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Cardiogenesis impairment promoted by bisphenol A exposure is successfully counteracted by epigallocatechin gallate

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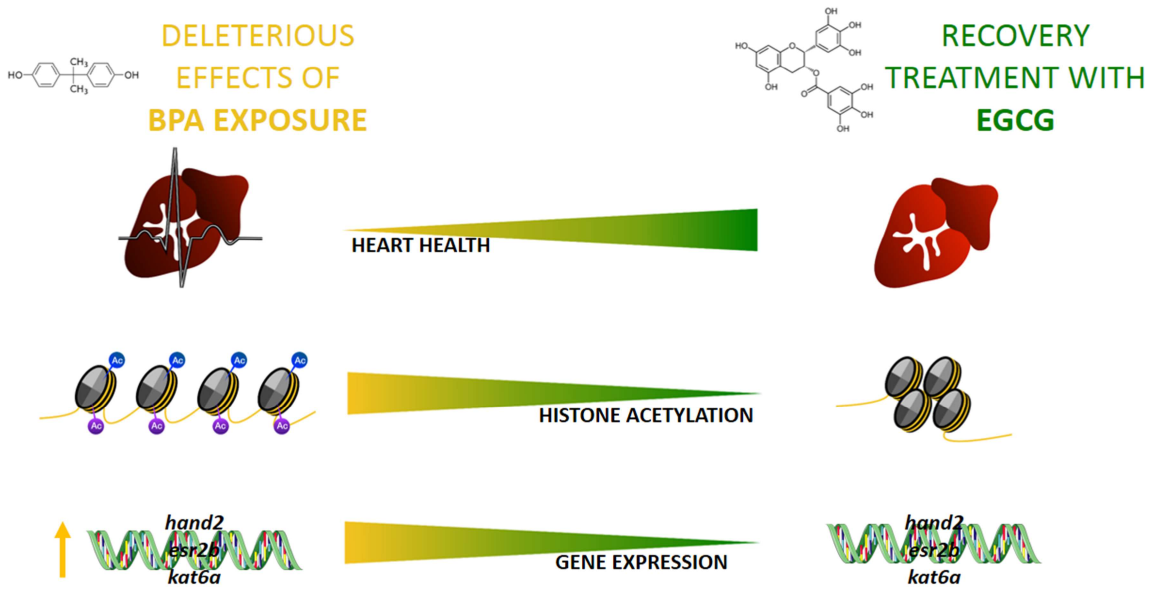

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1 **TITLE PAGES**

2 **Title:** Cardiogenesis impairment promoted by Bisphenol A exposure is successfully
3 counteracted by Epigallocatechin Gallate

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12 **ABSTRACT**

13 Exposure to the emerging contaminant bisphenol A (BPA) is ubiquitous and
14 associated with cardiovascular disorders. BPA effect as endocrine disruptor is widely
15 known but other mechanisms underlying heart disease, such as epigenetic
16 modifications, remain still unclear. A compound of green tea, epigallocatechin gallate
17 (EGCG), may act both as anti-estrogen and as inhibitor of some epigenetic enzymes.
18 The aims of this study were to analyze the molecular processes related to BPA
19 impairment of heart development and to prove the potential ability of EGCG to
20 neutralize the toxic effects caused by BPA on cardiac health. Zebrafish embryos were
21 exposed to 2000 and 4000 µg/L BPA and treated with 50 and 100 µM EGCG. Heart
22 malformations were assessed at histological level and by confocal imaging. Expression
23 of genes involved in cardiac development, estrogen receptors and epigenetic enzymes
24 was analyzed by qPCR whereas epigenetic modifications were evaluated by whole
25 mount immunostaining. BPA embryonic exposure led to changes in cardiac phenotype,
26 induced an overexpression of *hand2*, a crucial factor for cardiomyocyte differentiation,
27 increased the expression of estrogen receptor (*esr2b*), promoted an overexpression of
28 a histone acetyltransferase (*kat6a*) and also caused an increase in histone acetylation
29 both mechanisms being able to act in sinergy. EGCG treatment neutralized all the
30 molecular alterations caused by BPA, allowing the embryos to go on with a proper
31 heart development. Both molecular mechanisms of BPA action (estrogenic and
32 epigenetic) likely lying behind cardiogenesis impairment were successfully
33 counteracted by EGCG treatment.

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34 **HIGHLIGHTS**

35 • Exposure to BPA during early embryo life impairs cardiogenesis
36 • BPA promotes changes in expression of estrogen receptor
37 • Histone acetylation pattern in embryos is deeply modified after BPA exposure
38 • Treatment with EGCG restores normal cardiogenesis
39 • EGCG neutralizes BPA modifications in estrogen receptors and histone
40 acetylation

41 **KEYWORDS:** Bisphenol A, Epigallocatechin Gallate, cardiotoxicity, histone acetylation

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48 have no actual or potential competing financial interests.

49 **1. INTRODUCTION**

50 Cardiovascular diseases (CVDs) are disorders of the heart and blood vessels,
51 including coronary heart disease, cerebrovascular disease, rheumatic heart disease
52 and hypertension among other conditions (World Health Organization, 2017). They
53 have been reported to cause around 17.7 million deaths in 2015 (45% of all non-
54 communicable diseases and 31% of global deaths) (Institute of Medicine, 2010).
55 Unfortunately, in 2030 cardiovascular deaths are likely to increase up to 23.3 million
56 (Mathers and Loncar, 2006). Hence, health care systems devote a huge economic
57 burden to CVDs, being close to €170 billion annually in the EU (Leal *et al.*, 2006).
58 Besides the biological factors involved in CVDs such as genetic predisposition, obesity,
59 blood lipids and pressure as well as diabetes, there are other factors contributing to this
60 illness: bad habits (tobacco and alcohol consumption), lack of physical activity,
61 unhealthy diet and environmental pollution (Institute of Medicine, 2010). As far as
62 pollutants are concerned, there is much evidence associating high urinary
63 concentrations of emerging contaminants, such as bisphenol A (BPA), with a more
64 frequent diagnosis of CVDs (Gao and Wang, 2014).

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65 Since it was first synthesized in 1890, the industrial production of BPA has
66 exceeded 2.4 million tonnes, as a result of its use in plastics manufacturing and epoxy
67 resins (Vogel, 2009). The versatility of this compound has made it ideal for beverage
68 and food cans, baby bottles, water pipes, thermal paper, medical devices, dental
69 sealants and so on (Wetherill *et al.,* 2007). Therefore, its ubiquitous presence and its
70 capacity to be released from these materials to food and liquids explained why human
71 exposure to BPA (through diet, inhalation of dust or even dermal contact) is
72 widespread, more than 90% of individuals in the United States, Canada and Germany
73 having detectable amounts of this toxic in urine (Acconcia *et al.*, 2015). Moreover, BPA
74 presence has also been detected in the serum of pregnant women, breast milk,
75 amniotic fluid and placental tissue (Rochester, 2013).

76 A large amount of *in vitro* and *in vivo* studies have linked BPA exposure to
77 adverse effects on heart health (Chapalamadugu *et al.*, 2014; Gao and Wang, 2014;
78 Melzer *et al.*, 2010). One of the main reasons is that cardiac tissue expresses several
79 receptors which have been reported as targets for BPA (Moreman *et al.*, 2017; Pugach
80 *et al.*, 2016). From the 1930s, the action of BPA as endocrine disruptor has broadly
81 been described due to the structural similarity of this toxicant to endogenous estrogens
82 (Baker and Chandsawangbhuwana, 2012). Furthermore, it has been proven that BPA
83 is able to interfere not only with estrogen-related receptors, such as GPER (Pupo *et al.*,
84 2012) or ERRγ (Tohmé *et al.*, 2014), but also with androgen, thyroid (Wetherill *et al.*,
85 2007) and insulin receptors (Fang *et al.*, 2015).

