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ORIGINAL RESEARCH

Ameliorative effects of chitosan in water remediation, endocrine disruption and reproductive impairment of *Solea solea* after exposure to Benzo (a) pyrene

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Abstract Water pollution has been increased by the discharge of several polycyclic aromatic hydrocarbons including Benzo (a) pyrene (BaP), which may affect aquatic animal lives and threaten human health. Common sole, *Solea solea* was selected as a bioindicator benthic fish to monitor pollution from El-Maadeya (one of the industrially polluted areas in Egypt). Concentration of "BaP" in collected samples of water, sediments and some organs of sole was detected using HPLC. A detected level of 21 µg ″BaP″ /L was reported in water samples from El-Maadeya. Analysis results of ″BaP″ in El-Maadeya water, sediment, and biota exceeded the permissible limits. In a new trial for water remediation from ″BaP″, chitosan (Cs) was experimentally applied. Moreover, the impact of ″BaP″ alone or in combination with Cs for two weeks on water quality, sole fish brooders and histopathological changes in their ovaries was studied. Fifteen fish/tank were randomly classified into five groups in triplicates exposed to: GI) sea water only, GII) sea water with "DMSO", GIII) 21 µg ″BaP″ /L (equivalent to the level detected in El-Maadeya), GIV) 50 µg ″BaP″ /L, GVa) 21 µg ″BaP″ /L & 0.5 g ″Cs″ /L for 24 hrs and GVb) 21 µg ″BaP″ /L & 0.5 g ″Cs″/L for one week. Obtained results showed that ″BaP″ accumulated in the liver and ovary of fish resulting in a negative impact on the reproduction due to the alteration of the ovarian architecture, atresia, and gonadosomatic index. Western Blot analysis showed also that ″BaP″ disrupted steroidogenic enzymes, upregulated the expression of Hsp-70, and forced the degradation of ERα. The addition of 0.5 g Cs/L has showed its efficiency against BaP toxicity, since it plays a role in ″BaP″ elimination by fast adsorption from water and sediments in time-dependent manner, improved fish health, restored reproductive characteristics, decreased the percentage of atretic oocytes and reduced fish mortality.

Keywords Chitosan . Erα . Hsp-70 . *Solea* . Ultrastructure . Water remediation . Reproduction

Introduction

Water supply has become an essential need for both developed and developing nations worldwide. However, conventional water resources alone cannot meet the growing demand for water in urban cities (Ma et al. 2022). One of Egypt's most significant challenges today is the increasing number of rural and urban households that need access to basic infrastructure, water supply, and sewage. It is noteworthy mentioning that water pollution improves gradually with industrialization, urbanization, and technological development as

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these processes increase the discharge of pollutants into the receiving water, causing undesirable effects on the aquatic environment (Lin et al. 2022). Polyaromatic hydrocarbons (PAHs) originate from natural and anthropogenic sources such as transport, incomplete combustions, uncontrolled spills or industrial incinerations (Nácher-Mestre et al. 2010) and may cause toxicity to fish by interfering with cellular membrane function and the associated enzyme systems (Zhao et al. 2014). Hence, PAHs in many aquatic environments are important risk factors for various health aspects of fish (Payne et al. 2003).

Fishes are bioindicators that are increasingly crucial in monitoring water pollution and contamination of marine species (El-Ghazaly et al. 2017). Furthermore, the shock proteins are cosmopolitan in all living organisms, including heat shock protein-70 (HSP-70) which have been suggested as exposure levels and toxicity biomarkers (Hu et al. 2022). These proteins are crucial in physiological processes, such as protein chaperoning activity, protection against apoptosis, steroidogenesis, and stress tolerance (Hu et al. 2022). The main function of HSP-70 is to maintain protein integrity in the presence of stressors. Hence, it can be used as biomarker of homeostatic alterations in polluted environments, whereas members of the cytochrome p450 family participate in metabolism, steroid hormone synthesis, and xenobiotic transformation (Ríos-Sicairos et al. 2010). In vertebrates, estrogen receptors (ER) which belong to the nuclear receptor superfamily, are able to regulate the transcription of target genes and contain a modular organization in six domains. An overlapping distribution of estrogen receptors (ER) forms within the brain, pituitary, liver, and gonads in zebrafish means that each ER subtype acts to regulate different genes linked to various physiological processes such as growth, development or cell differentiation (Ghanem 2021).

There is a gap of knowledge concerning the incidence of biochemical and histological alterations in Solea's gills, gonads, and liver collected from the marine environment. Batool et al. (2023) reported that enzymes are necessary for normal cellular metabolism in the liver. Degenerative changes due to the joint xenobiotic toxicity detected in the liver alter the level of its enzymes. Consequently, proper remediation is essential as it simultaneously removes organic and inorganic pollutants from mixed-contaminated sites (Maity et al. 2013). Chitosan adsorption technique has proven to be suitable, simple, efficient, and environmentally friendly for the removal of PAHs, since the production of chemical byproducts is minimum (Albayati et al. 2020). The significant advantage of this technique comprises a possible mechanism that includes the detection and rapid removal of PAHs involving "BaP" at low concentrations in the different water sources (Dai et al. 2020).Chitosan is a linear cationic polysaccharide considered as a crucial contribution for wastewater treatment due to the presence of large numbers of hydroxyl and amino groups which provide high availability of active sites to enhance the adsorption capacity (Rani et al.2020).Therefore, it can simply adsorb organic pollutants through hydrogen bonding and electrostatic interactions leading to the formation of more complex structures (Chelu et al. 2023 a,b). Worth pointing out that chitosan nanoparticles are found to be an attractive alternative to liposomes for the delivery of peptides, proteins, antigen oligonucleotides, and genes since they have the advantages of longer shelf life, and are known to have a higher drug-carrying capacity (Rashki et al. 2021). Chitosan particles have various characteristics depending on their purity, molecular weight, degree of deacetylation, quality, and viscosity (Koppolu and Zaharoff 2013).

The present study was carried out to generate mitigation for the problem of El Maadeya water pollution since it was selected as a heavily industrial polluted area due to its economic importance. To achieve this purpose, water quality was assessed in a field study and a laboratory one. The field study comprised two locations [Ratama farm (LI) water in Damietta which appeared free from contaminant used as reference area while, El-Maadeya (LII) seems to be highly contaminated with ″BaP″] for monitoring physicochemical parameters of the selected locations (LI & LII) and detecting elevated concentration of "BaP" in water, sediments, and some organs of *"S.solea"*. Besides, a laboratory study was designed to : measure ″ BaP″ concentration accumulated in water, sediment, and some organs when fish exposed to waterborne ″BaP″ for two weeks, study the effect of ″BaP″ at concentration equivalent to that detected in water of LII on mortality of *"S.solea"*, record cellular and subcellular changes induced in ovary of fish due to ″BaP″ exposure, evaluate the role of chitosan, as a new trial for water remediation from ″BaP″ and assess the hepatic enzymes activities and the expression of HSP-70 protein, and ER-α as a result of exposure to ″BaP″ alone or after addition of ″Cs″.

Materials and methods

Study area

Ratama farm (LI), and El-Maadeya (LII) were two water locations selected in the current study between December and the beginning of March 2019. Based on the chemical analysis of water, sediment, and fish, Ratama farm (LI) in Ezbet El Borg in Damietta, Egypt was selected as a reference site. El-Maadeya (LII) was selected for sample collection because it represented a highly industrial polluted site, especially with PAHs originating from the effluents of companies located there and numerated from 1 to 12 as illustrated in Fig.1. In addition, it is known as the most polluted part of the River Nile found between Damietta and Rosetta branches. LII runs about 220 Km in length, with an average width of 180 m and an average depth of 1.5- 16.0 m. It flows downstream from Delta Barrage to Northward along the west boundary of the River Nile, where it ends at Edfina Barrage. LII is in Abu Qir Bay, and extends from Nilson Island to Rosetta's mouth. The chief pollution sources of the River Nile and main canals are the effluents from agricultural drains and treated waste of industrialized companies. These sources of pollution potentially deteriorate freshwater quality causing serious environmental impact.

Chemicals

Benzo (a) pyrene "BaP" ($C_{20}H_{12}$) used, was purchased from Sigma Aldrich (B1760; Sigma–Aldrich, St. Louis, MO, USA). It was dissolved in a 10 ml organic dimethyl sulfoxide solvent (Sigma–Aldrich).

Chitosan (Cs) is a natural polysaccharide ion (poly-(1-4)-2-amino-2-deoxy-d-glucose) ($C_6H_{11}NO_4$) derived from deacetylated chitin, which is the building material that gives strength to the exoskeletons of crustaceans (crab, lobster, and shrimp), insects, and the cell walls of fungi. It was purchased from Carl Roth Company (Cat. No. C 0108, Karlsruhe, Baden-Württemberg, Germany). Through enzymatic or chemical deacetylation, chitin can be converted to chitosan. The degree of deacetylation (%DD) can be determined by NMR spectroscopy, and the %DD in commercial chitosan ranges from 60 to 100%. The molecular weight of commercially produced chitosan is between 3,800 and up to 400,000 Daltons.

