment. Such organizations are United States Environmental Protection agency (USEPA), National Institutes of Health (NIH), Organisation of Economic Cooperation and Development (OECD), European Food Safety Authority (EFSA), The French Agency for Food, Environmental and Occupational Health & Safety (ANSES) and others.
- Identification of adrenal disrupting chemicals (ADCs) is challenging as they can act at multiple sites of hypothalamus-pituitary-adrenal (HPA)-axis modulating stress response. However, adrenal histology/histochemistry is a powerful and sensitive tool for evaluating adverse effects of chemicals on adrenal gland. As a test organism, fish are unique and this study highlights the value of Japanese medaka fish as a sentinel model for ADCs found in the environment.
- Due to the extensive use of engineered nanomaterials in human health, it is necessary to evaluate the potential health hazards of GO by using unique models living in different ecosystems. The cutting-edge data generated in this study are valuable in sharing the knowledge on the potential EDC effects of GO targeting adrenal gland of a model fish species, Japanese medaka, which has unique features of sexual dimorphism both at phenotypic and genotypic levels.

1. Data Description

Breeding pairs of reproductively active adult male and female medaka were exposed to 20 mg/L GO by continuous immersion (IMR) for 96h in BSS or 100 μg/g GO by intraperitoneal (IP) administration. Also, 1 d post-hatch (dph) medaka larvae were exposed to different concentrations of GO (2.5–20 mg/L) for 96h in ERM and then transferred to BSS (Fig. 1). Control (no GO) adults and larvae were maintained in identical conditions. Histopathological evaluation of IRG of adults were made on 21 day post-treatment, however for larvae after 6 weeks of depuration (Fig. 1). The location of interrenal gland (adrenal homolog) in the kidney of the adult fish and larvae was detected histologically by HE (Fig. 2a and b; supplementary Fig. ST1a, ST1b, ST1c; https://doi.org/10.6084/m9.figshare.20660412v1) and Masson’s Trichrome (MT) staining (Fig. 2c and d), histochemically by PAS staining (Fig. 2e and f), and immunohistochemically by using rabbit polyclonal antityrosine hydroxylase as primary antibody (Fig. 2g–j). Cellular distribution in IRG and the nuclear area of the IRG cells were also determined (Tables 1–6; supplementary Tables ST1–15; https://doi.org/10.6084/m9.figshare.20660412v1). Study results indicate that the IRG is distributed adjacent to the posterior cardinal vein and its branches within the head kidney. Columnar or oval shaped cells, arranged either in a single, or in groups, sometimes encircling a sinusoid, or in a straight chord, are adjacent to the endothelium of the cardinal vein with round or oval basophilic nucleus.
Fig. 1. Schematic diagram of the GO exposure paradigm to adults and larvae of Japanese medaka fish. Adult, reproductively active mature male and female medaka fish were maintained as a breeding pair (one male and one female) in a one L glass jar containing 500 ml BSS and kept in standard laboratory conditions (light cycle 16h light 8 h dark; 25 ±1 °C). The eggs were collected from the breeding pairs for 7 consecutive days before GO exposure. The breeding pairs were exposed to GO either by IP-injection (100 μg/g; single injection) or by IMR (20 mg/g; 96 h continuous with refreshing of medium every 24h), and the collection of eggs were continued (both fertilized and unfertilized). The fish were fed during the exposure period. Control fish were injected with vehicle (water) or immersed in 500 mL BSS only (no GO). The medium was refreshed every day. After GO exposure (either by injection or immersion) the survived breeding pairs were transferred to 500 mL of fresh BSS (no GO) and allowed breeding (same pair), and maintained 21 days in a GO-free environment with regular feeding and refreshing of the medium; the collection of the eggs were continued. After 3 weeks depuration (21 days post treatment) the fish were sacrificed and the trunk was fixed in 4% PFA in 20 mM PBS for histological examination of the IRG (adrenal homolog in mammals) as well as gonads (testis and ovary) (Fig. 1). For larvae, one day post hatch (dph) medaka fries were exposed to different concentrations of GO (0, 2.5, 5.0, 10.0, and 20.0 mg/L) continuously for 96 h in 1 mL ERM. The larvae were kept fasting during the exposure period and maintained as one larva/mL ERM/tube. The medium refreshed every 24 h. After treatment the larvae were transferred to 500 mL glass jars in 200 mL BSS (4 larva/ jar) and maintained in the laboratory with feeding for six weeks (42 days) and frequent change of medium, depending on the pH and ammonia content. After six weeks of depuration (47th dph), the larvae were sacrificed and trunk regions were fixed in 4% PFA in 20 mM PBS and used for histological analysis of IRG and gonads. The tail were used for DNA extraction and determination of the genetic sex of the larvae by genotyping the male-specific dmy gene.
**Table 1**
Effect of GO on the cellular distribution on the IRG (adrenal homolog) of Japanese medaka adults.

