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1 **Effects of Benzo[a]pyrene on DNA Damage and Histological**

2 **alterations in Gonad of Scallop *Chlamys farreri***

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6

7 **Abstract**

8 To investigate the biological impact of polycyclic aromatic hydrocarbons (PAHs) on
9 reproductive system, scallops *Chlamys farreri* were continuously exposed to benzo[a]pyrene
10 (BaP) (0.5, 3, 10 $\mu\text{g L}^{-1}$) during 15 days. DNA damage and histological alterations were
11 examined in female gonad. DNA strand break levels significantly increased after 12 h
12 exposure, and remained high and significantly different from control values until the end of
13 the exposure. In the ovaries of the scallops exposed to 10 $\mu\text{g L}^{-1}$ BaP for 10 days, histological
14 analysis showed that the cytoplasts of the oocytes in the outer layer of the ovaries became
15 sparse, and the nuclear membranes were obscure. Damaging effects on ovaries and oocytes
16 were more severe after 15 days exposure. Degenerating oocytes increased and the connective
17 tissue of the ovary envelopes became loose. Electron microscopic examination revealed
18 ultrastructural aspects of degenerating oocyte and degenerated oocyte after 15 days exposure.

19 **Keywords:** Benzo[a]pyrene; Scallop; DNA strand break; Genotoxicity; Endocrine;

20 Histopathology; Ultrastructure; Gonad; Oocyte

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21 1. Introduction

22 In recent years, special attention has been given to the disruption of reproduction by
23 organic compounds and the possible consequences on wildlife populations, because
24 reproductive systems appear to be particularly vulnerable to environmental contaminants
25 (Harries et al., 1997; Kovacs et al., 1997). Several studies have demonstrated that endocrine
26 disturbances due to xenobiotics in wildlife include sex changes in marine gastropods
27 (Horiguchi et al., 2002), delay in oocyte maturation and sperm release and perturbation of the
28 neuroendocrine control of spawning in mussels (Binelli et al., 2004; Gagné et al., 2004). Field
29 observations of reproductively abnormal organisms and populations decline in polluted sites
30 stimulated major research efforts to understand stressor-induced changes of some of the
31 reproductive parameters within an organism that could be valuable as early warning indicators
32 of the potential long-term hazard of environmental deterioration at the population level
33 (Thomas, 1990).

34 Bivalves are particularly at risk to aquatic xenobiotic chemicals because they are
35 stationary and filter relatively high volume of water, including suspended particles. Many
36 different effects have been recorded in bivalves exposed to chemical contaminants. Among
37 them, histological alterations have been widely employed as a method of assessing the
38 disruption of reproduction systems (Ketata et al., 2008). Binelli et al. (2004) reported
39 degenerating oocytes in relation to spawning delay in the zebra mussel (*Dreissena*
40 *polymorpha*) exposed to DDT. Numerous degenerating ovarian follicles and atretic oocytes
41 have also been observed in female mussels (*Mytilus edulis*) after exposure to bisphenol A
42 ($50\mu\text{g L}^{-1}$), BDE-47 ($1\mu\text{g L}^{-1}$), North Sea oil (0.5 mg L^{-1}) and a mixture of North Sea oil (0.5

43 mg L⁻¹), alkyphenols (0.1 mg L⁻¹) and polycyclic aromatic hydrocarbons (PAHs) (0.1 mg L⁻¹)
44 for three weeks (Aarab et al., 2004; Aarab et al., 2006). These results indicated dramatic toxic
45 effects on reproductive tissues in relation to environmental toxicants. PAHs, owing to their
46 highly mutagenic and carcinogenic features and widespread distributions in the global
47 environment, are considered as one of the major pollutants affecting marine organisms
48 (Menzie et al., 1992). In addition to genetic toxicity, PAHs are also listed by the US
49 Environmental Protection Agency (EPA) as compounds capable of disrupting the endocrine
50 system of animals. However, few laboratory studies of PAHs toxicity on the reproductive
51 system of bivalves have been carried out.

52 In the present study we used benzo[*a*]pyrene (BaP), a powerful carcinogen that is
53 included in the 16 PAHs designated as primary contaminants, as a representative of PAHs. We
54 investigated the female gonad damage induced by BaP exposure, including DNA damage and
55 histological perturbations in Zhikong scallop *Chlamys farreri*. The objective of our work was
56 to determine the basic characteristics of the endpoints of genotoxic effects and endocrine
57 disruption of BaP, and to compare the temporal and dose response relationship between BaP
58 exposure and the toxic effects. The study may provide preliminary information on evaluation
59 of gonadal status under the stress of PAHs.