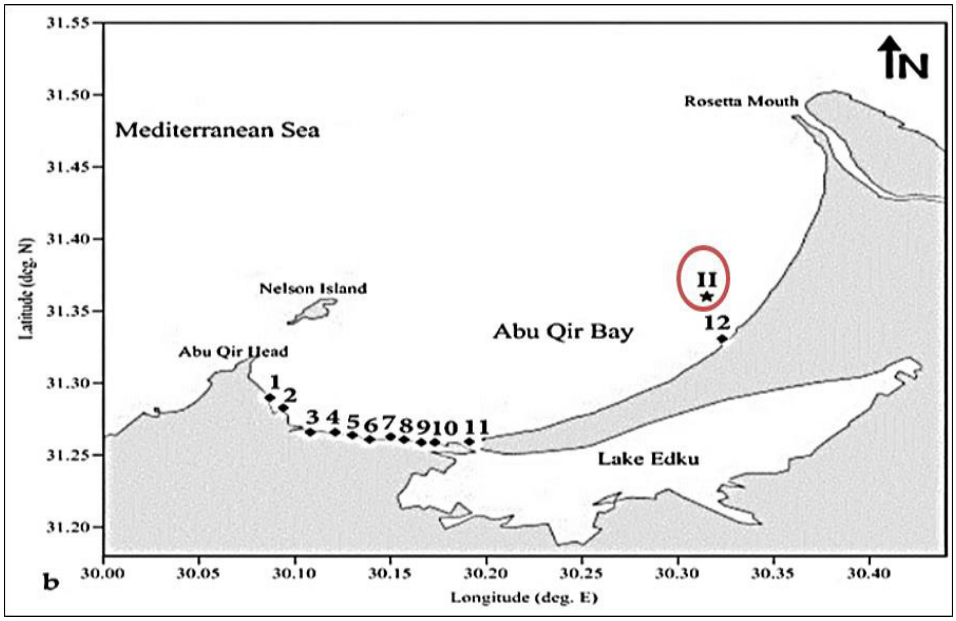


Fig. 1 A map Illustrating, a highly industrial polluted site in El-Maadeya (LII) due to the effluent discharge of many companies. Notice:1-12 pointed to 1: Qaha company, 2: Rakta company, 3: Raslan effluent, 4: El-Tarh effluent, 5: Abu Qir Old Electricity Company, 6: Abu Qir New Electricity company, 7: Petro jet company, 8: Petrol 2 company, 9: Gas company, 10: Boughaz El-Maadeya, 11: Gas company, 12: Rashid Petroleum company

Assessment of “BaP” in water, sediment, and selected fish organs

Assessment of “BaP” concentration in samples of water, sediment, and fish organs (liver, ovary, and muscle tissue) was performed using high-performance liquid chromatography (HPLC) (Shimadzu, Japan, SCL-10AVP) in the central laboratories unit of the National Institute of Oceanography and Fisheries, Alexandria, Egypt. Two water samples (1 Liter) and two sediment samples (each weighing 25 g) from each location as well as samples of *Solea* liver, ovary, and muscle tissue (each weighing 25 g) were collected from 5 fish samples from each location during and at the end of the study. A carbamate analysis column (150 mm X 4.6 mm, C18, 5 µm, Pickering laboratories, USA) was used for its separation. An aliquot (20 µL) of the acetonitrile solution was injected into the HPLC system and eluted with acetonitrile: water (80:20 v/v) at a constant flow rate of 2.0 ml/min. To quantify the “BaP” concentration, the detector was set at the excitation wavelength (290 nm) and emission wavelength (430 nm).

Chitosan concentration used in water remediation of ″BaP″

In the current laboratory experiment, several attempts were performed during the pilot study in order to detect the concentration level of ″Cs″ which gave the maximum adsorption and removal percentage of ″BaP″ from sea water. This was carried out using different concentrations of ″Cs″ (0.1, 0.5 and 1gm/L) dissolved in waterborne ″BaP″ (10 µg/L and 21 µg/L) at time intervals corresponding to 0, 30 and 60 minutes (Table 2).

Experimental design and fish groups

Ripe females caught from the two studied areas: Ratama farm (LI) in Ezbet El Borg in Damietta, Egypt (a reference site) and El-Maadeya (LII) the selected polluted location (with 86 - 120g body weight and 22 - 24 cm length) were captured simultaneously with water and sediment samples between December and the beginning of March 2019 for both the field and experimental studies. It is worth to mention that for the laboratory study, fish samples and sediments were collected from LI as a reference unpolluted site. Fish were transported alive to the marine hatchery of the National Institute of Oceanography and Fisheries, Alexandria, Egypt.

After acclimation, the experiment was conducted following the guidelines of Directive 2010/63/EU for animal experiments. The fish were held in tanks for two weeks for acclimation before being allocated to tanks connected to a common biological filter, oxygen pump, and circulation pump. Fish were offered dry shrimp ad libitum as fish feed. Before the experiment, hatchery seawater was treated with chlorine to remove bacterial contamination, sodium thiosulfate to dechlorinate it and ethylene diamine tetra acetic acid to remove heavy metals. Sediment samples that were placed in the different tanks were analyzed before and at the end of experiment. The experiment was performed on five groups treated as follows:
GI: Control fish exposed to waterborne free from “BaP″.
GII: Fish exposed to sea water with “DMSO”; vehicle of the “BaP″.
GIII: Fish exposed to 21 µg ″BaP″ /L; equivalent to the level detected in LII.
GIV: Fish exposed to 50 µg ″BaP″ /L sea water; equivalent to the double level detected in LII.
GVa: Fish exposed to 21 µg ″BaP″ /L sea water & 0.5 g ″Cs″ /L sea water for 24 hrs.
GVb: Fish exposed to 21 µg ″BaP″ /L sea water & 0.5 g ″Cs″/L sea water for one week.

The experiment was terminated after 15 days except for GIV (ended at the 5th day) and GVa which ended on the eighth day to examine the effect of ″Cs″ after 24 hrs from its addition.

The experiment was performed on five groups of *Solea solea* (n= 15 fish/tank) in triplicates. The research system consisted of 6 polyethylene tanks (240 L X 60 W X 33 cm H) filled with ~ 475 L filtered seawater of NIOF hatchery. During the experiment, fish tanks were placed under physicochemical conditions that mimic their natural habitat in LI (adjusted daily using YSI-556 multi-parameter equipment (Yellow Spring Instruments Co., USA), with a pH level of 7.22 ± 1.06, temperature equal to 16.01±0.58 ˚C, a dissolved oxygen of 5.63±0.72 mg/L, 0.02±0.91 mg/L of ammonia level and salinity corresponding to 28%. Photometer was used to measure ammonia (Hanna Instruments RI, USA), and water was replaced once/ week (Table 1). The YSI ammonia test is based on an indophenol method (Aminot et al.1997). Ammonia reacts with alkaline salicylate

in the presence of chlorine to form a green-blue indophenol complex. Catalysts were used to ensure complete rapid colour development. The reagents were provided in the form of two tablets for maximum convenience. The test is simply carried out by adding one of each tablet to the water sample. The intensity of the colour produced in the test was proportional to the ammonia concentration. After two weeks, fish were harvested, individually weighed, measured, rapidly anesthetized with 120 mg l^{-1} amino-benzoic acid (Sigma-Aldrich and they were dissected directly. Then, fish were dissected quickly and chosen organs; liver and ovaries were excised and fixed in liquid nitrogen for RNA extraction, or in 10% neutral formalin and in $_4F_1G$, (4 Formaldehyde: 1 Glutaraldehyde) either for histological or ultrastructural studies, respectively. The ovaries of 5 fish from each tank were removed and weighed to calculate gonadosomatic index (GSI) as follows:
GSI (%) = 100 (gonads weight (g)/gutted body weight (g)).
Moreover, non-edible tissues; liver and ovaries and edible ones; muscles were homogenized and analyzed by HPLC to detect the presence of ″BaP″ accumulated in these tissues. It is of importance to mention that fish individuals belonging to GV were dissect at two time intervals. Fish were dissected at the 8th day (i.e. 24 hrs from exposure of fish to ″Cs″) and 15th day to give an idea about short & long duration treatment.

Light and electron microscope specimens' preparations

For light microscopy, small pieces of the ovary specimens were fixed in 10% neutral formalin for 24 hrs., washed in distilled water, dehydrated in ascending grades of ethanol at 4˚C, cleared by methyl benzoate, and embedded in paraffin. Sections of 5µm were stained with Haematoxylin and Eosin and examined using a light microscope in the microscopic unit at the National Institute of Oceanography and Fisheries, Alexandria, Egypt (Radwan et al. 2023). On the other hand, for the transmission electron microscopy (TEM) slices of ripe ovaries were fixed for 24 hrs. in $_4F_1G$, (4 Formaldehyde: 1 Glutaraldehyde) in phosphate buffer (pH 7.4) at 4°C, post-fixed in 2% OsO4 in phosphate buffer for 2 hrs., washed, dehydrated through a series of ethanol, and embedded in Epon-Araldite mixture. LKB ultramicrotome was used to obtain semithin 1µm and ultrathin (50 nm) sections. The semithin sections were stained with Toluidine Blue, while the ultrathin sections were double stained with Uranyl acetate for ½ hr. and Lead citrate for 30 min and then examined using Joel 100 CX TEM in the microscopic unit at faculty of Science, Alexandria, Egypt (Reynolds 1963)

Cytochrome p450 and Glutathione-S- Transferase (GST) determination

The determination of cytochrome p450 concentration was conducted on microsomal preparations, which are turbid and require a spectrophotometer that performs accurately and reliably at high absorbance values (UV-72). The Cary range of 3 spectrophotometers can be used to determine cytochrome p450 concentration with OS2ADL24, a specially designed software program, developed using Cary's Applications Development Language following Huggett (1992) method. P450 concentration was measured by mg/mg protein unit. The GST Assay Kit defines total GST activity (cytosolic and microsomal) by measuring the conjugation of 1-chloro- 2,4- dinitrobenzene (CDNB) with reduced glutathione using the UV method (2518 catalog number, Cayman Chemical,MI,USA), according to the manufacturer's protocol. A procedure for tissue homogenates results in assaying total GST activity was utilized (Habig et al.1974).