| Cell types (number of cells/mm²) | IMR (20 mg/L) | IP (100µg/g) |
|----------------------------------|---------------|---------------|
|                                  | Male          | Female        | Male          | Female        |
|                                  | Control       | GO            | Control       | GO            | Control       | GO            |
| _DSN_                           | 12419         | 5975          | 7692          | 6713          | 9600          | 4548          | 5440          | 5490          |
| (n = 10)                        | ±2091         | ±709*         | ±589          | ±617          | ±1361         | ±426*         | ±990          | ±723          |
| _PSN_                           | 17617         | 18861         | 14479         | 14662         | 16620         | 15567         | 20545         | 15421         |
| (n = 10)                        | ±2110         | ±850          | ±923          | ±790          | ±1349         | ±973          | ±993          | ±1354*        |
| Total                           | 30033         | 24836         | 22688         | 21375         | 26784         | 20115         | 24622         | 20917         |
| (n = 10)                        | ±2558         | ±797          | ±1029         | ±940          | ±1376         | ±1182*        | ±904          | ±1623*        |

IMR= immersion; IP= intraperitoneal injection; DSN= cells with deep-stained nuclei; PSN= cells with pale-stained nuclei; GO= graphene oxide. Data with asterisk (*) indicate that the values are significantly different (p < 0.05) from the corresponding control group.

**Table 2**
Effect of GO on the nuclear area of the cells found on the IRG (adrenal homolog) of Japanese medaka adults.

| Cell types (nuclear area in µm²) | IMR (20 mg/L) | IP (100µg/g) |
|----------------------------------|---------------|---------------|
|                                  | Male          | Female        | Male          | Female        |
|                                  | Control       | GO            | Control       | GO            | Control       | GO            |
| _DSN_                           | 7.25          | 9.45          | 8.72          | 9.51          | 8.70          | 11.03         | 8.80          | 9.20          |
| (n = 36)                        | ±0.26         | ±0.30*        | ±0.20         | ±0.18*        | ±0.26         | ±0.22*        | ±0.26         | ±0.25         |
| _PSN_                           | 6.99          | 9.17          | 8.65          | 9.51          | 8.68          | 10.91         | 8.05          | 9.20          |
| (n = 59)                        | ±0.20         | ±0.20*        | ±0.17         | ±0.18*        | ±0.22         | ±0.20*        | ±0.17         | ±0.22*        |

IMR= immersion; IP= intraperitoneal injection; DSN= cells with deep-stained nuclei; PSN= cells with pale-stained nuclei; GO= graphene oxide. Data with asterisk (*) indicate that the values are significantly different (p < 0.05) from the corresponding control group.

**Table 3**
Effect of GO on the distribution of IRG (adrenal homolog) cells in XY and XX genotypes of Japanese medaka larvae.

| Cell types (number of cells/mm²) | XY                | XX                |
|----------------------------------|-------------------|-------------------|
|                                  | Control           | 2.5#              | 5.0              | 10.0             | 20.0             |
| _DSN_                           | 14898±1700        | 9253              | 28655            | 9375             | 13991            |
| (n = 19)                        | ±963*             | (n = 9)           | (n = 4)          | (n = 19)         | (n = 25)         |
| _PSN_                           | 28049±14080       | ±1058*            | ±4156            | ±2036            | ±1609*           |
| (n = 21)                        | ±1743             | (n = 9)           | (n = 3)          | (n = 19)         | (n = 25)         |
| Total                           | 41035±23333       | 47039             | ±6731            | ±2066            | ±1385*           |
| (n = 20)                        | ±1654             | (n = 9)           | (n = 4)          | (n = 19)         | (n = 25)         |

Genotypes

| XY | XX |
|----|----|
| 2.5 | 5.0 | 10.0 | 20.0 |

#Concentration of GO= mg/L. Data with asterisk (*) indicate that the values are significantly different (p < 0.05) from the corresponding control group.
Table 4
Effect of GO on the distribution of IRG (adrenal homolog) cells in the larvae of Japanese medaka having testis or ovary as gonads.