86 More recently, scientific reports about BPA have taken into account the emerging
87 role of epigenetics on cardiovascular disease (Baccarelli *et al.*, 2010; Patel *et al.*,
88 2013). Epigenetic modifications consist of chromatin covalent modifications involving
89 both the DNA itself and/or histone proteins, which have an impact on gene expression
90 and which may be inherited from one generation to the following when affecting germ
91 cells (McCarrey, 2012). These changes include DNA methylation (mainly in cytosine
92 residues), post-translational modification of histones (such as methylation and
93 acetylation), chromatin organization and non-coding RNAs. They influence gene
94 expression by excluding transcription factors, recruiting binding proteins which take
95 part in the transcription machinery (either inhibiting or activating) and altering DNA-
96 protein affinity. Therefore, all these mechanisms take over the increase or decrease in
97 chromatin compaction (Labbé *et al.*, 2016). Epimutations are caused by a variety of
98 agents, factors and compounds: insufficient nutrition, exposure to endocrine disrupting
99 chemicals, toxicants, biotic and abiotic stress, parental behavior and metabolic
100 syndromes (McCarrey, 2014). Indeed, epigenetic effects of bisphenol A have been

101 claimed to be involved in several disorders (Chao *et al*., 2012; Kundakovic *et a*l. 2013;
102 Laing *et al*., 2016), being the cardiovascular ones among them (Patel *et al*., 2013).

103 In contrast, tea plant (*Camellia sinensis*) has been cultivated for thousands of
104 years and its leaves have been used for medicinal purposes (Wolfram, 2007).
105 Polyphenols, especially flavanols and flavonols, represent approximately 30% dry
106 weight of the fresh leaf. Catechins, the predominant flavanols, are: epigallocatechin
107 gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin
108 (EC) (Balentine *et al*., 1997). EGCG, the most abundant tea cathechin, is increasingly
109 being considered as beneficial for cardiovascular and metabolic health (Wolfram,
110 2007). It has been reported to cause an increase in the activity of enzymes involved in
111 cellular protection against reactive oxygen species (superoxide dismutase and
112 catalase) and to prevent the appearance of atherosclerotic plaque by modifications of
113 lipid metabolism (Khan and Mukhtar, 2013). Moreover, EGCG has also been claimed
114 to have an antiestrogenic activity (De Amicis *et al*., 2013; Farabegoli *et al*., 2007) and
115 the capacity to inhibit the enzymes in charge of histone acetylation (Choi *et al*., 2009).
116 Taking all these data into account EGCG arose as a promising substance to counteract
117 the impact of BPA on cardiac health.

118 Due to the great challenge of studying the *in vivo* effects of BPA on human
119 cardiovascular development, we have used a model species which has emerged as a
120 high-throughput organism: the zebrafish. 70 % of annotated human genes have a true
121 orthologue in the zebrafish genome with significant homology at protein level.
122 Consequently, this high homology between two genomes is reflected as a strong
123 conservation of drug responses (Bournele and Beis, 2016). Moreover, zebrafish
124 embryos lack blood circulation so oxygen and nutrients reach all tissues by passive
125 diffusion, this fact allowing the analysis of embryos even if they suffer from severe
126 cardiovascular defects, since they manage to survive several days (Asnani and
127 Peterson, 2014; Bakkers, 2011).

128 The aim of this work is to analyze some molecular mechanisms which could be
129 involved in BPA impairment of heart development and to prove the ability of EGCG as
130 a compound able to neutralize the deleterious effects of BPA on cardiac health.

131 2. MATERIALS AND METHODS

132 2.1. *Ethics statement*

133 This work is included in a project from the Spanish Ministry of Economy and
134 Competitiveness (Project AGL2014-53167-C3-3-R) specifically approved by the

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135 University of León Bioethical Committee as well as by the competent body of Junta de
136 Castilla y León (proyect number: ULE009-2016). All the animals were manipulated in
137 accordance with the Guidelines of the European Union Council (86/609/EU, modified
138 by 2010/62/EU), following Spanish regulations (RD 1201/2005, abrogated by RD 53/
139 2013) for the use of laboratory animals.

140 **2.2. *Zebrafish maintenance and embryo collection***

141 4-month-old zebrafish (*Danio rerio*), AB strain (wildtype), were maintained in 2.5
142 L aquaria (ZebTEC, Tecniplast System) with a recirculating water system (pH 7.0–7.5,
143 30 mg/L Instant Ocean, at 27–29ºC, 14:10 light-dark cycle). Animals were fed twice a
144 day with dry food (Special Diets Services®).

145 In order to obtain the embryos, adults were mated according to a *sex ratio* 1:2
146 (male:female). Embryos were immediately rinsed 2 min in 0.5% (vol/vol) bleach and 10
147 s in 70% (vol/vol) ethanol. Then, they were transferred to egg water containing 0.038
148 mM $CaCO_3$, 0.446 mM $NaHCO_3$, 1.025 mM sea salt and 0.005% (vol/vol) methylene
149 blue.

150 **2.3. *BPA embryonic exposure***

151 After washing the embryos, 80 per replicate and treatment were transferred to a
152 Petri dish containing: egg water with 0.0175% (vol/vol) ethanol, vehicle for BPA
153 exposure, (control embryos) or 2000 µg/L and 4000 µg/L BPA (exposed embryos).
154 Embryos were incubated with the vehicle and the toxicant from fertilization up to 24
155 hours post fertilization (hpf) (treatments being renewed at 12 hpf). At this point, both
156 ethanol and BPA were removed and the embryos were maintained in egg water at
157 28ºC until further analysis (Fig.S1A).

158 **2.4. *EGCG embryonic treatment***

159 For EGCG treatment, 80 embryos per replicate and treatment were incubated in
160 egg water with 0.0175% (vol/vol) ethanol (control embryos), ethanol plus 50 and 100
161 µM EGCG and 4000 µg/L BPA plus 50 and 100 µM EGCG, until reaching 3.3 hpf. From
162 3.3 hpf (blastula stage, when cardiac progenitors start to differentiate) to 24 hpf
163 (pharyngula period when heartbeat starts), these embryos were kept in dishes
164 containing ethanol and 4000 µg/L of BPA, respectively. After 24 hpf embryos were
165 maintained in egg water under the same conditions as those previously described (Fig.
166 S1B).

167 **2.5. *Evaluation of embryo mortality and malformations***

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168 Embryo mortality was evaluated from 3.3 hpf each day until they reached 120
169 hpf. At this time, phenotype of control and exposed larva were assessed under a
170 stereomicroscope (Leica MZ16F).
171 For histological insight to the heart, 4-μm-section of control and treated larvae
172 were stained with hematoxylin and eosin and observed under light microscope (Nikon
173 Eclipse E400).
174 As for confocal images of the heart, a whole-mount immunostaining protocol was
175 carried out. Zebrafish control and treated larva were fixed at 120 hpf in 4% (wt/vol)
176 paraformaldehyde overnight at 4ºC. After washing twice in PBS 1X, larva were
177 incubated in 6% (vol/vol) H_2O_2, until pigmentation vanished, and permeabilised with
178 methanol during 2 h at -20ºC. Then, all larva were washed 3 times with TBS-T 1%
179 (19.8 mM Tris hydrochloride, 0.15 M NaCl (pH 7.6) plus 1% (vol/vol) Triton X-100) and
180 transferred to blocking solution (3% (wt/vol) BSA in TBST-1%) for 1 h at room
181 temperature. Primary antibody (anti-Myl7, Table S1) was diluted in blocking solution, in
182 which the larvae were incubated for 2 days at 4ºC. Next, incubation in fluorescence-
183 conjugated secondary antibody (goat anti-rabbit AlexaFluor®488 (Invitrogen)) was
184 carried out at 4ºC overnight. Nuclei were stained with 180 μM DAPI for 8 min. Finally,
185 larva were mounted with ProLong® Gold Antifade Mountant (Thermo Scientific) and
186 observed under confocal microscope LSM 800 (Zeiss).