Assessment of the relative density of HSP-70 & Estrogen receptor alpha (ER-α) bands

Collected tissues from the tested group were subjected to homogenization using ultrasonic in Radio-immunoprecipitation Assay (RIPA) buffer containing 1X cocktail of protease inhibitors (Complete Mini, Roche, Mannheim, Germany), and phosphatase inhibitors (phosphatase inhibitor cocktail I and II, Sigma) on ice for 1 hr. for whole tissue-bound protein extracts. The lysed samples were centrifuged at 15000 rpm for 30 min, and the supernatant was aspirated carefully and placed on ice. Then, protein concentrations were determined following Bolt and Mahoney (1997) and Hames and Rickwood (1998) methods, using "Bicinchoninic Acid, BCA" (Pierce Biotechnology, Rockford, USA) according to manufacturer's protocol in the biochemistry department at faculty of Science, Alexandria, Egypt.

The protein samples were subjected to 10% polyacrylamide gel electrophoresis for separation and transferred onto 0.22-mm nitrocellulose membranes (Bio-Rad, USA). The membrane was probed with specific

monoclonal anti-HSP-70 and monoclonal anti- ER-α as primary antibodies with a concentration of 1:1000. After membrane washing with Tween-Tris-buffered saline (TTBS), it was incubated with their respective secondary antibodies. For detection, an ECL kit was used according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). Membranes were stripped using blot stripping buffer [Thermo Scientific Restore™ (Thermo Scientific, lL, USA)] and re-probed with anti-β-actin as a control for equal loading (Bolt and Mahoney 1997; Hames and Rickwood 1998). Quantity One Software calculated the corresponding relative band density of HSP-70 and ER-α bands.

Statistical analysis

Results were expressed as mean ± standard error (SEM). Multiple comparisons were performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A P-value ≤0.05 was considered statistically significant. All statistical tests were carried out using the statistical package for the social sciences (SPSS) version 18.0 for Windows. The relative band density of western blots was measured using Quantity One analysis software (Bio-Rad). All experiments were repeated three times in triplicates.

Results

Environmental study

Measurement of physicochemical parameters of water

Table (1) illustrates the physicochemical parameters and comparison of the degree of water pollution between Ratama farm (LI) and El-Maadeya (LII). Chemical analysis of water revealed that LII showed a higher level of pH with 8.86± 0.36. The temperature in LII exhibited 16.48± 0.33˚C. The dissolved oxygen in LII was 3.91± 1.08 mg/L. LII also revealed a higher ammonia (NH_4) value (0.71±0.03 mg/L). The salinity % was increased to 36.7 % in LII. (*these mean values exceeded the permissible limits specified by Egyptian Law for protection (48/1982) of the River Nile). However, the chemical analysis of the sea water in Ratama farm (LI) demonstrated normal parameters with a pH level of 7.22 ± 1.06, temperature equal to 16.01±0.58 ˚C, a dissolved oxygen of 5.63±0.72 mg/L, 0.02±0.91 mg/L of ammonia level and salinity corresponding to 28% (Table 1).

"BaP" concentration in water, sediment, and fish organs

Using HPLC chromatography analysis, it was noticed that the concentration of "BaP" in water, sediment, and fish organs showed great difference in their level among the two chosen sites (Ratama farm and El-Maadeya). Since data obtained showed that the concentration of "BaP" was found to be = 21 µg/L in water samples collected from El-Maadeya (LII), while Ratama farm water (LI) revealed complete absence of "BaP".

On the other side, close inspection of the chemical analyses of sediment collected from location I and location II showed that "BaP" was found to be =76.85µg/L in "LII" while in Ratama farm (LI) as expected no concentration of "BaP" could be recorded. Therefore, the chromatograph revealed much more pronounced accumulation of "BaP" in sediment samples collected from El-Maadeya "LII", if compared to its corresponding in the reference location.

It was also reported that in fish collected from LII ″BaP″ accumulated in ovaries, liver, and muscles and its concentration was found to be equal to 15.68 µg/Kg in ovary, 16.35 µg/Kg in liver and 8.77µg/Kg in muscles, respectively. However, data analysis of the concentration of ″BaP″ in samples of fish ovaries, liver, and muscles collected from Ratama farm (LI), revealed complete absence of ″BaP″ concentration.

Experimental Study

Chitosan induced adsorption and removal of "BaP" from water

Table (2) shows that the concentration level equivalent to 0.5 g/L of Cs is considered the most effective con-

Table 1 Mean values (mean ±SE) of physicochemical parameters the study area (LI & LII) during exposure to BaP alone or in combination with Cs.

	Location	Level of BaP	BaP at day 0	BaP On the 15th day	pH	Temperature	Dissolved oxygen (mg/l)	Ammonia	Salinity (%˚)
Field study	I	Ratama water	0 µg/l	0 µg/l	7.22± 1.06	16.01±0.58	5.63±0.72	0.02±0.91	28 %˚
	II	El-Maadiya water	21 µg/l	21 µg/l	8.86±0.36	16.48± 0.33	3.91± 1.08	0.71±0.03	36.7%˚
	Group	Tank containing	BaP on day 0	BaP on the 15th day	pH	Temperature	Oxygen Dissolved (mg/l)	Ammonia (mg/l)	Salinity (%˚)
Experimental study	GI	Seawater only	0 µg /l	0 µg BaP /l	7.22± 1.17	15.02± 0.38	7.96± 0.08	0.48± 0.01	
	GII	Seawater with 10 ml DMSO	0 µg /l	0 µg BaP /l	7.43± 0.77	15.89± 0.62	7.02± 0.08	0.47± 0.02	
	GIII	Seawater & BaP	21 µg /l	16.8 µg /l	7.52± 0.72	16.13±0.61	6.95± 0.08	0.48± 0.01	38 (%˚)
	GVI	Seawater & BaP	50 µg /l	21.3 µg/l	7.33± 0.91	15.8±0.59	7.01± 0.07	0.46± 0.03	
	GV a	Sea water & BaP & Cs 24h	21 µg /l & 0.5 g/l Cs	2.8 µg /l	7.01± 1.14	15.65± 0.62	7.44± 0.08	0.47± 0.02	
	GV b	Sea water & BaP & Cs 7 days	21 µg /l & 0.5 g/l Cs	0.00 µg/l	7.01± 1.14	15.65± 0.62	7.44± 0.08	0.47± 0.02	
		Results of one-way ANOVA			F=2.71 p= 0.06	F= 0.65 p= 0.59	F= 0.68 p= 0.56	F= 2.00 p=0.15	

Note: Data presented as mean ± SE, in the same column and are statistically insignificant ($p > 0.05$), compared to values of the control group using one-way ANOVA. Note that the Permissible limits:1 µg/l (water); 10 µg/Kg (biota & sediments; pH 7-8.5; Temperature 5˚C above average; Dissolved oxygen ≤ 5 mg/l; Ammonia o.5 mg/l (aquatic environment), 0.2 mg/l (fish); Salinity (35%).

Table 2 The efficiency of chitosan "Cs" on BaP adsorption (µg/L) in water after different time intervals.

Time intervals (min)	Water with BaP (µg/l) only	Chitosan concentration (g/l) dissolved in waterborne BaP	BaP concentration (µg/l) in water after adding chitosan	Maximum BaP removal percentage (%)
0	10	0.1	8.67	13.3
0	21	0.1	17.42	17.05
0	10	0.5*	5.19	48.12
0	21	0.5	13.23	37
0	10	1	7.43	25.7
0	21	1	13.64	35.05
30	10	0.1	1.426	86
30	21	0.1	2.470	83.5
30	10	0.5*	0.054	99.46
30	21	0.5	0.824	94.5
30	10	1	1.234	76.2
30	21	1	2.930	80.46
60	10	0.1	4.606	53.9
60	21	0.1	6.686	55.4
60	10	0.5	2.757	72.4
60	21	0.5*	0.264	98.2
60	10	1	1.234	87.6
60	21	1	3.606	75.9

Notice: the concentration of Cs equivalent to 0.5* gm/l gave the maximum removal percentage; 48.12%, 99.46 %, 98.2 % of BaP at a concentration equivalent to 10 µg/l, 10 µg/l and 21 µg/l of water at time intervals corresponding to 0, 30 and 60 min, respectively.

centration of “BaP” adsorption. The maximum removal percentage was 48.12% at a concentration equivalent to 10 µg/L, 99.46 % at a concentration equivalent to 10 µg/L, and 98.2% at a concentration equivalent to 21 µg/L from the water media at time intervals related to 0, 30 and 60 min., respectively. Notably, the sub-lethal concentration of 21µg/L is equivalent to the concentration assessed and quantified using HPLC and imitates the reality of the “BaP” level in the polluted water column of El-Maadeya (LII).

Physicochemical parameters of hatchery seawater of experimental tanks

Parameters of seawater in different tanks were assessed during the experimental study as shown in Table 1. It is worth noting that an insignificant difference was noticed in pH and temperature between the experimental tanks and the values detected in field locations (LI and LII). Moreover, the mean value of DO_2 (mg/L) was maintained between 6.95±0.08 and 7.96±0.08, and the mean value of ammonia (mg/L) varied from 0.46± 0.03 and 0.48± 0.01, and salinity was maintained at 38%.

“BaP” concentration in water, sediment, and fish muscles

HPLC chromatography analysis of water from different tanks revealed that the tank containing 21µg “BaP”/L at the beginning of the experiment showed 16.8 µg “BaP” /L at the end of the experiment. While, the tank having 50 µg/L “BaP” at the beginning of the experiment possesses 21.3 µg/L “BaP” at the end of the experiment. Concerning the tank with 21µg “BaP”/L dissolved in water and on the 7th day 0.5 g Cs /L was added for 24 hrs., only 2.8 µg “BaP” /L was found at the 8th day of the experiment (i.e., ~ 87% of “BaP” adsorbed by Cs). However, the tank with 21µg “BaP” /L dissolved in water and on the 7th day 0.5 g Cs /L was added for a week showing 0.0 µg/L “BaP” at the end of the experiment. Thence, Cs possess a 100% removal percentage after one week, i.e., the efficiency of Cs was time-dependent.