| Cell types (number /mm^2) | Testis | Ovary |
|---------------------------|--------|-------|
|                           | Phenotypes | Phenotypes |
|                           | Control | 2.5# | 5.0 | 10.0 | 20.0 | Control | 2.5 | 5.0 | 10.0 | 20.0 |
| DSN                       |         |      |     |     |      |         |      |     |     |      |
|                           | 15554 ±1949 | 8408 ±730* | 12875 ±973 | 9265 ±849* | 15950 ±1646 | 16792 ±2002 | 10026 ±1295* | 12924 ±505 | 8593 ±631* | 15753 ±3440 |
|                           | (n = 25) | (n = 42) | (n = 20) | (n = 31) | (n = 9) | (n = 9) | (n = 19) | (n = 5) | (n = 4) | |
| PSN                       | 26379 ±1794 | 16674 ±1301* | 17652 ±741* | 26511 ±2035 | 19504 ±1602* | 21240 ±3150 | 22179 ±1241 | 19448 ±2925 | 25851 ±3637* | 13237 |
|                           | (n = 24) | (n = 41) | (n = 20) | (n = 30) | (n = 10) | (n = 10) | (n = 19) | (n = 5) | (n = 4) | |
| Total                     | 38555 ±1949 | 25082 ±1374* | 30808 ±1160* | 35715 ±2099 | 34825 ±1324 | 30294 ±3106 | 33925 ±1709 | 33439 ±3045 | 34445 ±6307 | 28990 |
|                           | (n = 25) | (n = 42) | (n = 20) | (n = 31) | (n = 12) | (n = 10) | (n = 19) | (n = 5) | (n = 4) | |

*Concentration of GO= mg/L
Data with asterisk (*) indicate that the values are significantly different (p < 0.05) from the corresponding control group.

Table 5
Effect of GO on the nuclear area of the cells found on the IR-axis (adrenal homolog) of Japanese medaka larvae as XY and XX genotypes.

| Cell types (nuclear area in μm^2) | Genotypes |
|-----------------------------------|-----------|
|                                   | XY        | XX       |
|                                   | Control | 2.5# | 5.0 | 10.0 | 20.0 | Control | 2.5 | 5.0 | 10.0 | 20.0 |
| DSN                               |         |      |     |     |      |         |      |     |     |      |
|                                   | 8.41 ±0.44 | 7.56 ±0.23 | 8.61 ±0.52 | 9.91 ±0.30* | 10.16 ±0.24* | 11.98 ±0.61 | 10.26 ±0.35* | 8.57 ±0.15 | 8.88 ±0.48* | 9.03 ±0.35* |
|                                   | (n = 48) | (n = 53) | (n = 18) | (n = 66) | (n = 92) | (n = 47) | (n = 63) | (n = 221) | (n = 18) | (n = 53) |
| PSN                               | 8.41 ±0.26 | 7.86 ±0.35 | 9.49 ±0.45* | 9.98 ±0.28* | 9.99 ±0.19* | 10.67 ±0.44 | 9.99 ±0.36 | 8.33 ±0.14* | 7.89 ±0.52* | 10.13 ±0.51 |
|                                   | (n = 84) | (n = 47) | (n = 16) | (n = 94) | (n = 116) | (n = 58) | (n = 74) | (n = 267) | (n = 32) | (n = 37) |

*Concentration of GO= mg/L
Data with asterisk (*) indicate that the values are significantly different (p < 0.05) from the corresponding control group.

Table 6
Effect of GO on the nuclear area of the cells found on the IR-axis (adrenal homolog) of Japanese medaka larvae having testis and ovary as gonads.