60

61 **2. Materials and methods**

62 *2.1 Scallops*

63 Scallops (*C. farreri*) used for this experiment were rope-cultivated scallops, aged 2 years
64 and with shell length of 5.8±0.8 cm, collected from Taiping Bay (Huanghai Sea, Qingdao,

65 China) on April 10th, 2006. The sunken ventral portion of the anterior ear of the right shell of
66 the scallop *C. farreri* provides an aperture when the two shells are closed. Female scallops
67 were identified after gently stimulating the aperture of the scallop to make it gape widely
68 using forceps, and observing the colour of the gonad. Mature female scallops have a red
69 gonad, whereas a male gonad is white. Female scallops held in aquaria (1 L water per scallop)
70 were acclimated to laboratory conditions for 7 days in filtered seawater pumped from Taiping
71 Bay. The sea water was continuously aerated, and salinity, temperature and pH were
72 respectively maintained at 31‰, 17(±1)°C and 8.1. The water was renewed every 24 hours.
73 The scallops were fed daily with dried powder of *Spirulina platensis* (30 mg for each
74 individual).

75 2.2 Experimental design

76 The background concentration of BaP in seawater from Taiping Bay was analysed by HPLC
77 before the experiment.. The effects of three waterborne BaP concentrations were studied (0.5, 3
78 and 10 µg L⁻¹). BaP was first dissolved in acetone, and then added to seawater to achieve final
79 acetone concentration of 0.0025%. Control groups included a seawater control and an acetone
80 control. There were three aquaria used as replicates per group. Experimental conditions (salinity,
81 pH, temperature, scallop density and feeding) were the same as those used for acclimation, and the
82 exposure media were renewed daily. The exposure experiment lasted for 15 days. During this
83 period, we determined the BaP concentration of the exposed groups every day before renewing the
84 water during the experiments. The analyzed BaP concentrations of each group were 0.52±0.07 µg
85 L⁻¹, 2.93±0.13 µg L⁻¹, 9.15±0.11 µg L⁻¹. Female gonads of scallops were excised and frozen
86 immediately at -80°C at days 0, 0.5, 1, 3, 6, 10 and 15 for the subsequent examination of DNA

87 integrity, two to three scallops were sampled per replicate. Female gonads from sea water control,
88 acetone control and $10 \mu\text{g L}^{-1}$ BaP treatment groups were sampled at day 10 and day 15 for
89 histological evaluation, and sampled for ultrastructural evaluation at day 15. Gonad tissues from
90 six scallops were sampled as 6 replicates. No mortalities were observed during the exposure
91 experiment.

92 *2.3 Alkaline unwinding assay*

93 DNA was extracted from female gonads by mashing the tissues from two to three scallops
94 with plastic sticks and homogenizing the tissue in 3 mL TE buffer (50 mM Tris, 100 mM
95 EDTA, pH 8.0). 0.45 mL lysate was transferred into 1.5 mL Eppendorf tubes. Then 0.05 mL
96 10% SDS (Sodium dodecyl sulfate) and $3 \mu\text{L } 20 \text{ g L}^{-1}$ proteinase K were added to it and the
97 mixture was incubated for 5 h at 55°C . An equal volume of buffered
98 phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v/v/v, pH 8.0) was then added to the
99 sample. The sample was gently mixed for 15 min and then centrifuged for 10 min at 13 000
100 rpm at 4°C . The aqueous layer was transferred to a new tube and digested with $0.8 \mu\text{L}$ Rnase
101 (10 mg mL^{-1}) for 30 min at 37°C and the digesta was extracted twice with PCI. DNA was
102 precipitated from the resulting aqueous layer by adding 2 volumes of absolute ethanol and
103 1/10 volume of 3 M sodium acetate, pH 5.2. The samples were allowed to settle for 2 h at
104 -20°C , and then centrifuged for 15 min at 13 000 rpm. The resulting pellet was rinsed with 1
105 mL 70% ethanol and air-dried, then dissolved in 1 mL of TE buffer (10 mM Tris, 1 mM
106 EDTA, pH 8.0). The amount of DNA was quantified using a UV/Visible spectrophotometer
107 (Ultraspec 2100 pro, Amersham Biosciences, Sweden).