187 **2.6. *Gene expression analysis***

188 Total RNA extraction from a randomly selected pool of 30 control and 30
189 exposed/treated embryos at 3.3 hpf, 12hpf, 24 hpf and 48 hpf, cDNA synthesis and
190 primers (shown in Table S2) design were done as previously published by our group
191 (Lombó *et al.* 2015). Quantitative PCR (qPCR) amplification was carried out in a
192 StepOnePlus™ System (Applied Biosystems) according to the guidelines provided.
193 Reaction mixtures (total volume = 20 μL) contained 700 ng cDNA, 10 μL of 1X SYBR
194 Green Master mix (Applied Biosystems) and 2 μL of a 5 μM mix of forward and reverse
195 primers. qPCR was initiated with a preincubation phase of 10 min at 95 °C followed by
196 40 cycles of 95 °C denaturation for 15 s and the temperature for primer annealing for 1
197 min. Product size was visualized by electrophoresis on agarose gel and dissociation
198 curves analysis were also performed (data not shown). *18S rRNA* and beta actin
199 (*actb2*) were used as endogenous reference genes after checking their stability with
200 BPA exposure and EGCG treatment (Fig. S2). The normalized mRNA levels were
201 compared using $2^{-\Delta Ct}$ (ΔCt=Ct target−Ct reference) whereas fold changes were
202 expressed as $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

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203 **2.7. *Analysis of global DNA methylation by UPLC-MS***

204 DNA was extracted from 3.3 hpf, 24 hpf and 120 hpf control and exposed/treated
205 embryos following the protocol previously described in our group (Lombó *et al.* 2015).
206 DNA concentration, as well as purity, were measured using the NanoDrop ND-1000
207 UV-Vis Spectrophotometer (Thermo Scientific). All samples showed high purity
208 (A260/A280>1.8).

209 Global DNA methylation (5mC) analysis was performed following the method
210 described by Le *et al.* (2011) at indicated stages of development.

211 **2.8. *Whole mount immunostaining of cytosine methylation (5mC) and histone***
212 ***acetylation***

213 Zebrafish control and treated embryos at 3.3 hpf were fixed in 4%
214 paraformaldehyde overnight at 4ºC. After washing twice in PBS 1X and removing both
215 chorion and yolk sac, embryos were permeabilised as previously described. For 5mC
216 analysis, an extra step of 2 h DNA denaturalization with 2N HCl, followed by 15 min of
217 neutralization with 1 mM Tris HCl was performed. Then, all embryos were washed 3
218 times with TBS-T 1% and transferred to blocking solution (the same recipe as indicated
219 in section 2.5) for 1 h at room temperature. Embryos were incubated for 2 days at 4ºC
220 in blocking solution with the primary antibodies described in Table S1. Next, they were
221 incubated with fluorescence-conjugated secondary antibodies (goat anti-mouse
222 AlexaFluor®568 and goat anti-rabbit AlexaFluor®488 (Invitrogen)) at 4ºC overnight.
223 For nuclei staining and mounting the same protocol was followed. Relative quantity of
224 5mC and histone acetylation in prophase and interphase nuclei was quantified as
225 mean of intensity using ImageJ software.

226 **2.9. *ChIP-qPCR***

227 Chromatin immunoprecipitation was done at 24 hpf using 150 embryos per
228 replicate and dose, following the protocol described by Bogdanović *et al.* (2013).
229 Primers for H3K9ac enriched areas in transcription start sites (TSS) as well as for
230 intergenic region (lacking acetylation) were designed with Primer3Plus and are listed in
231 Table S3. Data were normalized to standard curves of input DNA in order to ensure
232 quantitative measurements and are relative to intergenic regions.

233 **2.10.*Statistical analysis***

234 Statistical analysis were performed with SPSS version 24.0 (IBM). For parametric
235 data one-way ANOVA followed by DMS post hoc test was used whereas for non-
236 parametric data a Kruskal-Wallis test was applied. All data in bars are represented as
237 mean ± SE and boxes represent median ± maximum and minimum.

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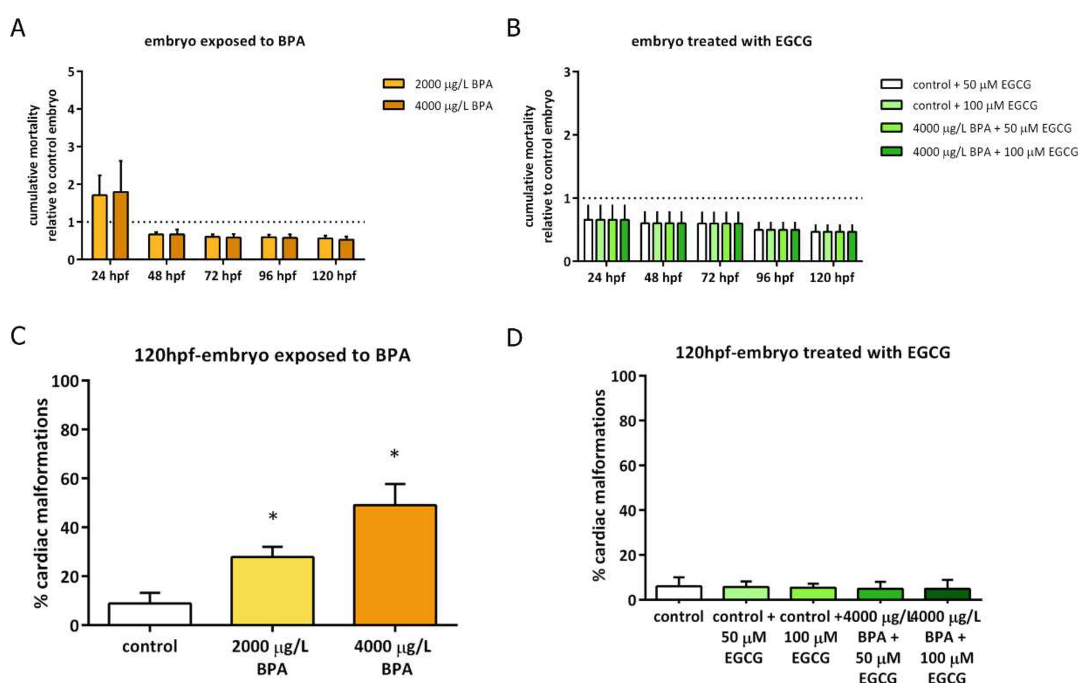
238 **3. RESULTS**