| Cell types (nuclear area in μm^2) | Phenotypes |
|-----------------------------------|------------|
|                                   | Testis | Ovary |
|                                   | Control | 2.5# | 5.0 | 10.0 | 20.0 | Control | 2.5 | 5.0 | 10.0 | 20.0 |
| DSN                               |         |      |     |     |      |         |      |     |     |      |
|                                   | 9.69 ±0.46 | 9.52 ±0.40 | 8.77 ±0.29 | 9.95 ±0.27 | 9.93 ±0.29 | 11.24 ±0.77 | 9.42 ±0.40* | 8.25 ±0.23 | 8.38 ±0.50* | 10.76 ±0.42 |
|                                   | (n = 68) | (n = 82) | (n = 161) | (n = 70) | (n = 128) | (n = 27) | (n = 41) | (n = 79) | (n = 14) | (n = 22) |
| PSN                               | 8.75 ±0.25 | 9.34 ±0.37 | 8.60 ±0.16 | 10.09 ±0.26* | 9.82 ±0.18* | 11.12 ±0.64 | 8.87 ±0.41* | 8.12 ±0.25* | 6.85 ±0.45* | 12.12 ±0.63 |
|                                   | (n = 102) | (n = 74) | (n = 182) | (n = 101) | (n = 130) | (n = 41) | (n = 47) | (n = 102) | (n = 25) | (n = 23) |

*Concentration of GO= mg/L
Data with asterisk (*) indicate that the values are significantly different (p < 0.05) from the corresponding control group.
2. Experimental Design, Materials and Methods

All the experimental animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Jackson State University, Jackson, MS, USA. Protocol number (#IBC 08-01-17 and IBC 09-01-17) following the guide lines of National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.1. Animal Maintenance and Exposure to GO

The adult Japanese medaka fish colony (Orange red varieties, Hd-rR strain), we maintained at the Jackson State University, Jackson, MS, USA was described previously [1–6]. Briefly, in September 2017, fifty Adult fish were initially obtained from the Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi, University, Mississippi, USA, by a protocol transfer agreement and set up a medaka culture facility at the Plant Science Building of Jackson State University, Jackson MS, USA. We used 35 L tanks for breeders (6 females and 4 males) containing 25 L of BSS and the tanks were maintained at 25 ±1 °C with light cycle 16h L: 8h D. The pumps and disposable bio and carbon filters (That Fish Place-That Pet Place, Lancaster, PA, USA) were used for recirculation and purification of the media. Fish fed twice daily with brine shrimp nauplii (reared in the laboratory) and Tetramin flakes (That Fish Place-That Pet Place, Lancaster, PA, USA); the morning feeding is scheduled between 9 and 10 a.m. and the afternoon feeding is between 4 and 5 p.m. Depending on the pH (7–8.5) and the ammonia concentration (1–3 ppm), BSS was changed every two/three weeks or as and when necessary. With these conditions, the fish breed successfully and laid good quality eggs. The collection of the eggs from the culture (abdominal region of the female fish) was done within 1–3 h after the light turned on. ERM was used for embryo culture and the embryos generally hatched out within 10–12 days in culture. The development and staging of the experimental fish were classified following Iwamatsu [7].

2.2. GO Preparation

The procedure of GO synthesis, dilution, sonication, application to the animal was described previously [1,2,8]. Briefly, the GO used in the experiment was either synthesized in the laboratory or obtained from a commercial source (Sigma-Aldrich, St. Louis, MO, USA). Before used in the exposure, GO (a dispersed solution on water with a concentration of 2 mg/mL or 2000 mg/L; Sigma-Aldrich, St. Louis, MO, USA) was diluted to desired concentration (20 mg/L which is equivalent to 20 μg/mL; 100-fold dilution of the GO received from the commercial source) either by nanopure water (Barnstead Nanopure, model D11901, Thermo Fisher Scientific, Waltham, MA, USA) for IP or in BSS for IMR. Moreover, a stock solution of 2 mg/mL GO which was synthesized in the laboratory [1,2], was also dispersed in nanopure water. The GO either in aqueous solution (2000 mg/L) or in BSS (20 mg/L) was sonicated for 5 min (2s on-1s off pulse, 225 w) using a probe sonicator (ultrasonicator LPX 750, Cole Palmer, Chicago, IL, USA) in ice temperature. The sonicated nanomaterial was further checked by TEM at the Electron Microscopy Core laboratory at the Jackson State University, Jackson, MS, USA [1,2].