108 The alkaline unwinding assay used in the study was adapted from Ching et al (2001). In

109 the assay, the rate of transition of double-stranded DNA (dsDNA) to single-stranded DNA
110 (ssDNA) under pre-defined alkaline denaturing conditions was proportional to the number of
111 breaks in the phosphodiester backbone, and thus was used as a measure of DNA integrity
112 (Daniel et al., 1985). The amounts of various types of DNA were quantified by measuring the
113 varying degrees of fluorescence resulting from a DNA-binding dye, bisbenzamide. After
114 reaction with the dye, the fluorescence of dsDNA is double that of the ssDNA (Cesarone et al.,
115 1979).

116 The DNA sample was diluted and divided into three equal portions for fluorescence
117 determination of dsDNA, ssDNA and alkaline unwound DNA (auDNA). The fluorescence of
118 the initial DNA or dsDNA was determined by placing 100 μL of the DNA sample, 100 μL of
119 25 mM NaCl and 2 μL of 0.5% SDS in a pre-chilled test tube, followed by the addition of 3
120 mL 0.2 M potassium phosphate (pH 6.9), and 3 μL bisbenzamide (1 mg mL⁻¹). The contents
121 were mixed and allowed to react in darkness for 15 min. The fluorescence of the sample was
122 measured using a spectrofluorimeter (Molecular Spectroscopy LS 55 from Instruments, P.E.,
123 MA, USA) with an excitation wavelength of 360 nm and an emission wavelength of 450 nm.
124 The fluorescence of ssDNA was determined as above but using a DNA sample that had
125 already been boiled at 80°C for 30 min to completely unwind the DNA. auDNA samples were
126 made by subjecting initial DNA samples to alkaline treatment, i.e. 50 μL of 50 mM NaOH
127 was rapidly mixed with 100 μL of DNA sample in a pre-chilled test tube and incubated on ice
128 in darkness for 30 min, followed by a rapid addition and mixing of 50 μL of 50 mM HCl and
129 2 μL of 0.5% SDS then the mixture was forcefully passed through a 21G needle several times.
130 The fluorescence of the alkaline unwound DNA sample was measured as described above.

131 The ratio between double-stranded DNA to total DNA (F value) was determined as

132 follows: F value = $(\text{auDNA} - \text{ssDNA}) / (\text{dsDNA} - \text{ssDNA})$

133 *2.4 Histological evaluation*

134 The female gonads were excised and fixed with Bouin's fixative for 18 hours. Following
135 dehydration through an ascending ethanol series, the tissues were embedded in paraffin,
136 sectioned at 5 μm , stained with Ehrlich's haematoxylin and eosin (H&E), and observed with
137 an Olympus BX-41 microscope (Olympus, Japan).

138 For transmission electron microscopy (TEM), tissues were prepared as follows: selected
139 female gonads of scallops were cut at 1 mm^3 , fixed in 2.5% glutaraldehyde in phosphate
140 buffer (pH 7.2) at 4°C, postfixed in 1% osmium tetroxide, dehydrated in an ethanol series,
141 and embedded in Epon 812. Ultrathin sections were cut on an LKB ultramicrotome, stained
142 with uranyl acetate and lead citrate. Sections were examined in a Hitachi H-7000 transmission
143 electron microscope (Hitachi, Japan).

144 For quantifying the oocyte degeneration, the degenerating oocytes were counted in the
145 histological sections of each ovarian piece. The number of the degenerating oocytes was
146 determined in a 90,000 μm^2 field at a magnification of x400.

147 *2.5 Statistical analysis*

148 All values were expressed as mean \pm SD of three replicates in one representative
149 experiment. Experiments were performed three times to confirm the results. The analyses
150 were carried out using SPSS software (Version 11.5). Data were compared with one-way
151 ANOVA, and Newman-Keuls posthoc test when appropriate. The α level used was 0.05. All
152 data were checked for normality and homogeneity of the variances, and transformed when

153 necessary.