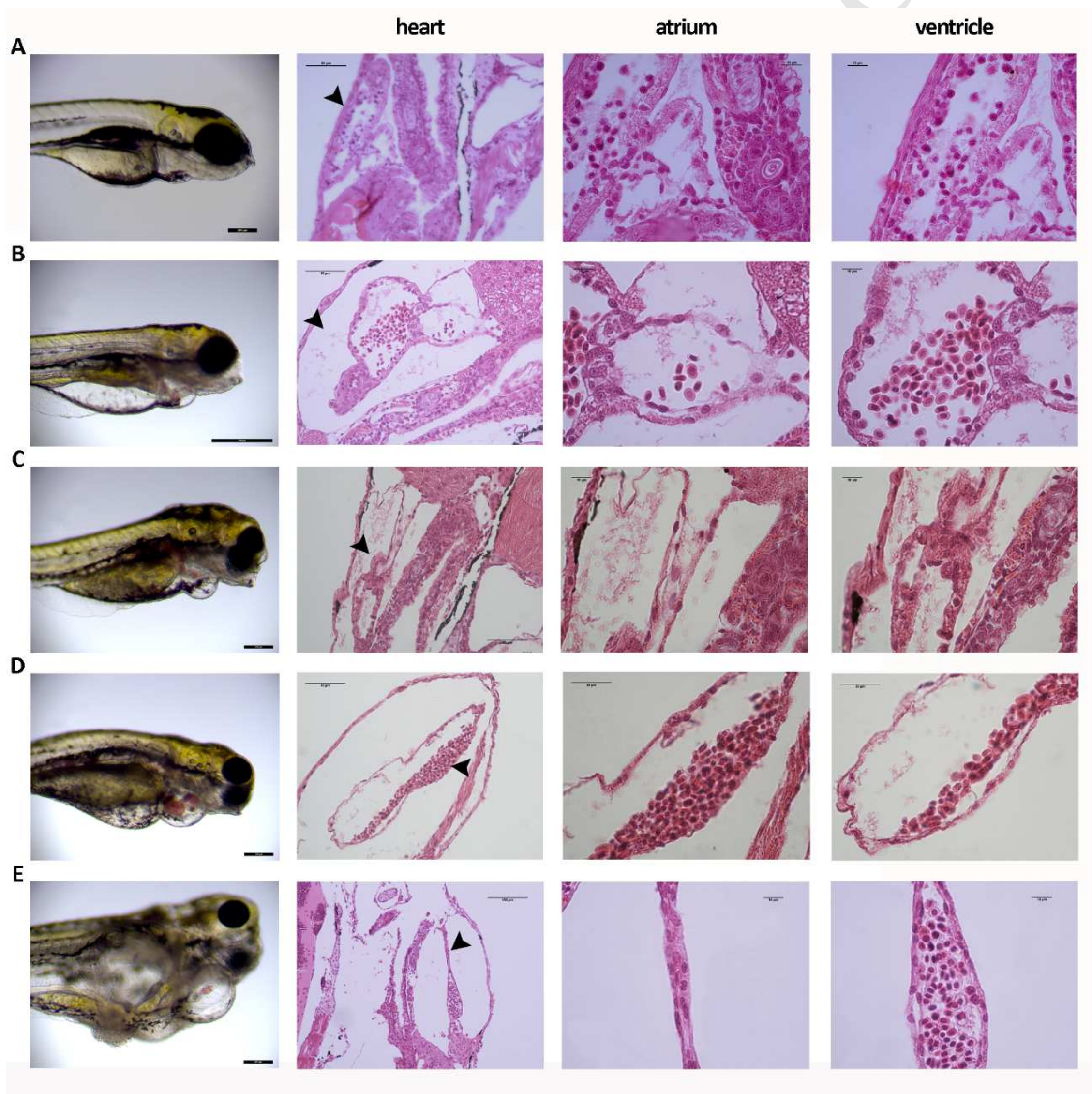
239 **3.1. *Embryo survival and heart development***

240 Embryo mortality was assessed throughout the first 5 days of development. Both
241 control and BPA-exposed embryos displayed a similar pattern of mortality which did not
242 change when treating the embryos with EGCG (Fig.1A and 1B). Phenotype was
243 evaluated at 120 hpf. The exposure to all doses of BPA led to phenotypical aberrations:
244 control embryo developed properly whereas more than 30% of exposed embryo
245 displayed cardiac malformations, being the ratio even higher (49.12%) in embryo
246 batches exposed to the highest dose. EGCG treatment reverted malformations caused
247 by BPA exposure to levels similar to those of control group (6.01%) (Fig.1C and 1D;
248 Fig. S3).



249 **Fig. 1 I Index of mortality relative to control batches during early embryo**
250 **development in: BPA exposure (A) and EGCG treatment (B). Percentage of**
251 **cardiac malformations in embryo exposed to BPA (C) and treated with EGCG (D)**
252 **at 120 hpf.** Bars represent 4 batches of control and treated embryos (n=4) and
253 asterisks indicate significant differences ($p < 0.05$) when comparing to control group.

254 Observed cardiac malformations mainly consist of: cardiac edema (increased
255 volume of the pericardial cavity); defects in looping (incorrect asymmetric position of
256 heart chambers which prevent the future ventricle from reaching the right side, whereas
257 the future atrium is not able to remain at the left side of the embryonic mid-line);
258 ballooning (lack of heart bulging which blocks the formation of two distinct surfaces
259 within the chambers: an inner curvature and an outer curvature); accumulation of blood
260 as a result of problems in heart beating and elongation of heart chambers (Fig.2). The
261 distribution of Myl7 (cardiac myosin light chain 2, also known as Cmlc2) throughout
262 both chambers in confocal images provided more accurate information of heart shape
263 in both control (Fig.3A) and malformed larvae, displaying again problems in chamber's
264 proper looping (Fig.3B and C) and ballooning (Fig.3D).



265 **Fig. 2 I Longitudinal histological sections of 120 hpf control (A) and BPA-**
266 **exposed embryos displaying different types of heart malformations: cardiac**
267 **edema (B), defects in looping and ballooning (C), blood accumulation and**

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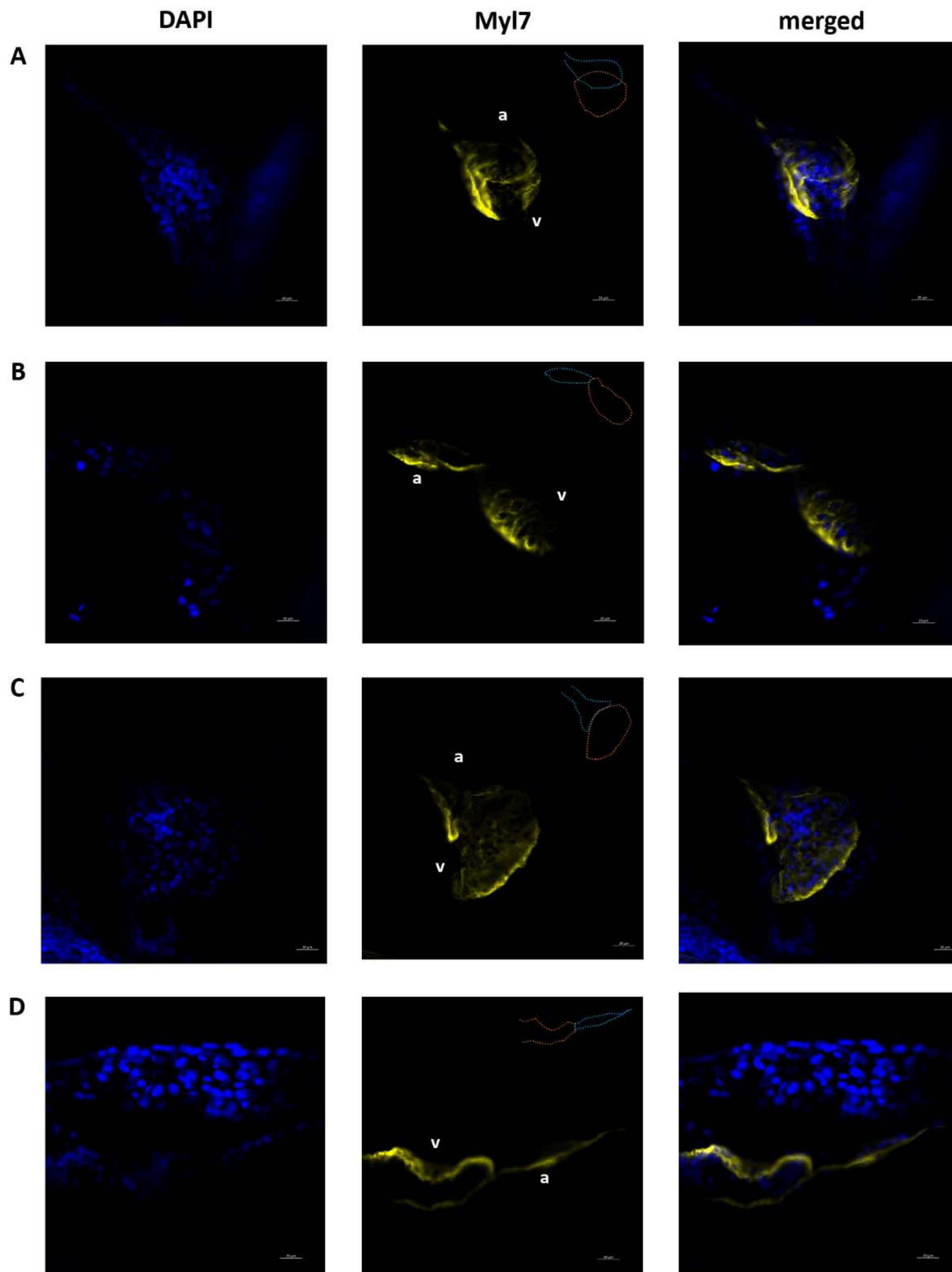
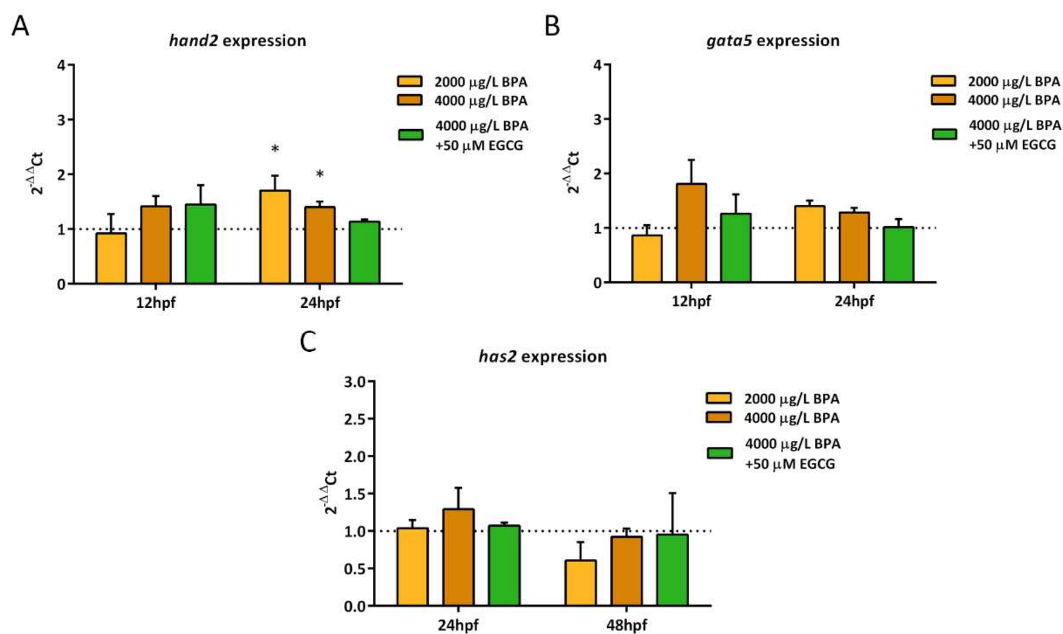
268 **elongation of heart chambers (D).** Images on the left side are pictures of control and
269 malformed living larva (from which the sections were obtained), whereas images on the
270 right side show proper and abnormal heart morphology at histological level (40x
271 magnification), splitting up atrium and ventricle (100x magnification). Arrow heads point
272 to the normal heart and the cardiac malformations described for each larvae.