2.3. Exposure of Adult Fish to GO

The adult medaka fish we used for histological/histochemical/immunohistochemical evaluation of interrenal gland (adrenal homolog) were obtained from our previous GO experiments on Japanese medaka adults (IMR fish [8], IP fish [1,2]). We have used two methods of GO exposure (IMR and IP) for adult fish. Briefly, before exposure to GO (either by IMR or IP), the fish were maintained as a breeding pair (one male and one female) in 500 mL BSS in 1L glass jar
in the laboratory conditions (25±1 °C and 16h light: 8h dark Light cycle). When they breed successfully for one week (7 days), the fish were used for GO exposure either by IMR (20 mg/L) or by IP (100 μg/g) [1,2,8]. For IMR experiment (body weight of control male and female fish is 228.8±25.61 mg, n = 4 and 323.8±14.7 mg, n = 4, respectively; and GO-exposed fish, male 229±10.98 mg, n = 5; female fish 280.2±14.65 mg, n = 5) we have used only one concentration of GO (20 mg/L) for exposure (96h in GO with refreshing of media every 24 h and then depurated for 21 days in a GO-free environment) (please see the schematic diagram in Fig. 1) obtained from commercial source (Sigma-Aldrich, St. Louis, MO, USA). The selection of GO to 20 mg/L used in the experiment was based on our previous studies on larvae [3,4,5,6]. For IP experiments, although in our previous studies, we have injected 4 doses of GO (25, 50, and 200 μg/g; single intraperitoneal injection) to the sexually mature and productively active adult medaka male and female fish [1,2], in the present study, we have considered only one IP group (100 μg/g) along with the controls (vehicle-injected) for the immunohistochemical evaluation of the interrenal (adrenal homolog) gland (Fig. 1). To avoid movement during IP injection, the fish were anesthetized in MS 222 (100 mg/L in ice cold BSS) and after successful injection returned to BSS until complete recovery (back to normal swimming activity) (the IMR fish did not anesthetized before exposure to GO as controls). The injected volume of the GO (2 mg/mL or 2μg/μL) or vehicle (nanopure water) is 1μl/ 10 mg body weight (adjusted to the nearest 10 mg body weight). The body weight of the male fish used in IP injections (0–200 μg/g) was 304.2 ±11.04 mg (n = 46); and female fish 348.3±11.83 mg (n = 58) [2]. The volume of the injected material never exceeds 50 μl/fish. Among the IP-injected fish, 4 pairs injected with GO (100 μg/g) obtained from a commercial source (Sigma-Aldrich, St.Louis, MO) and 3 pairs injected with GO (100 μg/g) synthesized in the laboratory [1,2]. After IP injection the surviving fish (the same breeding pair) maintained 1 L glass jars in 500 mL BSS and were allowed for 21 days depuration in a GO-free environment in the laboratory. Both IMR and IP fish were allowed breeding during depuration period (same partners used before GO exposure) and the eggs were collected and the media refreshed every day. On 21st day the fish, for histopathological observations, were sacrificed and the trunk tissue was preserved in 4% PFA in 20 mM PBS containing 0.05% Tween 20. The histopathology of IRG of the fish exposed to GO by IMR [9] were also compared with the fish administered GO (100 μg/g) either synthesized in the laboratory or obtained from commercial source (Sigma- Aldrich, St. Louis, MO) by IP-injection [1,2]. The gonad histology was used to confirm the phenotypic sex of the breeding pairs following OECD guidelines [9].

2.4. Exposure of Larvae

In our previous experiments, 1 dph medaka fries (Stage 40, 1st fry stage) were exposed to different concentrations (2.5, 5, 10, 20 mg/L in ERM) of GO (Sigma-Aldrich, St. Louis, MO) continuously by IMR for 96 h and assessed the EDC activities of GO after 6 weeks of depuration by studying gonads (testis and ovary) and thyroids [3–6]. In the present study, the IRG of some of these larvae was evaluated by histological/histochemical/ immunohistochemical methods. We are unable to track the IRG of all the experimental larvae we have used in our previous experiments due to some unavoidable technical reasons. Briefly, the experimental fries (1 dph larvae) were exposed to different concentrations of GO (control; no GO, treatments 2.5, 5.0, 10.0, and 20 mg/mL GO; 96 h continuous; 1–5 dph). No external food was given to the larvae during the exposure period. Survived fries/larvae were transferred to BSS on 5 dph, and maintained in 500 mL glass jars (4 fries/ 200 mL BSS) in the laboratory conditions (25 ±1°C; 16h light and 8 h dark light cycle). The larvae were fed during the depuration period. Larval mortality, if any, was checked every day and frequently changed the media as and when it is necessary (based on pH and ammonia concentration). We did not see any mortality of the larvae (n = 40; 8 controls, 8 per GO-treated groups; 8 × 4 = 32 treatments) during the depuration period. The larvae were sacrificed after 42 days (6 weeks) depuration at the stages 42–43 [7] of development (47 dph). The trunk tissues excluding tail were preserved in 4% paraformaldehyde (PFA) containing 20 mM PBS and 0.05% Tween 20 used for histological evaluation of the IRG of medaka larvae; the DNA
extracted from post anal tail of the larvae were used for genotype (XY/XX) identification [3–6]. The larval phenotypes (testis and ovary) was determined by gonad histology [3–6] following OECD guidelines [9].