154 3. Results

155 3.1 DNA strand breaks

156 The results of mean F values of each individual treatment group are presented in Figure
157 1. No significant difference in F values was observed over the 15-day exposure period for the
158 control and acetone control groups ($P > 0.05$). F values of control and acetone control groups,
159 respectively, were 0.640 ± 0.084 and 0.633 ± 0.050 . F values of samples from 3 and $10 \mu\text{g L}^{-1}$
160 treatment groups showed marked decreases (0.516 ± 0.014 and 0.462 ± 0.063 , respectively) (P
161 < 0.05) after 12 h of exposure. While the $0.5 \mu\text{g L}^{-1}$ BaP treatment groups showed marked
162 decreases in mean F values (0.495 ± 0.074 , $P < 0.05$) after 1 day exposure. The F values
163 remained low and significantly different from the control values ($P < 0.05$) until day 15 for the
164 $0.5 \mu\text{g L}^{-1}$ (0.369 ± 0.024), $3 \mu\text{g L}^{-1}$ (0.259 ± 0.035) and $10 \mu\text{g L}^{-1}$ (0.270 ± 0.020) BaP treatment
165 groups. There were no significant differences between the F values of $3 \mu\text{g L}^{-1}$ and $10 \mu\text{g L}^{-1}$
166 BaP exposed groups from day 6 to day 15 ($P > 0.05$).

167 3.2 Histological evaluation

168 Histological and ultrastructural studies of female gonads from both seawater control and
169 acetone control scallops showed the same histological features. The female gonad
170 development of *C. farreri* can be divided into five stages: proliferation, growth, maturation,
171 spawning and recovery. Histological sections of female gonad displayed numerous ovarian
172 follicles filled with mature oocytes, the interspace among follicles disappeared, indicating that
173 the female gonads of scallops were at the late pre-spawning stage (Figure 2a, 2b). Electron
174 microscopic examination showed that most of the oocytes were at the vitellogenic stage.

175 Oolemma of oocytes formed microvilli, and the oocytes' ooplasm was rich in mitochondria,
176 cisternae of rough surfaced endoplasmic reticulum and ribosomes, vast amounts of yolk
177 bodies and cortical granules. Nucleoli were visible in nucleus, nuclear membrane
178 protuberated at some site, and the euchromatins uniformly distributed in nucleus (Figure 3a,
179 3b).

180 Histological analysis of the ovaries indicated that after 10 and 15 days exposure of $10 \mu\text{g}$
181 L^{-1} BaP degenerating oocytes increased especially in the outer layer of the ovaries. The
182 degenerating oocytes appeared irregular or polyhedric, and were deformed by compression of
183 the oocyte (Figure 2c, 2d, 3c, 3f). Cytoplasts of the degenerating oocytes were sparse, and the
184 nuclear membranes were obscure compared with the control. More severe damaging effects
185 on ovaries and oocytes were observed after 15 days exposure. Degenerating oocytes increased,
186 meanwhile the connective tissue of the ovary envelops became loose in most of the ovaries
187 observed (Figure 2c, 2d). Oocyte count indicated that degenerating oocytes increased
188 significantly ($P < 0.05$) after 10 days and 15 days exposure compared with control (Table 1).
189 Ultrastructural sections of female gonad from $10 \mu\text{g L}^{-1}$ BaP treatment group after 15 days
190 exposure displayed degenerating oocyte and degenerated oocyte. The degenerating and
191 degenerated oocytes appeared irregular or polyhedric, and were deformed by compression of
192 the oocyte (Figure 2c, 2d, 3c, 3f). During the gradual disintegration of the oocytes, numerous
193 vacuoles appeared in the ooplasm of oocytes. A few lysosomes (phagosomes) and vacuoles
194 also appeared in the cytoplasm of the follicle cells which were attached to the degenerating
195 oocyte. (Figure 3c, 3d, 3e). In degenerated oocytes, endoplasmic reticulum, mitochondria, and
196 the quantity of yolk bodies decreased, resulting in the electron density reduction of the

197 ooplasm. The microvilli were shed and numerous glycogen particles occurred in cytoplasm.
198 Nuclear membranes were anomalous, and the distribution of euchromatins in nucleus was not
199 uniform (Figure 3f).