273
274 **Fig. 3 I Confocal images of control (A) and abnormal hearts (B and C: problems**
275 **in looping, D: defects in ballooning) at 120 hpf.** Nuclei were stained with DAPI (in
276 blue) whereas Myl7 protein was labelled with Alexa Fluor®488 (in yellow). Heart shape
277 was drawn in dashed lines: blue from atrium (a) and orange for ventricle (v). Scale bar
278 20 µm.

279 So as to deeper assess the impairment of heart formation, expression of two
280 transcription factors involved in cardiac development (*hand2*: heart and neural crest
281 derivatives expressed 2; *gata5*: GATA-binding protein 5) was analysed during
282 cardiomyocyte differentiation (12hpf) and heart tube formation (24 hpf). Moreover, the
283 expression of one enzyme which takes over cell matrix synthesis (*has2*: hyaluronan
284 synthase 2) was analyse at 24 hpf and at 48 hpf (when heart chambers are acquiring
285 shape and size). Embryo exposed to the highest doses of BPA showed an upregulation
286 of *hand2* at 24hpf, but no changes in *gata5* or *has2* were observed in none of the
287 stages they were studied. This overexpression in *hand2* was no longer detected after
288 treating BPA-exposed embryo with both 50 and 100 µM EGCG (Fig.4).

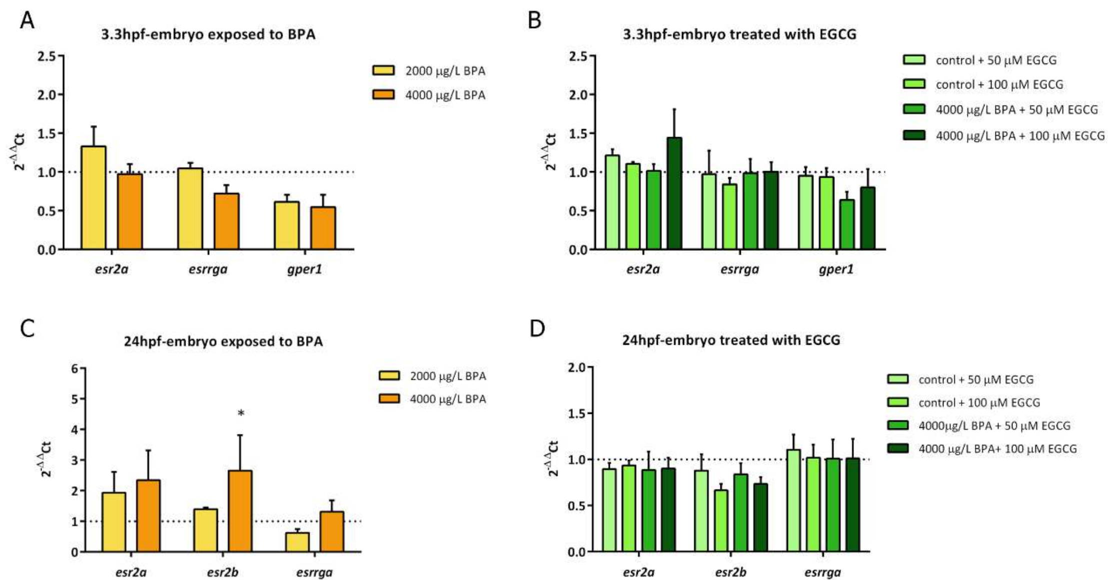


289 **Fig. 4 I Relative expression of *hand2* (A) and *gata5* (B) at 12 and 24hpf, as well as**
290 **of *has2* (C) at 24 and 48hpf in embryos exposed to both doses of BPA and**
291 **exposed to 4000 µg/L BPA and treated with 50 µM EGCG.** Expression levels relative
292 to *18SrRNa* (12hpf) and *actb2* (24 and 48hpf) were calculated using $2^{-\Delta\Delta Ct}$ method of
293 three independent experiments (n=3).

294 **3.2. *Endocrine receptors***

295 As for the expression of endocrine receptors, no changes were observed in
296 estrogen receptor alpha (*esr2a*), estrogen-related receptor gamma (*esrrga*) or G
297 protein-coupled estrogen receptor 1 (*gper1*) at 3.3 hpf when comparing BPA-exposed
298 embryos to the control group. Neither did the treatment with EGCG caused changes in
299 the expression of these genes at this stage (Fig.5A and B). However, the study of
300 these receptors at 24 hpf revealed an upregulation of estrogen receptor beta (*esr2b*) in

301 embryos exposed to 4000 µg/L BPA, this effect being reverted when treating these
302 embryos with both doses of EGCG (Fig.5C and D).



303 **Fig. 5 I Relative expression of estrogen and estrogen related receptors at 3.3 hpf**
304 **in BPA exposed embryo (A) and embryo treated with EGCG (B) and at 24 hpf (C**
305 **and D, respectively).** Expression levels relative to *18SrRNA* and *actb2* were
306 calculated using $2^{-\Delta\Delta}$Ct method of three independent experiments (n=3). Asterisks
307 indicate significant differences ($p < 0.05$) when comparing to control embryos (dashed
308 line).