Besides, HPLC analysis revealed that the sediment of the control group was free from ”BaP”. However, the average concentration of “BaP” in the sediment of water of tanks containing 21µg/L “BaP” and 50 µg/L “BaP” at the end of the study possess 3.12µg/L and 19.2µg/L, respectively. Moreover, the average concentration of “BaP” in the sediment of water with 21µg/L and Cs added to water on the 7th day for 24 hrs. = 2.78 µg/L. “BaP” average concentration in the sediment of “GV b” (with 21µg/L “BaP” and Cs added on the 7th day for a week) is equal to 2.46 µg/L. This indicates that Cs eliminates “BaP” from sediment in GVa and GVb water tanks compared to the concentration detected in sediment placed in water of GIII. Furthermore, HPLC was used to measure the concentration level of ”BaP” in fish muscles of the experimental groups where it was undetected in the muscles of all experimental fish groups.

Effect of "BaP" on *S. solea*

Mortality among fish of the different treatments were recorded daily. Control fish exposed to seawater (GI) or DMSO (GII) exhibited zero mortality. While, fish exposed to 21µg/L “BaP” (GIII) at the end of the study showed 27% mortality. Fish group (GIV) possessed 60% mortality. It is of interest that fish exposed to 21µg/L “BaP” and Cs added on the 7th day for 24 hrs. (GVa) didn’t record any mortality percentage since this group ended on the eighth day. Furthermore, fish exposed to 21µg/L “BaP” and Cs for a week revealed a general amelioration and marked fish health regarding motility; their mortality percentage decreased to 20%, indicating the efficacy of Cs.

Gonadosomatic index (GSI) changes in fish of diverse groups

Obtained data showed an insignificant difference ($P > 0.005$) in the gonadosomatic index (GSI) mean value between control fish (GI &GII) at the end of the experiment. However, the GSI mean value of fish group (GIII) =2.77 ± 0.71, while it exhibited 2.59 ±0.83 in fish group (GIV). This recorded a dramatically significant decrease compared to that of GI ($P < 0.05$) and an insignificant difference between GIII and GIV. Data also revealed that the mean value of GSI in the fish treated with 21 µg/L ”BaP” and Cs for 24 hours and fish treated with 21 µg/L “BaP” and Cs for 1 week was insignificant ($P > 0.05$) compared to that of the controls and designates that the use of Cs was independent of time.

Hepatic biotransformation enzymes analysis

The current results indicate that there is a significant difference in GST (mg/g protein) among the mean values of the different fish groups (P = 0.001). Data obtained showed that the mean value of GST in the liver of the fish group (GIII) = 27.5 ± 2.47 mg/ g protein (Fig. 2), indicating a highly significant decrease compared to its corresponding in GI, possessing 58.8 ± 4.52 mg/g protein. An insignificant decrease between hepatic GST means values of GIII and GII was observed (Fig. 2). Additionally, concentrations of "BaP" and/or Cs treatment for one week (GVb) revealed a normal GST level.

Data from the present study showed that p450 aromatase exhibited a significant difference among groups. The mean value of p450 aromatase in the liver of fish exposed to the low "BaP" concentration

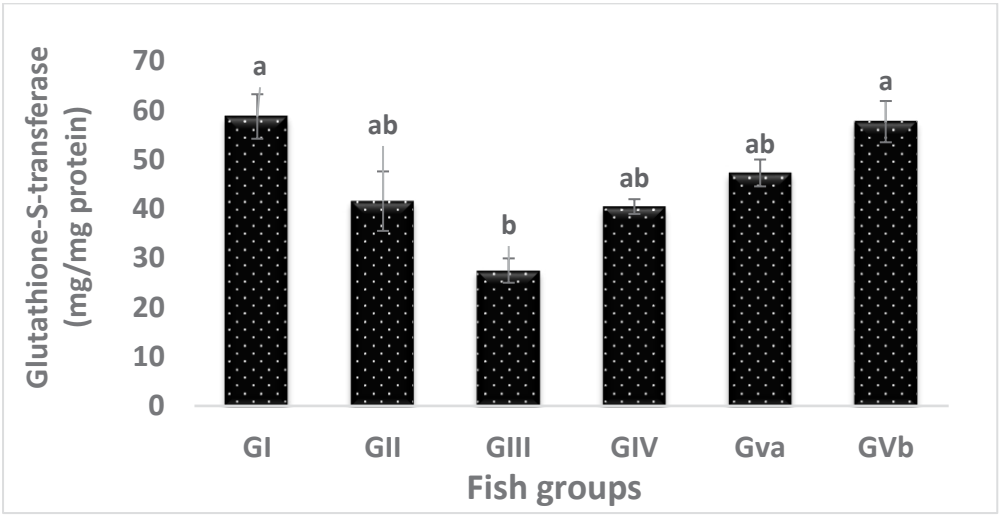


Fig. **2** Histogram showing, a significant decrease in GST of ripe and spawning female *Solea* in GIII exposed to water with low concentration BaP, an insignificant decrease in GST of fish of GIV exposed to water with high concentration BaP, in comparison to control, Cs restores the normal level of GST in a concentration-dependent manner. GI: Fish exposed to seawater only, GII: Fish exposed to water with ″DMSO″, GIII: Fish exposed to water with low concentration ″BaP″, GIV: Fish exposed to water with high concentration ″BaP″, GVa: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for 24 hrs., GVb: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for one week].

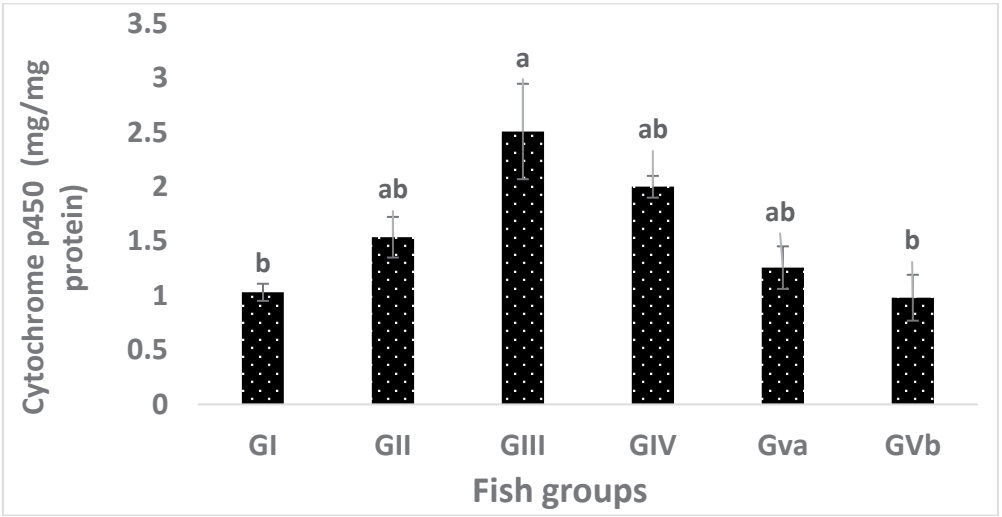


Fig. 3 Hepatic cytochrome p450 activity: quantification of ripe and spawning Solea represents the most remarkable increase in cytochrome p450 activity (M±SE) quantification belonging to GIII fish exposed to water with low concentration BaP, compared to control fish exposed to seawater (GI), and exposed to water with DMSO (GII). Note also fish exposed to water with a low concentration of BaP & Cs for 24 hrs., (GV a) or one week (GVb) restore normal microsomal p450 activity (M±SE) quantification. GI: Fish exposed to seawater only, GII: Fish exposed to water with ″DMSO″, GIII: Fish exposed to water with low concentration ″BaP″, GIV: Fish exposed to water with high concentration ″BaP″, GVa: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for 24 hrs., GVb: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for one week].

(GIII) displayed a significant increase compared to its corresponding in controls (GI). Moreover, an insignificant increase compared to GII fish (Fig. 3) was detected. Worth pointing out that fish exposed to water with low "BaP" concentration and exposed to Cs for one week (GVb) possess 0.980±0.21 mg/g protein indicating that Cs restored average microsomal p450 value and exhibited an insignificant change compared to control (i.e., Cs is a potent inhibitor of fish liver phase I p450). Interestingly, the enzymatic results of GIV (which ended on the fifth day of exposure due to the high mortality) and GVa fish (ended on the 8th day of the experiment since the study aimed to determine the effect of Cs related to time) were excluded.

Liver ER-α and HSP-70 protein expression

The mean values of ER-α and HSP-70 protein expression levels differed among the fish groups are presented in Figs. 4. The present data indicated that the expression level of ER-α protein in the liver of fish exposed to "DMSO" (GII) represents insignificant decrease (by 3%) and appears nearly similar to the expression level of ER-α in liver of GI. The current results also showed that the liver of *S. solea* fish exposed to a low "BaP" concentration (GIII) exhibited 20% downregulation in ER-α protein expression level compared to fish exposed to only seawater (GI).While, the liver samples of the fish group GIV showed downregulation by 44% compared to GI. Furthermore, the expression levels of ER-α protein in the liver samples of fish