2.5. Phenotype and Genotype Determination of the Adult and Larvae

In adults, the phenotypic sex of the fish was based on external secondary sex characters (papillae of anal fin, furrow in dorsal fin and the shape of the fish) and also confirmed internally by the histological evaluation of the gonads as testis or ovary after HE staining [9]. In larvae, the secondary sex characters were yet to appear, and we have determined the phenotypic sex by gonad histology and the genotypic sex by genotyping the male-specific gene dmy as we described previously [3,4]. We have observed several of the larvae have sex reversal (male phenotype with XX genotype); we therefore categorized the larvae into three different groups: male (testis) phenotypes with XY genotype, male (testis) phenotypes with XX genotype (sex reversed), and female (ovary) phenotypes with XX genotype. Our studies did not find any female (ovary) phenotypes showing sex reversal (with XY genotype). Due to limited number of sex-reversed larvae, during data analysis, the XX genotypes having phenotypic testis (sex-reversed) were included in male (testis) group during phenotypic analysis, and as XX genotypes in genotypic analysis. However, for adults, based on their phenotypic features (externally by fin features and internally by gonad histology) they were grouped as male (testis) and female (ovary).

2.6. Histology of Interrenal Gland

The trunk region of the medaka adults and larvae containing head kidney was fixed in 4% PFA in 20 mM PBS containing 0.05% Tween 20. After 48 h of fixation (changed PFA once after 24 h), the fixed tissue was washed thoroughly in water for 2–3 days with change of water once in every 24 h and then dehydrated in graded alcohols (30–100%), cleared in xylene and embedded in paraffin (58–60 °C); Using a manual rotary microtome (Olympus cut 4055), 5μm thick paraffin sections were cut and stained on glass slides. Some of these sections were used for hematoxylin and eosin (HE), Masson’s Trichrome (MT), and Periodic acid staining (PAS) for characterization (Fig. 2a–f; supplementary Fig. SF1a–1c). The digital images of the stained sections were taken in an Olympus CKX53 inverted microscope attached with a DP22 camera with CellSens software (Huntopics Imaging, Pittsburg, PA, USA). The images captured in randomly in selected regions which have head kidney with IRG and used for counting the cells (HE stained). To determine the specific area of the IRG where cell counting was made, we used imagej software (http://www.imagej.nih.gov/ij). For quantitative analysis, the data were expressed as number of cells/mm² area of the IRG (Tables 1, 3, and 4). Although the IRG of medaka consist both IR-cells and chromaffin cells [10], based on the staining intensity of the nuclei, in the current study, we have further classified the IRG cells stained in HE as the cells with deep-stained nuclei (DSN) and the cells with pale-stained (PSN) nuclei (Tables 1, 3 and 4; supplementary Tables ST1–3, ST6–11). The cell counting data were expressed as number of cells (either DSN or PSN)/ mm² area of the IRG. The DSN and PSN cells are added together and expressed as total IRG cells/mm² in a particular region of IRG (Table 1). Moreover, the same imagej software was used for the determination of the nuclear area of DSN or PSN cells (Tables 2, 5, and 6; ST4–5, ST12–15).