309 **3.3. *DNA methylation***

310 The assessment of global DNA methylation did not reveal any difference among
311 control and BPA-exposed embryos at 3.3, 24 or 120 hpf, neither by UPLC-mass
312 spectrometry (Fig. S4A) nor by whole mount immunocytochemistry (supplemental
313 material Fig. S4B). This method allows to distinguish the different stages of cell cycle in
314 which the blastomeres are, excluding from the analysis the highly methylated mitotic
315 cells which could mask subtle differences among treatments. Moreover, the expression
316 of a maintenance DNA-methyltransferases (*dnmt1*) and three *de novo* DNA-
317 methyltransferases (*dnmt3*, *dnmt5* and *dnmt8*) remained the same in both control and
318 embryo exposed to BPA at 3.3 hpf and 24 hpf (supplemental material fig.S4C and D).

319 **3.4. *Histone acetylation***

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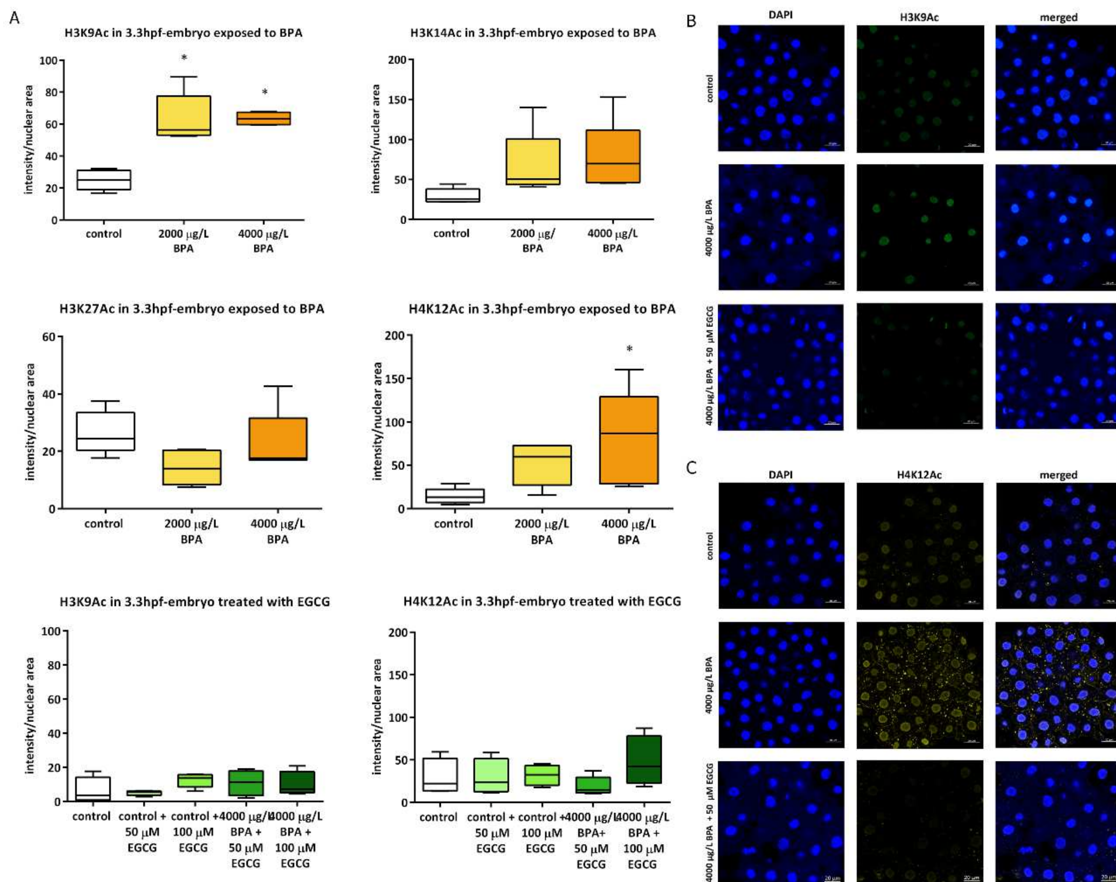
320 Acetylation of lysine 9 and 14 in histone 3 and lysine 12 in histone 4 was highly
321 increased after embryonic exposure to BPA, whereas no changes in the acetylation of
322 lysine 27 in histone 3 were observed (Fig.6A).

323 The assessment of the marks affected by exposure to 4000 µg/L BPA (H3K9 and
324 H4K12) after treatment with both 50 and 100 µM EGCG showed a decrease in histone
325 acetylation up to control levels as shown in fig.6B and C).



326 **Fig. 6 I Quantification of acetylation by whole mount immunofluorescence in 3.3**
327 **hpf control embryo, embryo exposed to BPA and treated with EGCG. A:** boxes
328 represent intensity of fluorescence related to acetylation in lysine 9 in histone 3
329 (H3K9Ac), lysine 14 in histone 3 (H3K14Ac), lysine 27 in histone 3 (H3K27Ac) and
330 lysine 12 in histone 4 (H4K12Ac); all labelled with AlexaFluor®488, relative to nuclear
331 area of around 200 cells per replicate (n=5). Asterisks indicate significant differences (p
332 < 0.05) when comparing to control embryos **B** and **C** are pictures of representative
333 images of H3K9Ac and H4K12Ac, respectively, in control embryo, embryo exposed to
334 4000 µg/L BPA and embryo exposed to 4000 µg/L BPA and treated with 50µm EGCG.
335 Different channels for DAPI and Alexa Fluor® 488 appeared separated and merged,
336 scale bar represents 20 µm.

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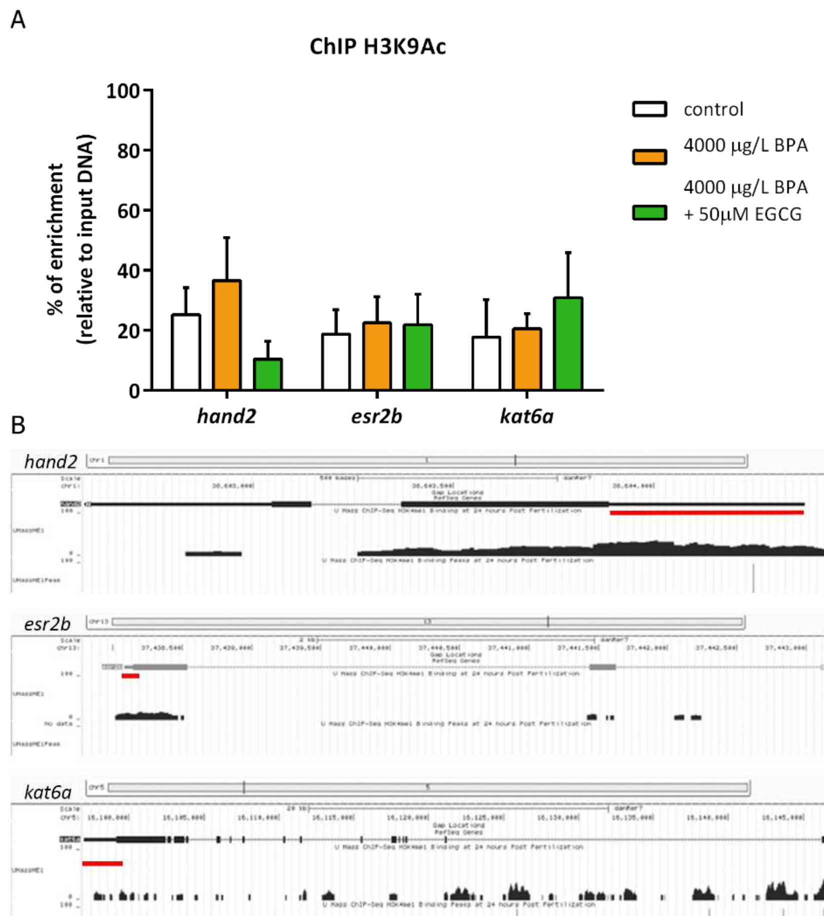
337 Acetylation in lysine 9 of histone 3 of gene promoters (whose expression was
338 increased by BPA exposure) was also analyzed by ChIP-qPCR. Results indicated that
339 BPA exposure did not change the pattern of histone acetylation near TSS of studied
340 genes (Fig.7).