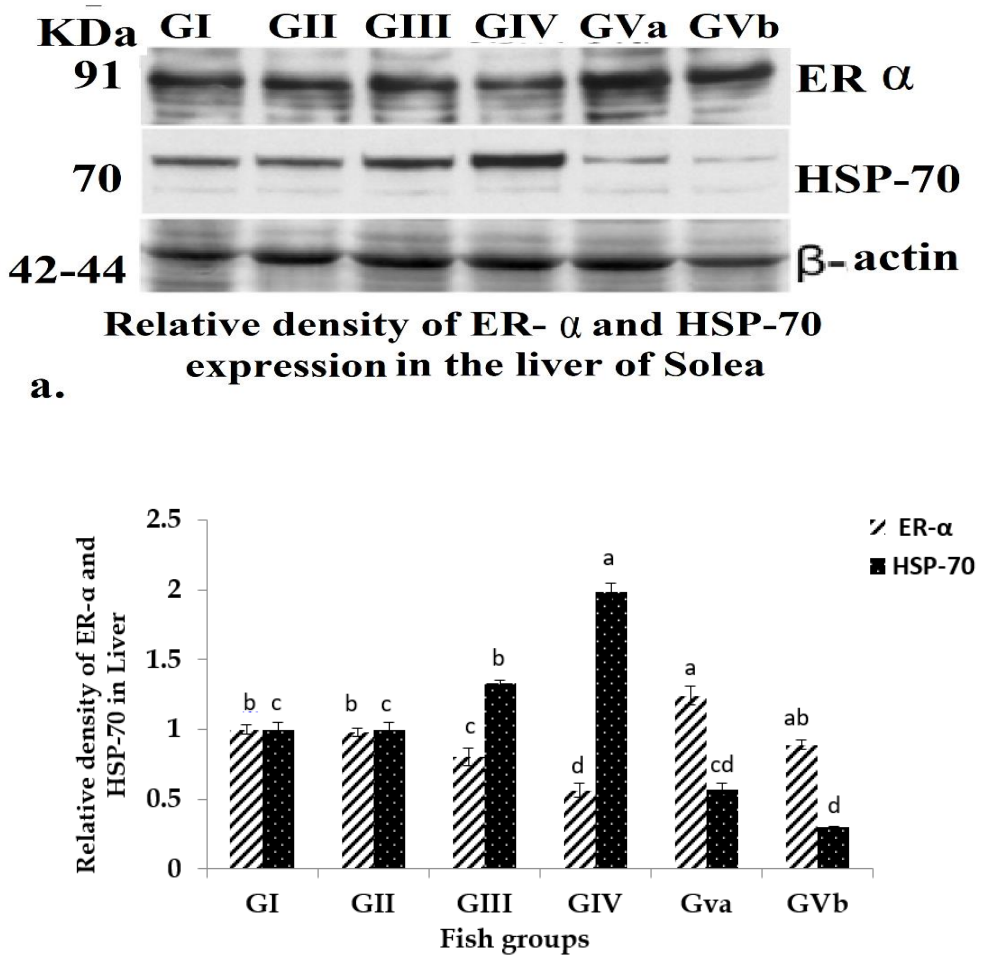


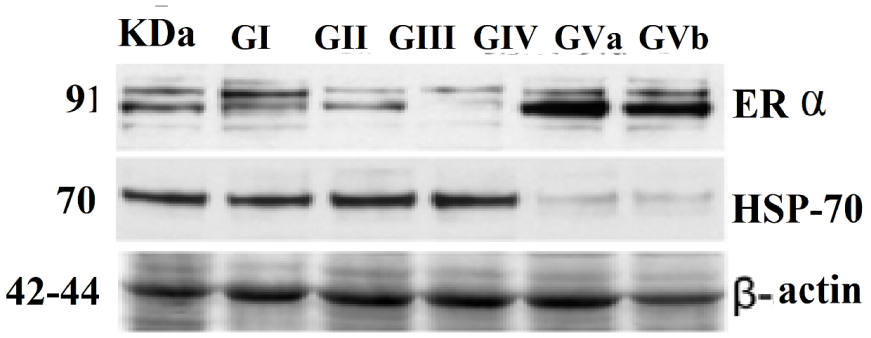
Fig. 4 a) Western blot analysis illustrating, the effect of BaP on ER-α and HSP-70 expression in liver tissues of female Solea. b) The relative density of ″ER-α″ and ″HSP-70″ expression in the liver of ripe and spawning female Solea. GI: Fish exposed to seawater only, GII: Fish exposed to water with ″DMSO″, GIII: Fish exposed to water with low concentration ″BaP″, GIV: Fish exposed to water with high concentration ″BaP″, GVa: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for 24 hrs., GVb: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for one week.

group that was exposed to the low concentration of "BaP" and Cs for 24 hrs. (GVa) showed a significant upregulation by 24% if compared to that of G1. Whereas, the expression level of ER-α protein of the liver of the fish group (GVb) was 22% upregulated compared to fish of G1 (Fig. 4a).

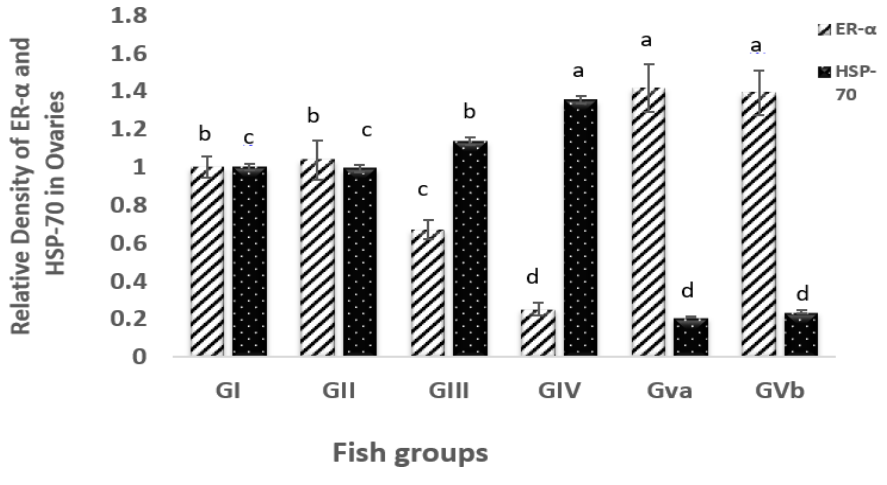
The expression level of liver HSP-70 of fish exposed to DMSO (GII) was found to be similar (99%) to its expression level in the liver of GI. Moreover, fish from the treated group (GIII) exhibited an upregulation in HSP-70 expression level by 33% compared to that of GI fish. While in fish exposed to the high concentration of GIV, there was an upregulation in HSP-70 protein expression level by 98% compared to that of control (GI) as shown in Fig. 4b. However, the expression level of HSP-70 in the liver of fish group (GVa) showed significant downregulation by 43% compared to that of control in GI. The expression level of HSP-70 in the liver of fish group (GV b) revealed a significant downregulation by 70% compared to control (GI) as shown in Fig. 4b.

Ovarian ER-α and HSP-70 protein expression

Results of the present study indicate that exposure to DMSO showed no changes in the expression level of ovarian ER-α protein in GI. The fish group (GIII) exhibited a significant decrease by 33% in ER-α expression level compared to the control group (GI). On the other hand, exposure to high concentrations of "BaP" (GIV) resulted in a substantial decrease in ovarian ER-α expression levels by 75% compared to the control group (Figs.5a,b).



a. Relative density of ER- α and HSP-70 expression in the Ovaries of Solea



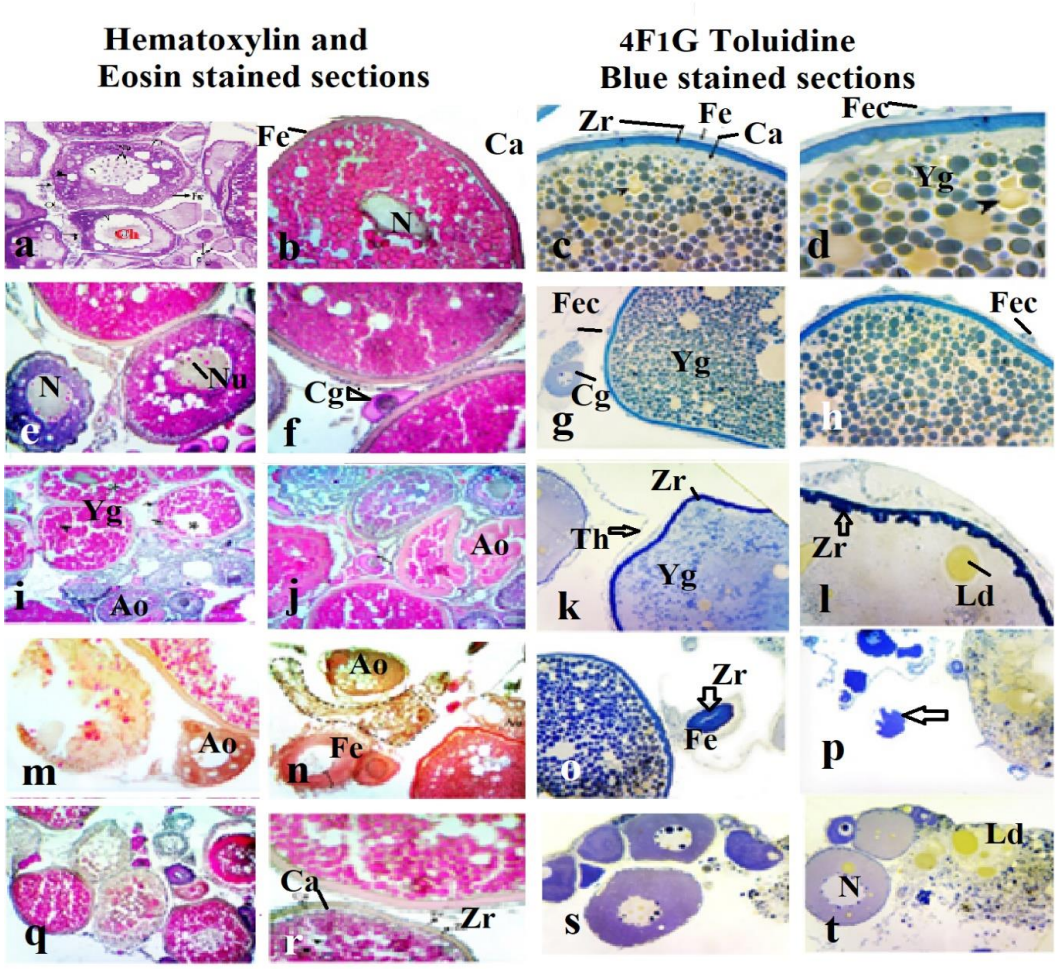
b.