Tyrosine hydroxylase immunohistochemistry: The enzyme tyrosine hydroxylase (TH) is expressed in the medullary cells of the adrenal gland. For identification of the IRG in medaka head kidney, we followed immunohistochemical detection of TH enzymes in the medaka kidney by a method described by Shin et al [11] with some modifications (Fig. 2g–j) [8]. In brief, tissue sections (5 μm thickness) on glass slides were deparaffinized in xylene rehydrated in graded alcohols and brought to tap water. Antigen retrieval (unmasking) of IRG was done for 40 min in citrate buffer (pH 6.0) at 85–90 °C in a water bath. After washing the slides in 0.1 M phosphate
Fig. 2. Identification of interrenal gland (adrenal homolog) in Japanese medaka adults. **Fig. 2a–d**: histological identification of the interrenal gland (IRG) of Japanese medaka adult (male and female) fish by HE (Fig. 2a and b; Female) and Masson’s Trichrome (MT) (Fig. 2c and d; male) staining; 2a and 2c = low magnification; 2b and 2d = higher magnification. **Fig. 2e and f** (female): histochemical identification of the IRG of Japanese medaka female by PAS staining (2e = low magnification; 2f = higher magnification). **Fig. 2g–j** (male): immunostaining of adrenal homolog cells by rabbit antityrosine hydroxylase antibody. **Fig. 2g and h**: IRG sections of a male medaka adult fish are immunostained with rabbit polyclonal antityrosine hydroxylase antibody; **Fig. 2i and j**: IRG sections are immunostained without antityrosine hydroxylase antibody. **Fig. 2g and i** low magnification; **Fig. 2h and j** = higher magnification. Black arrows indicate the position of IRG around the cardinal vein (CV) passing through head kidney of Japanese medaka.
buffered saline-tween 20 (PBST; PBS with 0.3% Tween 20; Sigma-Aldrich, St. Louis, MO), for blocking of endogenous peroxidase and alkaline phosphatases, the sections were treated for 10 min at room temperature with bloxall (Vector laboratories, Burlingame, CA). Next, the sections, after a brief wash in PBST, were incubated with 3% normal goat serum in PBST for 1 h at room temperature and finally with primary antibody (polyclonal rabbit-derived anti-TH obtained from Sigma-Aldrich, St. Louis, MO, Catalog # AB-152 with a final dilution of 1:200 in 3% goat serum in PBST) overnight at 4°C (Fig. 2g and h). Parallel control sections on glass slides were incubated in identical condition in 3% goat serum-PBST only without the primary antibody (Fig. 2i and j). Next day, sections were washed several times in PBST and incubated with biotinylated goat anti-rabbit secondary antibody (Vector laboratories, Burlingame, CA) diluted (1:200) with 3% goat serum-PBST, for 1 h at room temperature. Sections were washed again in PBS and incubated with avidin-biotin complex in PBS for 1 h at room temperature as recommended by the manufacturer (Vector laboratory, Burlingame, CA, USA). The sections were washed several times in PBS and incubated again for 1 h at room temperature with vectastain elite ABC reagent (Vector laboratory, Burlingame, CA, USA). After incubation, sections were washed several times in PBS and incubated again at room temperature for 10 min with freshly prepared peroxidase substrate (vector DAB) as recommended by the manufacturer (Vector laboratory, Burlingame, CA, USA). After incubation, the sections were washed in tap water and counter stain with hematoxylin, dehydrated in graded alcohols, clear in xylene and mount in permount. The sections were evaluated and photographed under microscope (Olympus CKX53 inverted microscope). Antibody positive cells in IRG were brown in color (Fig. 2g and h).
2.7. Statistical Analysis

Data were analyzed using GraphPad prism version 7.04 (GraphPad Prism, San Diego, CA). We used descriptive analysis to evaluate nuclear area (μm²) of IRG cells, and the distribution (number/mm²) of these two cell types either alone (DSN and PSN) or total cells (DSN+PSN) in IRG of Japanese medaka adults and larvae (47 dph) after HE staining. We used Grubbs’ test to identify the data as outliers as recommended by the GraphPad Prism. The data identified as outliers are included in the raw data tables (ST1–ST15) but not in the final summary tables (Tables 1–6) D’Agostino-Pearson (DP) or Shapiro-Wilks (SK) tests were used to determine the normality of the data. We used one-way ANOVA followed by unpaired parametric “t” test including Welch’s correction as a post-hoc test, if the data were normally distributed and meet the percepts of using a parametric test. The Statistical significance was defined as p < 0.05. We performed the Kruskal-Wallis test followed by Mann-Whitney’s test (nonparametric test) as a post-hoc test and p < 0.05 was considered as the level of significance, if the normality test indicated that the data did not meet the criteria of using a parametric test. All the numerical data were expressed as mean ±SEM.

Ethics Statement

No human subjects were involved in this research. The experiments conducted in this study were approved by the Institutional Animal Care and Use Committee (IACUC Protocol number IBC 08-01-17 and IBC 09-01-17) of the Jackson State University, Jackson, MS.

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.