341 **Fig. 7 I ChIP-qPCR analysis.** A: H3K9ac of *hand2*, *esr2b* and *kat6a* promoters in 24
342 hpf embryos from control, 4000 µg/L BPA and 50 µM EGCG batches. Acetylation
343 levels are relative to input DNA and normalized with non-acetylated regions of the
344 genome (n=4). B: red line represents enriched areas in activation marks, obtained from
345 UCSC Genome Browser, from which acetylation was analysed.

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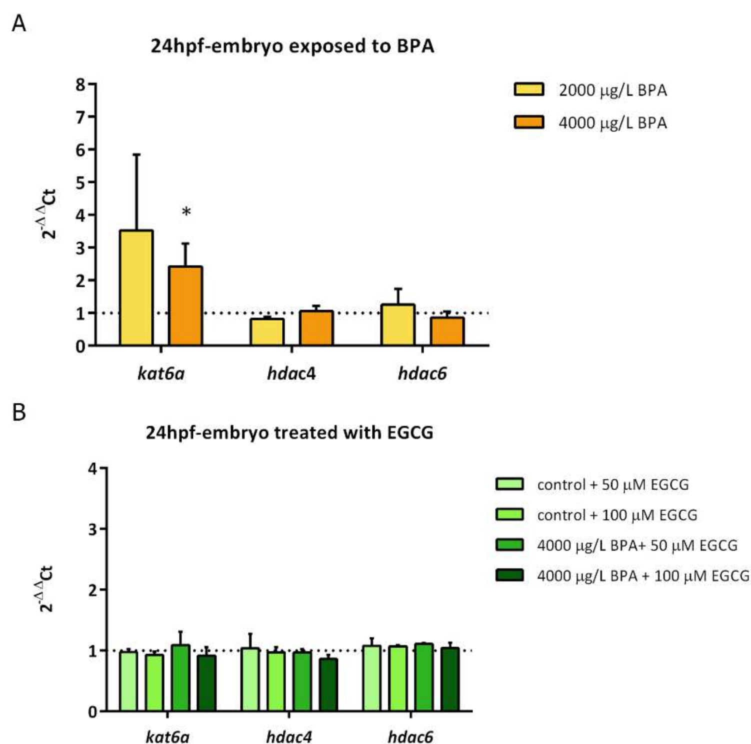
346 Regarding the expression of epigenetic enzymes involved in histone acetylation
347 (*kat6a*) and deacetylation (*hdac4*, *hdac6*) was analyzed in BPA-exposed and EGCG-
348 treated embryos at 24 hpf. Results did not show any changes in *kat6a* expression at
349 3.3 hpf (Fig. S5) but revealed an upregulation of this gene at 24 hpf after exposing the
350 embryos to 4000 µg/L of BPA (Fig.8A), this change being faded when EGCG treatment
351 was applied (Fig.8B).



352 **Fig. 8 I Relative expression of epigenetic enzymes at 24 hpf in embryos exposed**
353 **to BPA (A) and embryos treated with EGCG (B).** Expression levels relative to *actb2*
354 were calculated using $2^{-\Delta\Delta Ct}$ method of three independent experiments (n=3). Asterisks
355 indicate significant differences ($p < 0.05$) when comparing to control embryos (dashed
356 line).

357 ## 4. DISCUSSION

358 Exposure to toxic substances during early development potentially influences
359 health and disease in adulthood, especially during critical windows of organogenesis
360 such as cardiogenesis. In mammals, several works have shown that exposure to BPA
361 during pregnancy leads to heart disease in the offspring by altering glucose metabolism
362 (Alonso-Magdalena *et al*., 2010) and by changing gene expression in the fetal
363 cardiovascular tissue (Chapalamadugu *et al*., 2014). In zebrafish, a large number of
364 studies have reported that exposure to BPA during early life leads to several
365 developmental disabilities. Both exposures to higher (15 and 20 mg/L) and to lower

366 concentrations (from 1500 to 4500 µg/L) of BPA during embryo development caused
367 problems in pigmentation, skeletal malformations (Duan *et al*., 2008) and cardiac
368 edema (Lam *et al*., 2011; Makarova *et al*., 2016; Moreman *et al*., 2017). The dose of
369 2000 µg/L BPA selected for this experiment was the same that led to cardiogenesis
370 impairment by paternal BPA exposure (Lombó *et al*., 2015) and a higher one of 4000
371 µg/L was also tested. Our results confirmed that embryonic exposure to this toxicant,
372 even to the lowest dose, but mainly to the highest one, increased the percentage of
373 heart malformations such as cardiac edema, defects in looping and ballooning, blood
374 accumulation and elongation of heart chambers. Bearing in mind that heart tube
375 formation in zebrafish is formed by the first 28 hours (Bakkers, 2011; Stainier, 2001;
376 Staudt and Stainier, 2012), exposure to BPA from fertilization upon this stage is likely
377 to affect molecular mechanisms underlying cardiac development. In fact, the
378 transcription factor *hand2* was overexpressed at 24hpf in embryos exposed to both
379 2000 and 4000 µg/L BPA. This gene encodes a beta helix–loop–helix (bHLH) protein
380 involved in the regulation of cardiac progenitors differentiation (starting at late blastula
381 stages) as well as morphogenesis of the myocardial cells, and it is likely to take part in
382 heart tube formation at later times (24hpf) (Bakkers, 2011; Stainier, 2001). As noticed
383 by Schindler and colleagues *hand2* has a potent influence on cardiomyocyte production
384 between 10 and 24 hpf, after gastrulation and before the initial assembly of the heart tube.
385 Moreover, these authors associated *hand2* overexpression with an excessive
386 proliferation of cardiac progenitor cells, leading to the formation of an abnormally
387 elongated tract (Schindler *et al.*, 2014)..Therefore, the observed overexpression of this
388 gene might well lie behind the cardiac enlargement and elongated outflow tract of
389 embryo exposed to BPA,. Furthermore, exposure to 7 µM of 17β-estradiol, another
390 endocrine disruptor, relates the increase in *hand2* expression to a higher percentage of
391 cardiac deformities (Diamante *et al*., 2017).

392 It has been proven that estrogen receptor signaling is involved in the deleterious
393 effect of BPA on cardiovascular system (Gao and Wang, 2014). Zebrafish species
394 harbor three estrogen receptor genes: *esr1* encodes a protein corresponding to
395 mammalian ERα, whereas *esr2b* encodes ERβ1 and *esr2a* encodes ERβ2 *(Bardet et*
396 *al*., 2002; Menuet *et al*., 2002). Although BPA has an affinity for ERα/β 10,000-fold
397 lower than 17b-estradiol (Kuiper *et al*., 1998), it may also interact with the
398 transmembrane G protein coupled estrogen receptor 1 (GPER, a mediator of the non-
399 genomic effects) (Pupo *et al*., 2012) and with ERRγ, able to bind estrogen response
400 elements (EREs) (Acconcia *et al*., 2015). Taking all these data into account, BPA has
401 been claimed to fulfill the definition of endocrine disruptor since its interference with

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402 estrogenic pathways is directly linked to severe pathologies affecting different tissues
403 (Rubin, 2011). Besides, cardiovascular tissue has been reported to have estrogen
404 receptors which make it more susceptible to BPA (O. Lee *et al.*, 2012) The gene
405 expression analysis of estrogen and estrogen-related receptors during different periods
406 of development revealed a deep upregulation of *esr2b* in embryos exposed to 4000
407 µg/L BPA, which is in accordance with previous studies of adult male exposure and
408 embryonic exposure in which Esr2b showed the strongest response to BPA exposure
409 among all estrogen receptors (Ge *et al.*, 2015; Mu *et al.*, 2018).