Fig. 5 a. Western blot analysis illustrating, the effect of ″BaP″ on ″ER-α″ and ″HSP-70″ expression in ovarian tissue of ripe and spawning female S. vulgaris. b. Histogram illustrating, the relative density of ER-α and HSP-70 expression in ovarian tissue of ripe and spawning female S. vulgaris among the different fish groups. GI: Fish exposed to seawater only, GII: Fish exposed to water with ″DMSO″, GIII: Fish exposed to water with low concentration ″BaP″, GIV: Fish exposed to water with high concentration ″BaP″, GVa: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for 24 hrs., GVb: Fish exposed to water with low concentration ″BaP″ & ″Cs″ for one week.

While, the fish exposed to low "BaP" concentration and Cs for 24 hrs (GV a) showed a 41% increase in ovarian ER-α expression level compared control (GI). Also, the fish exposed to the same concentration and duration of exposure for a week (GVb) exhibited a 38% up-regulation in ovarian ER-α expression level compared to GI fish (Fig.5 a). The obtained results found that there was a significant variation in the expression level of ovarian HSP-70 among the different groups of fish. Interestingly, the expression level of HSP-70 in the ovary of fish exposed to DMSO (GII) was almost identical to that of control fish (GI). However, fish group (GIII) exhibited an upregulation of 13.5% in the ovarian HSP-70 expression level compared to the control group GI. The fish group (GIV) exhibited an even higher upregulation of 35% in the expression level of ovarian HSP-70 compared to the control fish of seawater (GI). In contrast, the expression level of ovarian HSP-70 of fish group (GVa) showed significant downregulation of 79% compared to that of control fish (GI). Similarly, the expression level of ovarian HSP-70 of fish (GVb) showed a downregulation of 76% compared to control fish, suggesting that the exposure of fish ovarian cells to Cs was time-dependent (Fig.5 b).

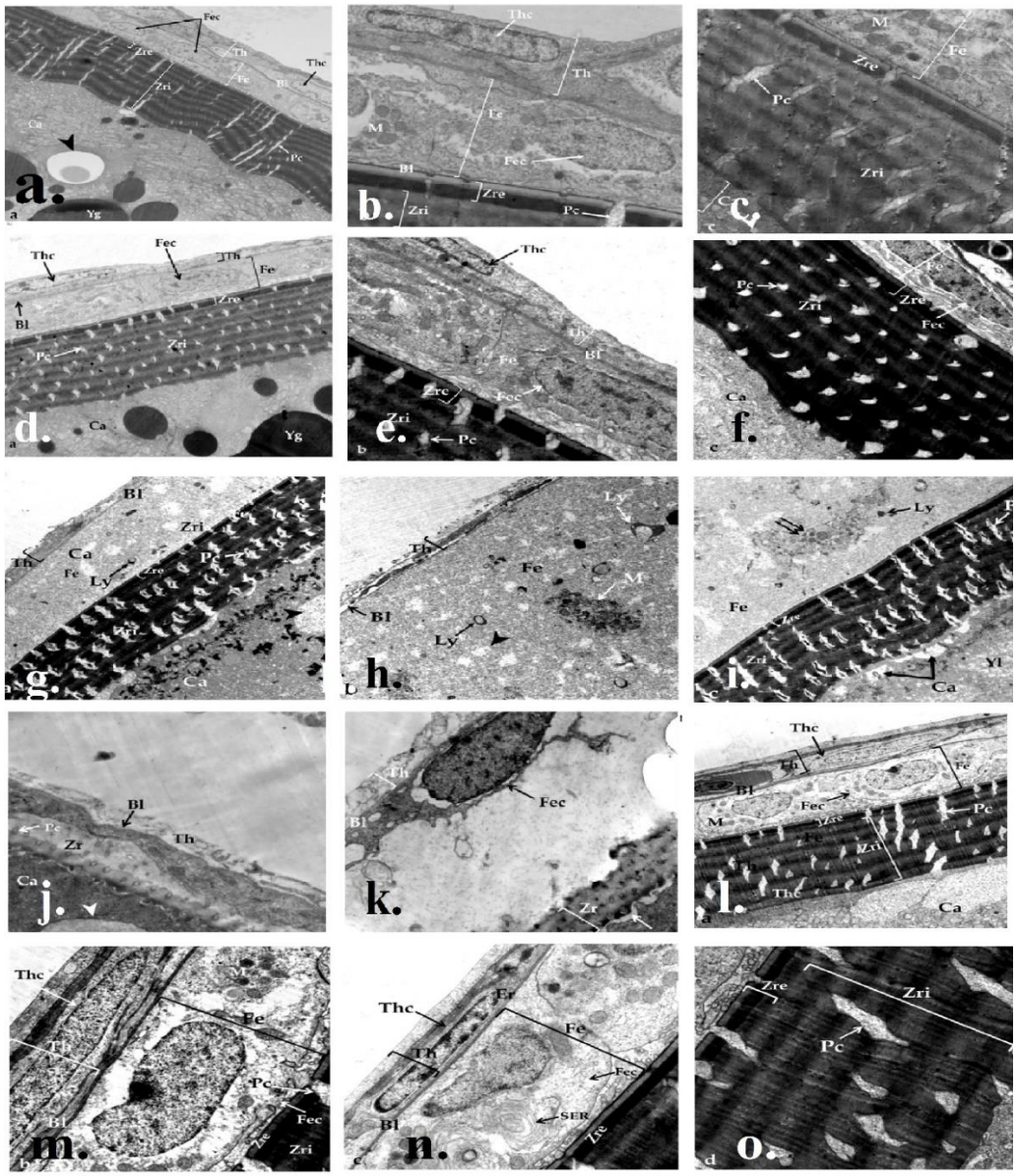
Cellular and subcellular features of ripe and spawning ovaries

First and foremost, it must be pointed out that the cellular and subcellular changes described were restricted only to the oocyte at the tertiary yolk stage of fish ovaries of diverse groups. Light micrographs of ripe oocytes revealed that the oocyte of the control fish (GI) is elongated with regular cellular boundaries and large-size eccentric amoeboid shape (Fig. 6 a,b). Nuclei were displaced towards the animal pole, with a reg-



Figs. 6 (a-t). Photomicrographs of T.S. of the ovaries of Solea at the ripening and spawning stage: a-d) Control fish GI); e-h) Control fish (GII); i-l) Fish exposed to low concentration of BaP (GIII); m-p) Fish exposed to a high BaP concentration (GIV); q-t) Fish exposed to low BaP concentration & Cs″ for a week (GVb). Notice, follicular epithelial cells (Fe), nucleus (N), peripheral dense nucleoli (Nu), follicular epithelial layer (Fe), zona radiata (Zr), cortical alveoli (Ca), yolk globules (Yg), lipid droplets (Ld)) (X 400).(H&E and Toluidine blue stains).

ular nuclear envelope, and contains numerous nucleoli (Nu) located at the periphery. The nucleoli reached 12-15 in number with 3-10 µm scattered in the nucleus (Fig.6b). Toluidine blue semithin sections of this stage (Fig.6 c, d) revealed that the oocytes were coated with a follicular epithelial layer and full of diverse sizes yolk globules (80-111 µm in diameter) interfered with many vacuoles (2-107 µm in diameter). The lipid droplets enlarged and scattered between the yolk granules. LM semithin sections (Fig.6 c, d) showed well-developed cell boundary, normal follicular epithelial cells, zona radiata layer, and cortical alveoli. While, yolk globules (Yg) appeared with less dense and exhibited small diameters and interfered with lipid droplets. Similarly, LM micrograph of the ovary of fish exposed to DMSO (GII) (Fig.6 e-h) revealed no abnormal cellular or subcellular change at different oocyte stages compared to those of their correspond-



Figs. 7 (a-o). Transmission Electron Micrographs of T.S of the ovaries of Solea at the ripening and spawning stage: a-c) Control fish (GI)(a: X 1500, b &c: X4000); d-f) Control (GII) (d: X 1500, e&f: X4000); g-i) fish exposed to low conc, of BaP (GIII) (g: X 1500, h &i: X4000); j, k) fish exposed to a high conc, of BaP (GIV) (j: X 1500, k: X4000); l- o) Fish exposed BaP & Cs for one week (GV b) (l: X 2500, m, n& o: X4000). Notice, the theca layer (Th), squamous theca cells (Thc), with a relatively large amount of cytoplasm, a euchromatic nucleus, follicular epithelial layer (Fe), round mitochondria (M), well-developed SER throughout the cytoplasm of the follicular cells (Fec), lysosomes (Ly), zona radiata (Zr), zona radiata externa (Zre), zona radiata interna (Zri), cortical alveoli (Ca), theca cells (Thc) located peripherally followed by basal lamina (Bl), pore canals (Pc) arranged perpendicularly on zona radiata, yolk globules (Yg) and vacuoles (arrowhead), liquification of yolk (Yl), aggregated organelles (double arrows).[Specimen fixed in $_4F_1G$, post-fixed in OsO_4, and stained with uranyl acetate and lead citrate].

ing control (GI). Additionally, observations revealed altered features of the ripe and spawning ovary of fish group (GIII) (Fig.6 i-l), indicating that all the atretic oocytes were found at the vacuolized, primary, secondary, and tertiary yolk stages. LM examination of the ripe and spawning ovary of fish group (GIV) (Fig. 6 m-p) revealed the presence of atretic oocytes surrounded by an altered wall, disintegrated nucleus, and resorbed cytoplasm. Many atretic oocytes exhibited shrinkage with a disintegrated nucleus and an enlarged follicular epithelial layer, indicating signs of infertility. The oocytes of fish group (GVb) (Figs. 6q- t) showed cellular features similar to those observed in controls.