Data Availability

Experimental data-sets on the histopathological and immunohistological assessment of the Interrenal gland (adrenal homolog) of Japanese medaka (Oryzias latipes) fish exposed to graphene oxide. (Original data) (Figureshare).

CRediT Author Statement

Asok K. Dasmahapatra: Conceptualization, Formal analysis, Visualization, Investigation, Methodology, Project administration, Software, Supervision, Writing – review & editing, Writing – original draft; Paul B. Tchounwou: Conceptualization, Funding acquisition, Visualization, Investigation, Project administration, Resources, Supervision, Writing – review & editing, Writing – original draft.

Acknowledgments

The research was supported by NIH/NIMHD grant # G12MD07581 (RCMI Center for Environmental Health), NIH/NIMHD grant #1U54MD015929 (RCMI Center for Health Disparities Research) and NSF grant #HRD 1547754 (CREST Center for Nanotoxicity Studies) at Jackson State University, Jackson, Mississippi, USA
Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.dib.2022.108693.

References

[1] A.K. Dasmahapatra, D.K. Powe, T.P.S. Dasari, P.B. Tchounwou, Assessment of reproductive and developmental effects of graphene oxide on Japanese medaka (Oryzias latipes), Chemosphere 259 (2020) 127221, doi: 10.1016/j.chemosphere.2020.127221.

[2] A.K. Dasmahapatra, D.K. Powe, T.P.S. Dasari, P.B. Tchounwou, Experimental data sets on the characterization of graphene oxide and its reproductive and developmental effects on Japanese medaka (Oryzias latipes) fish, Data Br. 32 (2020) 106218, doi: 10.1016/j.dib.2020.10628.

[3] A. Myla, A.K. Dasmahapatra, P.B. Tchounwou, Sex-reversal and histopathological assessment of potential endocrine disrupting effects of graphene oxide on Japanese medaka (Oryzias latipes) larvae, Chemosphere 279 (2021) 130768, doi: 10.1016/j.chemosphere.2021.130768.

[4] A. Myla, A.K. Dasmahapatra, P.B. Tchounwou, Experimental data sets on sex-reversal and histopathological assessment of potential endocrine disrupting effects of graphene oxide on Japanese medaka (Oryzias latipes) larvae at the onset of maturity, Data Br. 38 (2021) 107330, doi: 10.1016/j.dib.2021.107330.

[5] T.E. Asala, A.K. Dasmahapatra, M. Myla, P.B. Tchounwou, Experimental data sets on the evaluation of graphene oxide as a thyroid endocrine disruptor and a modulator of gas gland cells in Japanese medaka (Oryzias latipes) larvae at the onset of maturity, Data Br. 39 (2021) 107625, doi: 10.1016/j.dib.2021.107625.

[6] T.E. Asala, A.K. Dasmahapatra, M. Myla, P.B. Tchounwou, Histological and histochemical evaluation of graphene oxide on thyroid follicles and gas gland of Japanese medaka larvae (Oryzias latipes), Chemosphere 286 (2021) 131719, doi: 10.1016/j.chemosphere.2021.131719.

[7] T. Iwamatsu, Stages of normal development in the medaka Oryzias latipes, Mech. Dev. 121 (2004) 605–618, doi: 10.1016/j.mod.2004.03.012.

[8] A.K. Dasmahapatra, P.B. Tchounwou, Histopathological evaluation of the interrenal gland (adrenal homolog) of Japanese medaka (Oryzias latipes) exposed to graphene oxide, J. Envirop. Toxicol. (2022) July 09PMID 35809259, doi: 10.1002/tox.23610.

[9] OECDGuidance Document of the Diagnosis of Endocrine-Related Histopathology in Fish Gonads, OECD, Paris, 2010 OECD series on Testing and Assessment. No 123 https://www.oecd.org/chemicalsafety/testing/42140701.pdf.

[10] M. Oguri, Histo-morphology of the kidney and adrenal gland of medaka, Oryzias latipes (T.E.T S) Bull. Jap. Soc. Sci Fish. 17 (1961) 1058–1062.

[11] S.W. Shin, N.J. Chung, J.S. Kim, T.S. Chon, O.S. Kwon, S.K. Lee, S.C. Koh, Effect of Diazinon on behavior of Japanese medaka (Oryzias latipes) and gene expression of tyrosine hydroxylase as a biomarker, J. Environ. Sci. Health B36 (2001) 783–795.