200 **4. Discussion**

201 BaP is a genotoxic agent capable of binding to DNA via CYP transformation to arene
202 oxides, forming DNA adducts that are more effective sites for the production of strand breaks.
203 It may also increase the presence of reactive oxygen species (ROS) via redox cycling, which
204 can be responsible for lipid peroxidation processes and result in DNA strand break eventually
205 (Martinez and Livingstone, 1995; Canova et al., 1998). The micronucleus frequency observed
206 for adult oysters *Crassostrea gigas* contaminated for 48 h with BaP (0.05, 0.5, 1 and 500 μg
207 L^{-1}) showed the clastogenic action of BaP and a dose dependent effect. Siu et al. (2003, 2004)
208 and Ching et al. (2001) studied BaP (0.3, 3, 30 $\mu\text{g L}^{-1}$) effects on DNA strand breaks of
209 haemocytes and hepatopancreas in mussels *Perna viridis*, and the results suggested that the
210 response of DNA alteration upon BaP exposure may be tissue-specific and threshold
211 dependent in the species. In a previous study, Pan et al. (2007) reported that the *F* values in
212 gill and digestive gland of 0.5 and 3 $\mu\text{g L}^{-1}$ BaP treatment groups decreased until day 6 and
213 then showed a recovery. This was considered to be due to an early elicitation of the DNA
214 repair system. While, in this study, DNA strand breaks of gonads from 0.5, 3 and 10 $\mu\text{g L}^{-1}$
215 treatment groups showed marked decrease after 12 h or 1 day exposure, and no recoveries
216 appeared until the end, indicating a decreased capacity for DNA repair activity. The result
217 corresponds with the findings of Gagné et al. (2004) which suggests that ripe mussels whose
218 oocytes were meiose-arrested might have reduced DNA synthesis/repair activity during the

219 mature stage. However, further studies about the relationship between DNA repair activity of
220 oocytes and the gonad mature status are needed. In addition, a dose-dependent relationship
221 was observed between the DNA strand break levels and the BaP exposure dose (from 0.5 to 3
222 $\mu\text{g L}^{-1}$), while the DNA strand break levels had no marked difference between 3 and 10 $\mu\text{g L}^{-1}$
223 BaP from day 6 to day 15, indicating that the toxic effects on DNA in gonads may be
224 threshold dependent.

225 Microscopic observations in the present work showed time course of the BaP toxic
226 effects towards ovaries of scallops. During the 15 days exposure, degenerating oocytes
227 significantly increased in ovaries (Table 1). Ultrastructure of the ovaries was studied to find
228 out more detailed effects. Results indicated that oocytes were at the vitellogenesis stage and
229 yolk granules were accumulating. After 15 days exposure, degenerating oocytes increased in
230 the treatment group and ultrastructural sections displayed degenerated oocytes with reduced
231 density of the ooplasm and yolk granules, which could be the reason for the obscure nuclear
232 membrane observed under the microscopic. As a result of reductions in yolk bodies and cell
233 organelles in degenerated oocytes, and numerous glycogen particles appeared in cytoplasm,
234 there was a reduction in the electron density of the ooplasm, and the chromatin distributed
235 around the nuclear membrane. Consequently, the electron density of cytoplasm and nucleus
236 became similar and made the nuclear membrane appear obscure (Figure 2c, 2d, 3f). Oocyte
237 degeneration is a continuous process throughout the development of oocyte, and it seems to
238 be most important for vitellogenic oocytes (Gaulejac et al., 1995; Chung et al., 2007).
239 Degenerated oocytes may be observed in the ovaries amongst the normal oocytes at any stage
240 of development. It may be caused by overproduction of oocytes in spawners as a result of

241 environmental stress (Leino and Maccormik, 1997). Oocyte degeneration due to xenobiotic
242 and heavy metal contamination was reported in several bivalve species (Maung and Tyler,
243 1982; Rasmussen et al., 1983). Lowe and Pipe reported a high level of gamete atresia in *M.*
244 *edulis* treated with hydrocarbons, and presumed that the degeneration was due to the direct
245 action of these chemicals on gametes by the destabilization of lysosomes and yolk granules
246 which contain lysosomal enzymes (1986). The pathological effects in gill and digestive gland
247 of *C. farreri* exposed to $10 \mu\text{g L}^{-1}$ BaP were also reported (Miao et al., 2007). Considering the
248 results of DNA strand break in this work, it was suggested that the oocyte degeneration can be
249 considered to be subsequences of the effect of BaP reactive intermediates on the
250 biomacromolecules, which caused DNA, protein and the cell membrane system damages.