410 Chromatin remodeling complexes are aimed to assist ERs and other
411 transcriptional factors for activation or repression of gene transcription, since they
412 regulate the access of the transcriptional machinery to DNA (Kim, 2001). Since 1960s,
413 histone acetylation is known to promote chromatin remodeling by getting rid of positive
414 charges in the lysine residues which decreases the affinity of histones for DNA (Davie
415 2003). Histone acetyltransferases (HATs) are the enzymes in charge of this chromatin
416 relaxation (which results in an activation of gene expression) whereas histone
417 deacetylases (HDACs) revert this process (so they are related with gene repression).
418 In this study, it has been shown that BPA exposure increased the expression of *kat6a*
419 (also known as *myst3*) at 24 hpf, whose HAT activity has been reported to regulate
420 ERα expression by modifying its promoter in breast cancer cells (Yu *et al.*, 2017).
421 Epigenetic toxicity of BPA was firstly reported in yellow agouti mouse model when
422 maternal exposure to this toxicant was found to induce hypomethylation of IAP
423 (Intracisternal A-Particle) resulting in agouti gene overexpression (Dolinoy *et al.*, 2007).
424 From this moment onwards a great number of works have proven the ability of BPA to
425 modify DNA and histone methylation as well as expression of DNA-methyltransferases
426 (Laing *et al.*, 2016; Yin *et al.*, 2016; Zhao *et al.*, 2017). However, our results did not
427 show neither any effect of this toxicant on DNA methylation at different stages of
428 development nor on the transcription of maintenance and *de novo* DNA-
429 methyltransferases. As far as histone acetylation is concerned, few studies are focused
430 on the effect of this toxicant on this epigenetic modification and results depended on
431 the doses and the tissue of study. In male rats a remarkable reduction in acetylation K9
432 and K27 (in H3) and K12 (in H4) was detected in testes after long-term exposure to a
433 'safe' dose of BPA (Chen *et al.*, 2017). While on the contrary, perinatal exposure to
434 BPA increased H3K9 and H3K14 acetylation in cerebral cortex and hippocampus of
435 postnatal 3 and 8 week-embryo (Kumar and Thakur, 2017) and H3K14 in adult mice
436 (Q. Zhang *et al.*, 2014). In zebrafish, our results have revealed for the first time that
437 exposure to BPA during the first day of development highly rises the level of acetylation

438 of H3K9, H3K14 and H4K12 at 3.3 hpf but, since *kat6a* expression was affected after
439 BPA exposure we were not able to stablish a relationship between this enzyme and the
440 evident increase in histone acetylation. This rise may be related to the activation of
441 other histone acetyltransferases or the de-activation of deacetylases that have not
442 been evaluated in this study. Concerning cardiac disease, heart hypertrophy has been
443 linked to histone acetylation (Abi Khalil, 2014; Chang and Bruneau, 2012) and what is
444 more, congenital heart defects have been recently associated with mutations in genes
445 codifying histone acetyltranferases in microdeletion syndrome patients (León et al.,
446 2017). Even though an increase in H3K14 after prenatal alcohol exposure acetylation
447 has been reported to cause overexpression of EHAND and DHAND (also known as
448 HAND1 and HAND2) (W. Zhang *et al.*, 2014), we were not able to establish a
449 relationship between specific increase in lysine 9 acetylation of histone 3 and the
450 increase in gene expression of neither *hand2*, *esr2b* nor *kat6a*.

451 Once the effects of BPA on hearth development, estrogen receptors expression
452 and histones acetylation were identified, the next aim was to check the possibility to
453 counteract the deleterious impact of this toxicant. EGCG, whose agonist/antagonist
454 estrogen effects have been characterized (Goodin *et al.*, 2002; Kuruto-Niwa *et al.*,
455 2000) and which has already been described to reduce the HAT activity (Choi *et al.*,
456 2009), therefore emerged as a potential candidate. EGCG is the major catechin found
457 in tea, widely considered as a health-promoting beverage (Wolfram, 2007).

458 Association of green tea consumption with cardiovascular and metabolic health is
459 a result of the anti-inflammatory (Donà *et al.*, 2003), anti-oxidative (Zhang and Rock,
460 2004) and anti-angiogenic effects (Oak *et al.*, 2005) mainly attributed to its most
461 abundant catechin: EGCG (Wolfram, 2007). In fact, EGCG has been reported to
462 improve the prognosis in patients with chronic heart failure due to phosphorylation of
463 Akt and eNOS (reducing mortality in *in vivo* model) (Oyama *et al.*, 2017). Since only
464 few experiments have been done in zebrafish with this compound, we chose 50 µM
465 and 100 µM, doses which had previously been used in other studies with this species
466 (Wang *et al.*, 2009). Moreover, our results showed that EGCG was able to reduce the
467 sharp increase in cardiac malformations caused by BPA exposure to levels similar of
468 control batches. This healing effect might be explained by reversion of changes in
469 molecular mechanisms triggered when exposing the embryos and that will be hereafter
470 explained.

471 Firstly, it must be taken into account that BPA acts as an estradiol (E_2) antagonist
472 (Acconcia *et al.*, 2015). In fact, the first study published regarding the effects of EGCG
473 on endocrine system showed that cell BPA exposure decrease the E_2-induced
474 expression whereas co-treatment with BPA plus EGCG resulted in a slight decrease in

475 this expression (Kuruto-Niwa *et al.*, 2000). In addition, cell treatment with EGCG has
476 been linked to an increase in E_2-induced response (Goodin *et al.*, 2002). This is in
477 accordance with the reversion in *esr2b* upregulation caused by 4000 µg/L BPA
478 exposure after treating the embryo with both doses of EGCG. Indeed, concentrations
479 between 100 and 150 µM have already been reported to be anti-estrogenic (Farabegoli
480 *et al.*, 2007) and to decrease the transcription of ESRα gene (De Amicis *et al.*, 2013).

481 Regarding the epigenetic effects, although most catechins have low levels of anti-
482 HAT activity, EGCG acts in a dose dependent-manner as HAT-inhibitor because of its
483 specificity for the majority of HAT enzymes but not for other epigenetic enzymes (Choi
484 *et al.*, 2009). It has been proven that EGCG impaired the translocation of androgen
485 receptor to the nucleus since it significantly suppressed the acetylation of this receptor,
486 displaying a promising use for prostate cancer cure (Y. H. Lee et al., 2012). In our
487 experiment, EGCG treatment suppressed the sharp overexpression of the histone
488 acetyltransferase *kat6a* induced by BPA exposure. Hence, H3K9 and H4K12
489 acetylation were reduced after EGCG treatment comparing with the high level of
490 acetylation triggered by BPA exposure. During cardiac-specific differentiation of mouse
491 mesenchymal stem cells, the decrease in the expression of Gata4, Nkx2.5 and Mef2c
492 after treatment with 120 µM EGCG was attributed to a decrease in the H3 acetylation,
493 evaluated by western-blot (Yin *et al.*, 2014). Even if these authors hypothesized that
494 the dysregulation was specifically caused by an increase in H3Ac in the promoters of
495 those genes, in our study *hand2* upregulation caused by BPA and its reversion by
496 EGCG were not explained by specific changes in H3K9 acetylation of its promoter.

497 **CONCLUSIONS**

498 We have observed that BPA exposure during early stages of development
499 seriously affects heart development. This toxicant has two mechanisms of action
500 underlying cardiogenesis impairment: oestrogenic and epigenetic, which have been
501 identified by the overexpression of *esr2b* and the increase in histone acetylation
502 (specifically in H3K9 and H4K12). Both mechanisms, which are closely related, might
503 well act in synergy and could be responsible for the upregulation observed in the
504 transcription factor *hand2*, crucial for cardiac formation.. Remarkably, EGCG treatment
505 was able to recover the phenotype and reverse all the deleterious effects previously
506 displayed by BPA exposure. This healing outcome could be explained by the anti-
507 estrogenic and anti-HAT activity of this polyphenol, since both *esr2b* and *hand2*
508 expression as well as H3 and H4 acetylation returned to control levels.

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HIGHLIGHTS

- Exposure to BPA during early embryo life impairs cardiogenesis
- BPA promotes changes in expression of estrogen receptor
- Histone acetylation pattern in embryos is deeply modified after BPA exposure
- Treatment with EGCG restores normal cardiogenesis
- EGCG neutralizes BPA modifications in estrogen receptors and histone acetylation

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