In TEM preparations, the ripe oocyte is characterized by the presence of five ideal ovarian layers, (Fig. 7a) in which the outermost layer is the theca layer cells that possess flattened nuclei (Fig. 7b). The 2nd layer consists of one row of oval-shaped, follicular epithelial cells provided with oval centrally located nuclei with regular shape outlines, prominent nucleolus and few cytoplasmic organelles including mitochondria. The 3rd and 4th layers are zona radiata externa and interna. The 5th cortical alveoli layer was observed (Figs. 7 a, b, c). Similarly, TEM of the ripe and spawning ovary of GII (Figs. 6 d-f) showed a normal ovarian wall with normal ovarian layers. Most ripe oocytes at the tertiary yolk stage of fish of GIII showed oocytes with deflected, enlarged, or destructed ovarian walls. TEM of the GIII fish group (Figs.7. g- i) showed oocytes bound with a thin theca layer that lacked theca cells, a thin basal lamina enlarged follicular epithelial layer, zona radiata externa, zona radiata interna, and the cortical alveoli. Signs of atresia and infertility and liquefied yolk appeared also in TEM micrographs of GIII. Examination of TEM specimens of fish group GIV showed that the 2nd follicular epithelial layer interfered with the 3rd & and 4th zona radiata layers. Moreover, deformed and shrinked follicular epithelial cells with an amoeboid nucleus, liquefaction of the yolk, and disintegrated organelles were detected (Figs. 7 j, k).

Examination of ripe and spawning oocytes at the tertiary yolk stage of fish group (GVa), showed ideal ripe oocytes with outer follicular epithelial, zona radiata, cortical alveoli, and yolk globules among vacuoles. On the other hand, LM of the ovary of fish (GVb) (Figs. 7 l-o) revealed normal oocytes with ideal layers of the oocyte wall and the beginning of cortical alveoli arrangement. A few atretic oocytes exhibited deformed layers of the ovarian wall represented by deflection in the follicular epithelial and an enlargement in the zona radiata for phagocytosis of oocyte contents. TEM preparations of the female ripe and spawning oocytes of GVb represented ideal oocytes with cytoplasmic organelles, indicating a sign of oocyte activity and growth.

Discussion

In the present study as well as previously reported data (El-Amier et al. 2015; Mostafa and Peters 2016; El Batrawy et al. 2018) El-Maadeya water quality assessment indicated its deterioration compared to the reference site. Water temperature is an essential parameter in the aquatic environment as it affects growth rate, development, activity, reproduction, and susceptibility to diseases. It can also influence fish physiology, so energy consumption and metabolic demands will be affected (Byström et al. 2006). About 35 ºC is considered the maximum tolerance for the survival of aquatic life (Griffith 1993). In the present study, the water temperature in LII =16.48± 0.33ºC Vs./16.01±0.58 ºC in LI. This change may be influenced by variations in air temperature and different sampling times and seasons.

The water hydrogen ion concentrations (pH) play a key role in the biodiversity of fish fauna in the aquatic environment. Data obtained revealed that pH = 8.86± 0.36 in LII Vs./ 7.22 ± 1.06 pH in LI, (i.e., within the optimal range of pH). As pH increases, the proportion of un-ionized ammonia increases, which is toxic to fish while pH 4.0 is the acidic death point (Stone and Thomforde 2004). Moreover, it is known that DO in water is a principal factor for aquatic organisms, since it affects several biological processes. DO in water collected from LII was found to be =3.56 mg/LVs./ 3.91± 1.08 mg/L in samples collected from LII in 2016 (Mostafa and Peters 2016) (i.e., less than the permissible value). This decline in the present study may be due to the discharge of wastewater, high organic pollutants and might be related to sampling during different seasons. Besides, oxygen is vital for fish survival and growth, and as a result, it affects fish respiration and ammonia toxicity. In tilapia species, the minimum DO requirement is 5 mg l^{-1} and respiration and feeding activities decrease when the DO concentration decreases (Mallya 2007). Besides, it was reported that chronic exposure to ammonia is deleterious to marbled spine foot rabbit fish (*Siganus rivulatus)* and low concentrations of ammonia appear to kill the fish in <50 days whilst fish can survive for more than 50 days

at concentrations between 6 and 12 mg/L (Roumieh et al. 2013). Moreover, the mean values of ammonia in LII were found to be = 0.71±0.03 mg/L (exceeding the permissible limits). It is believed that the highest ammonia concentration values recorded during fall and winter may be related to the low water levels, the leaching of fertilizer residues used in agriculture and industrial effluents discharged into the Rosetta branch.

HPLC data analysis in this study showed that El-Maadeya (LII) exhibited 21.07µg/L "BaP" in water and 76.85µg "BaP" /kg in sediment, while Ratama farm (LI) water and sediment revealed its complete absence. Because of their excessive amounts in sediment compared to water, the determination of PAHs is valuable for assessing the potential danger to groundwater by waste materials. In addition, it was found that LII fish accumulated 16.35 µg "BaP" /kg, 15.68 µg "BaP"/kg, and 8.77µg "BaP" /kg in the liver, ovary, and muscles, respectively if compared to their complete absence in corresponding organs of LI fish, confirming data reported by Liang et al. (2007). At the end of the experimental study, a decrease in the level of "BaP" was noticed in tank water for both GIII and GIV. This may be due to its fast absorption by *Solea* and /or due to its fast metabolism by the biotransformation enzymes (Rodrigues et al. 2015). Interestingly, when chitosan was added to water containing 21µg ″BaP″ /L a decrease in "BaP" by a removal percentage = 86.67% after 24 hrs. and 100% after a week was noticed. This indicates that Cs efficiency is time-dependent, and it possesses a powerful effect in a manner that it can eliminate most "BaP" from water confirming data reported by Aranaz et al. (2021) who stated that Cs is utilized as adsorbents of organic trace pollutants from water. Researchers discovered that fish received "BaP" orally or through sediment accumulated in fish tissues in the order: of bile > blood > skin > muscle (Varanasi et al. 1989). Furthermore, it was reported that the hepatobiliary system accumulated higher PAHs and their metabolites than muscle tissues (Liang et al. 2007). These data are in accordance with our data since HPLC revealed that "BaP" accumulates in fish tissues collected from LII exhibited the order: liver > ovaries > muscle.

Histopathological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to pollutants (El-Ghazaly et al. 2017). Micrographs of control *S. solea* ripe oocytes revealed normal architecture with eccentric nuclei near the animal pole and housed several nucleoli arranged peripherally and centrally and had numerous yolk granules and different-sized vacuoles. This coincides with the description of the stages of oocytes of control hybrid red tilapia fish (Eissa et al. 2023).

The atresia that occurs under natural and experimental conditions in the vitellogenic oocyte has been classified as an important indicator of a pathological condition when fish are exposed to contaminants (Corriero et al. 2021). Moreover, signs of atresia have been reported in wild naturally spawned oocytes as a natural process of recycling, i.e., unused, or unspawned oocytes resorbed by phagocytosis between reproductive seasons (Ramsay and Witthames 1996). *S. solea* in late vitellogenesis, ripe, and spawning stages undergo atresia in polluted sites was discovered along the NW Mediterranean fishing grounds (Solé et al. 2016). The current data indicate that the increase in "BaP" concentration increases the incidence of atretic oocytes. Preovulatory atretic follicles have been identified as a key histological feature that shows endocrine disrupting chemicals (EDC) effects on ovarian development and spawning (Leino et al. 2005). Thence, fish exposed to either low or high concentrations of "BaP" revealed increased oocyte atresia in sole, supporting the conclusion that EDC exposure arrests oocyte development, induces oocyte death, and potentially reduces individual reproductive success (Wen and Pan 2015; Ghanem 2021). Similarly, ripe and spawning ovaries of goby (*Gobius niger*) from polluted sites showed a significantly higher follicular atresia percentage compared with the female collected from the unpolluted site (Louiz et al. 2009).The atretic oocytes exhibited an altered ovarian wall with increased ZR at certain sites. Moreover, the breakdown of follicular epithelial layers leads to structural impairment and functional loss (Mlambo et al. 2009). The atresia may be initiated by disturbances in environmental, endocrinological and metabolic factors and may result in reduced fecundity (Massar et al. 2014). In the current study, at the end of atresia, the histological preparations revealed that the ooplasm and its yolky content had completely resorbed and disappeared containing oocytes filled with a loose mass of phagocytes. The apoptosis contributed to follicular atresia in teleost ovaries plays a role in the removal of degenerating oocytes and dying follicular cells (Santos et al. 2008). The current study showed that atresia in female *S. solea* exposed to 21µg "BaP"/L is represented by distinctive features. Similar histological data were previously reported by Louiz et al. (2009) in female ovaries of goby collected from the polluted Bizerta lagoon. The aforementioned authors revealed that the oocytes showed retraction of the cytoplasm from follicular cells, karyoplasm clumping, generating a space between the cytoplasm and karyoplasm, and follicular atresia like the present data.

In the present study, TEM of fish exposed to "BaP" revealed numerous ripe oocytes possessed disrupted walls and deformed layers due to atresia, including disintegration of the outermost theca layer followed by an irregular basal lamina. The follicular epithelial layer interfered with ZR layers which were no longer differentiated and contained altered pore canals. Atretic oocytes in *Solea* in late vitellogenesis and ripe stages collected from polluted areas along the northwest Mediterranean fishing grounds were characterized by the presence of a fragmented ZR and vacuolated and hypertrophied cytoplasm (Solé et al. 2016). Absence and disintegration of the cortical alveoli layer and the theca cells were noted in the present study. Moreover, observations in the fish exposed to 50 µg/L "BaP" agreed with data reported by Miranda et al. (1999) about follicular atresia in female Astyanax *bimaculatus lacustris* and *Leporinus reinhardti* which revealed degeneration characterized by necrosis, dissolution and disappearance of the nucleus, changes in the ooplasm, dissolution of the mitochondria and organelles. Liquification of the yolk appeared to interfere with lipid droplets in the ovarian samples exposed to the "BaP" and disintegrated organelles appearing in the follicular epithelial layer as observed by Miranda et al. (1999). In the present results, ovarian samples of fish exposed to "BaP" displayed deformed and shrunken follicular epithelial cells with an amoeboid nucleus and heterochromatin clumps as opposed to the nuclear envelope. Damaged mitochondria were observed in many cells of the oocytes indicating the stress associated with the presence of toxic agents, which in turn generate free radicals causing molecular and cellular damage, making the tissues highly susceptible to other toxic agents (Massar et al. 2014). In TEM micrographs, follicular epithelial cells in the oocytes of fish exposed to 50 µg/L "BaP" appeared with ruminants of aggregated mitochondria in some samples and seemed to disintegrate in other ones. Furthermore, mitochondria seemed to be less electron-dense materials as compared with control, distorted and condensed in some oocytes. Likewise, the follicular lining lost its shape; it no longer enclosed the follicle and many empty follicles were observed as reported by Massar et al. (2014). Other ultrastructural preparations showed the absence of follicular lining in some places of the oocytes, possessing a few yolk globules and vacuoles in the vitellogenin oocytes (Booc et al. 2014).