251 On the other hand, BaP is also a compound capable of disrupting the endocrine system of
252 animals. Fong et al. (1994) observed that methiothepin, a serotonin (5-hydroxytryptamine,
253 5HT) receptor antagonist, caused oocyte degeneration, with morphological aspects similar to
254 those observed in this work. Degenerating oocytes in relation to spawning delay have also
255 been reported in association with endocrine disruption induced by DDT in zebra mussel
256 *Dreissena polymorpha* (Binelli et al., 2004). In our results, ultrastructure of the ovaries was
257 studied to find out more detailed effects. The ultrastructure characteristics of degenerating and
258 degenerated oocytes displayed the same aspects with the natural interdigitation phenomena
259 reported in the marine bivalve *Pina nobilis* (Gaulejac et al., 1995) and *Cyclina sinensis*
260 (Chung et al., 2007). Meanwhile, it was reported in *C. sinensis* that follicle cells appear to
261 play an integral role in oocyte degeneration (Chung et al., 2007), which were also observed as
262 a common phenomenon in our results (Figure 3d). Furthermore the ultrastructure observation

263 indicated that quantity of yolk granules in exposed samples decreased significantly during the
264 oocyte degenerating which suggested that oocyte degeneration caused by PAHs exposure
265 could be a reason for vitellogenin-like protein levels variation reported in mussels *M. edulis*
266 and *D. polymorpha* (Aarab et al., 2004; Lafontaine et al., 2000). A long-term and low-dose
267 effect of PAHs needs to be further studied to examine the endocrine effects and the
268 mechanisms of PAH impact on bivalve reproductive systems.

269

270 **5. Conclusion**

271 The results of the present study confirmed that BaP exposure resulted in DNA damage
272 and perturbations in histological structure of female gonad in scallops. According to the
273 results, the effect of BaP reactive intermediates on biomacromolecules, which caused DNA
274 and cell membrane system damage in scallop female gonad, were corroborated. The
275 experiment indicated that DNA damage in gonad was more sensitive than other tissues, and
276 gonad DNA strand break could be a potentially suitable biomarker as early warning indicator
277 for toxicity of PAHs. However, the mechanism of BaP effects needs to be studied further to
278 understand the phenomenon of increased oocyte degeneration.

279

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286

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371 Table 1

372 Effect of Benzo[a]pyrene on oocyte degeneration in mature ovarian tissues prepared from scallop

373 (*Chlamys farreri*)

374

Treatment	Rate of degenerating oocyte (%)		
	0 day	10 days	15 days
Seawater control	2.33 ± 1.55	2 ± 0.57	2.33 ± 1.52
Acetone control		1.67 ± 0.33	2 ± 1.15
10 µg L ⁻¹ BaP		9.67 ± 2.08	25.67 ± 4.04

375 Values are means ± S.E. (n=6)

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376 **Figure Captions**

377 Fig.1. Variations in the ratio between double-stranded DNA to total DNA (F value) (mean \pm
378 SD) in mature female gonads of scallop (*Chlamys farreri*) groups exposed to various
379 concentrations of BaP over a 15-day period (n=3).

380

381 Fig.2. Effects of BaP in female scallop *Chlamys farreri* gonad section. 2a and 2b - control; 2c
382 - exposed to $10 \mu\text{g L}^{-1}$ BaP for 10 days. Arrows indicate the sparse cytoplasm of oocytes; 2d -
383 exposed to $10 \mu\text{g L}^{-1}$ BaP for 15 days. Ovarian follicles filled with atretic oocytes. Arrows
384 indicate the sparse cytoplasm of oocytes. (CT- connective tissue; DO-
385 degenerating/degenerated oocyte; FC- follicle cell; Fw- follicular wall; MO- mature oocyte;
386 N- nucleus; Nu-nucleolus) (Paraffin sections, $5\mu\text{m}$ -thick, hematoxylin-eosin-saffron.)

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388 Fig.3. Effects of BaP on ultrastructure of scallop *Chlamys farreri* oocytes. 3a and 3b -control;
389 3c, 3d, 3e and 3f - exposed to $10 \mu\text{g L}^{-1}$ BaP for 15 days. 3c showed perivitelline space of
390 several degenerating oocyte. 3d showed part of a degenerating oocyte and a follicle cell. 3e
391 showed a degenerating oocyte with numerous vacuoles and modified mitochondrion. 3f
392 showed a degenerated oocyte with large quantity of glycogen in the cytoplasm. (CG- cortical
393 granules; DO- degenerating oocyte; FC- follicle cell; Fw- follicular wall; GP- glycogen
394 particle; L- lysosome; Mi-mitochondrion; Mv-microvillus; N- nucleus; Nu-nucleolus;
395 RER-rough endoplasmic reticulum; Va-vacuoles; Yg-yolk granules) (Epon 812 embedded,
396 stained with uranyl acetate and lead citrate.)

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399 **Figures**

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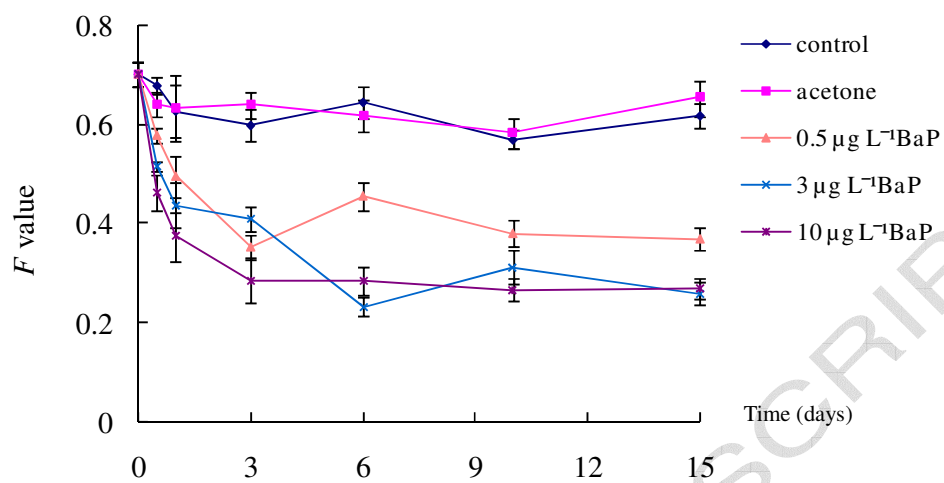
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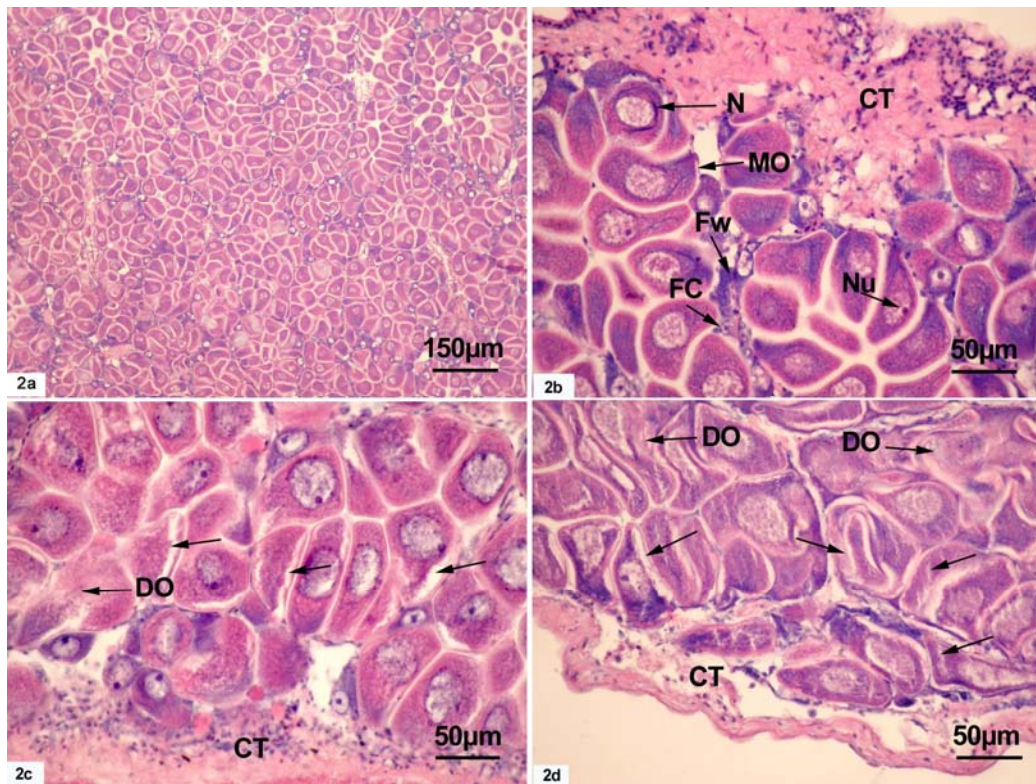
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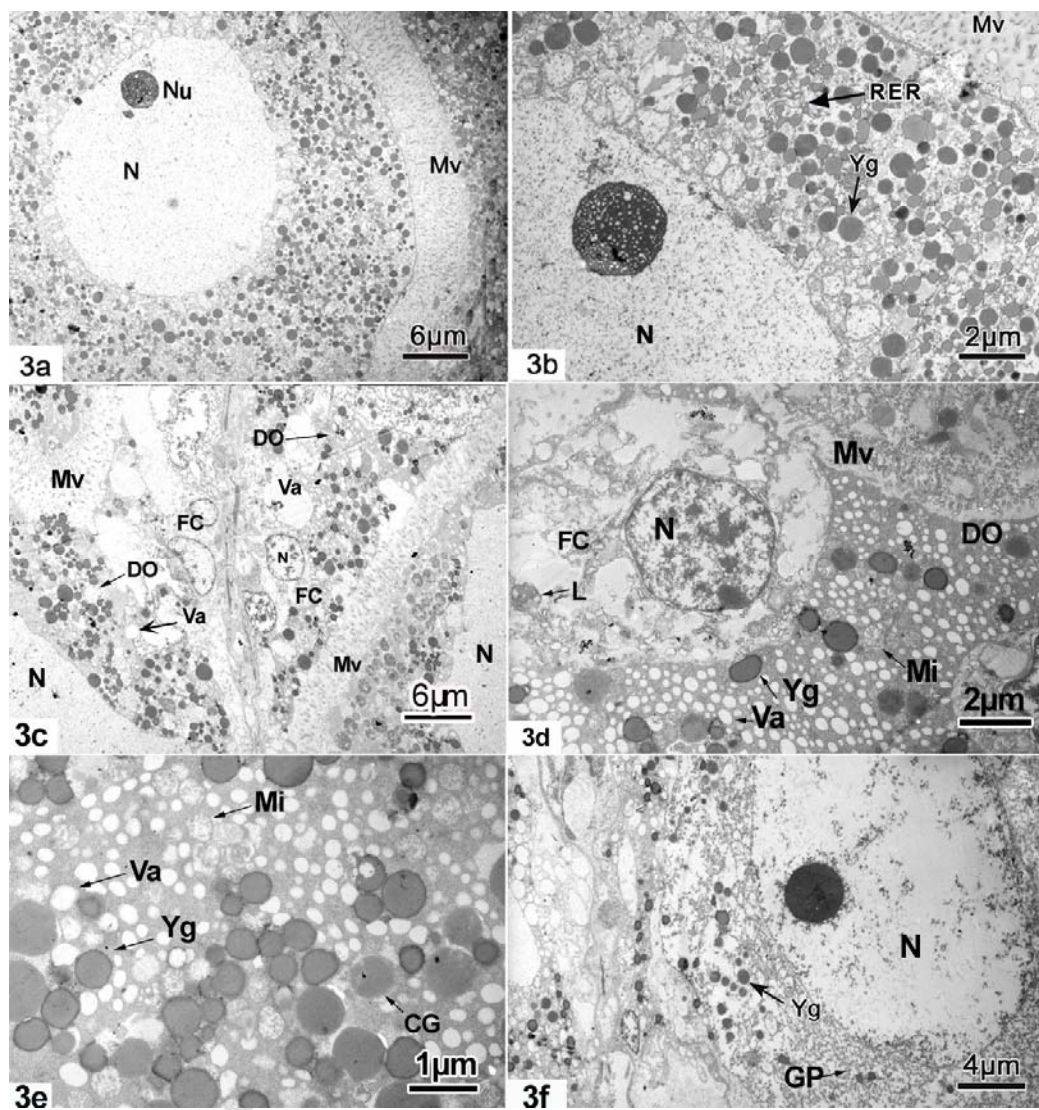


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