The current study is the first to focus on fish reproduction disturbance and diverse types of gonadal lesions in *S. solea* due to "BaP" exposure. "BaP" detoxification in fish involves the action of microsomal monooxygenases, especially from the cytochrome p450 and GST cytosolic enzymes (González et al. 2020), which are extensively used as effective biomarkers for "BaP" in fish (Rodrigues et al. 2015). "BaP" is a potent inducer of the 1A isoform of cytochrome p450 and GST in the Nile tilapia fish and plays a pivotal role in the metabolism of xenobiotics in fish (Yildirim et al. 2014). Important to point out that the present results showed that p450 aromatase activity exhibited a significant difference among groups. Various EDCs are known to mimic sex hormones and/or disrupt steroidogenic enzyme function, including aromatase and consequently, they can alter normal reproductive function in wildlife (Ghanem 2021). The effects of "BaP" exposure on *Fundulus heteroclitus* fish led to significant increases in the activities of phase I-type xenobiotic-metabolizing enzymes (Nacci et al. 2002). The inhibition of p450 enzyme activity by estrogens is linked to carcinogenesis and endocrine disruption in fish because of the bioaccumulation of procarcinogens and estrogen in the body (Celander 2011). Furthermore, it was reported that GST proteins play roles in normal cellular metabolism, the detoxification of xenobiotics (Yildirim et al. 2014) and served as a marker indicative of phase II metabolism (Rodrigues et al. 2015). In the present work, the significant decrease in the GST means value indicate that the low concentration of "BaP" suppressed the GST activity in the Solea liver detected. Evidence demonstrated that the transcriptional levels of GST-A and M-GST were inhibited significantly in the liver of the *Japanese medaka* exposed to "BaP" (Song et al. 2015). The absence of induction of GST activity explains that it is not a suitable indicator of contamination in the common sole, though it is frequently used as an ecotoxicology biomarker enzyme and has low sensitivity to PAHs (Trisciani et al. 2011). Moreover, the present results showed that phase I biomarkers (p450 aromatase) were more sensitive than phase II biomarkers (GST). While, GST biomarkers evaluated on various fish species have proved to be less dependable as an indicator of contamination (Van der Oost et al. 2003).

In the present study, fish exposed to "BaP" exhibited an upregulation of HSP70 in hepatic tissue compared to that of the control, suggesting that its higher levels may be considered an adaptive strategy to maintain native protein structures under environmental stress (Yusof et al. 2022). Therefore, sufficient HSP concentrations is crucial to cope satisfactorily with the levels of aberrant proteins generated by any given environmental stress. The present results are in accordance with Voznesensky et al. (2004), who mentioned that induction of HSP-70 mRNA and synthesis of HSP-70 protein in response to stressors have been docu-

mented in marine organisms. Moreover, the high expression of HSP-70 in the liver of *Solea* agrees with the results of white mullet (*Mugil curema*) presented in the three coastal lagoon systems receiving contaminants derived from local anthropogenic activities with high expression of liver CYP1A and HSP-70 (Ros-Sicairos et al. 2010). Additionally, a downregulation in the expression of HSP-70 in the solea liver was detected in fish groups exposed to "BaP" and chitosan. This indicate that exposure of hepatic tissue to Cs was time-dependent. Furthermore, after a week of Cs addition, the removal of "BaP" % by Cs in water was 100%, indicating that Cs have a powerful effect in eliminating most of the BaP from water.

The expression of HSP-70 in the ovarian tissue and the liver varied, which may be due to HSPs being hypothesized to play a role in metabolic activity and oocyte survival (Neuer et al. 1999). This increase may have occurred due to "BaP" exposure since HSP-70 is a highly conserved, ubiquitous cellular protein that acts as a molecular chaperone (Peng et al. 2000). Furthermore, it was mentioned that HSP-70 is associated with sex steroid receptors and participates in the modulation of steroid receptor function, which is important in ovarian physiology, particularly in follicular development and is related to fertility (Velázquez et al. 2011). Elevated levels of various HSP have been found in the tissues of fish exposed to contaminants such as PAH as in the study of Basu et al. (2002) thus, agree with the present results. However, HSP expression in the ovarian tissue of fish exposed to 21µg/L "BaP" and Cs for 24 hrs or one week revealed a downregulation compared to that of the control, which may be due to the inhibition effect of the Cs on "BaP" in time-dependent mode. Besides, these data were in accordance with another investigation concerning the effects of incorporating hydrolysed shrimp shell chitin into the diet of hybrid tilapia (Qin et al. 2014). All together reporting that HSP-70 is a useful biomarker for pollutants and that chitosan can improve fish health and points to a good relationship between HSP-70 expression and chitosan effect.

Estrogens (ER) are crucial during ovarian development, the production of gametes, and regulating female reproductive behaviours. Consistent with the current findings, female zebrafish were found to have increased hepatic ER and ER- gene expression after long-term exposure to low concentrations of tris (1,3-dichloro-2-propyl) phosphate (TDCPP) (an EDC), which could account for the increased 17-estradiol (E_2) levels affecting fish reproduction (Wang et al. 2015). In this respect, TDCPP can function as an ER agonist and induce estrogenic activity *in vitro* (Zhang et al. 2014). Therefore, it is believed that since "BaP" (EDC) can function as an ER agonist, since it can induce estrogenic activity either by working on ERs to motivate vitellogenin "VTG" gene transcription and synthesis in the liver or by increasing estradiol levels in the blood (Wang et al. 2015). q-PCR revealed a significant increase in ER gene expression and ER-mRNA transcription levels in sea bream (*Sparus aurata*) juveniles exposed to contaminants (Ribecco et al. 2011). It is known that the increase in the gene expression of ER in fish exposed to contaminants is considered a concise indicator of endocrine disruption. Furthermore, the current findings are consistent with other studies that investigated the effect of short-term "BaP" exposure on crabs (*Portunus trituberculatus*) in which elevated levels of "BaP" suppressed ER expression because it interferes with the ubiquitin-proteasome pathway, affecting ER and ER-mediated gene expression (Wen and Pan 2015).

In the present work, the use of chitosan helps in the recovery of the expression of ER- in the ovary to its basal level after the exposure of fish to the "BaP". Furthermore, the downregulation of ER- expression was found to be more pronounced in the ovary than in hepatic tissue due to the sensitivity of ovarian tissue to "BaP" exposure. In addition, it has been widely demonstrated that vitellogenin mRNA and protein expression are increased following exposure to environmental estrogens (Zhang and Zhou 2005). Moreover, it is believed that ER transcription may be due to "BaP" metabolites rather than the parent compound (Hoffmann and Oris 2006). One mechanism for "BaP"-induced reproductive toxicity in fish is explained by the binding of "BaP" metabolites to the estrogen receptor, which induces the expression of estrogen-responsive genes such as ER and vitellogenin (Hoffmann and Oris 2006). In the current study, the expression of ovarian ER- of fish exposed to "BaP" was also found to be lower than in control fish. Since "BaP" is an environmental endocrine disruptor, it can act through estrogenic or anti-estrogenic mechanisms (Patel et al. 2006). Moreover, the downregulation in the levels of ER protein expression noticed in the current study may be due to CYP1A induction, which might increase the metabolism of estrogens, suggesting another potential mechanism for endocrine disruption. It was suggested that EDCs may alter sex hormone levels through gene expression in the HPG axis and disrupt hormone homeostasis, which further alters gonadal development. Consequently, "BaP" may affect ovarian development and reproduction in this respect (Malott et al. 2022).

Conclusions

It is important to consider the potential impact of exposure to "BaP" on fish reproduction. The current data revealed that "BaP" may disrupt the steroid homeostasis and lead to reproductive deficits in fish by affecting the activity of CYP1A and steroidogenic enzymes. Additionally, the protein expression levels of HSP-70 and ER in *S. solea* may be affected leading to a negative impact on *Solea* reproduction. Moreover, the present research indicates that the fish under study is a tolerant species to changes in physicochemical parameters and elevated ″BaP″ concentration in water and sediments of El-Maadeya. Furthermore, chitosan possess a powerful efficiency against toxicity of ″BaP″ since it plays a role in elimination of ″BaP″ by fast adsorption from water and sediment placed at the bottom of tank in time- dependent manner. Moreover, chitosan's addition resulted in improvement in fish health, increased the ovarian weight to normal, succeeded to decrease the percentage of atretic oocytes in ovary in a time- dependent manner and reduced fish mortality.

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Authors' contributions: NEA, and SFG: conceptualization, formal analysis; NEA, ASS; supervision. NEA, ASS SSA, NM; resources and investigation. NEA, SFG, SSE, ASS, RF: methodology. SFG, ASS; data curation, writing-original draft, conduction and supervision the molecular data. NEA: validation and writing-review and editing. All authors have read and approved the final version of the manuscript. All authors read and approved the final manuscript.

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