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The PBDE metabolite 6-OH-BDE 47 affects melanin pigmentation and THRβ MRNA expression in the eye of zebrafish embryos

Wu Dong, **Laura J Macaulay**, **Kevin WH Kwok**, **David E Hinton**, **P Lee Ferguson**, and **Heather M Stapleton***

Nicholas School of the Environment; Duke University; Durham, NC USA

Abstract

Polybrominated diphenyl ethers and their hydroxyl-metabolites (OH-BDEs) are commonly detected contaminants in human serum in the US population. They are also considered to be endocrine disruptors, and are specifically known to affect thyroid hormone regulation. In this study, we investigated and compared the effects of a PBDE and its OH-BDE metabolite on developmental pathways regulated by thyroid hormones using zebrafish as a model. Exposure to 6-OHBDE 47 (10–100 nM), but not BDE 47 (1–50 μM), led to decreased melanin pigmentation and increased apoptosis in the retina of zebrafish embryos in a concentration-dependent manner in short-term exposures (4 – 30 hours). Six-OH-BDE 47 exposure also significantly decreased thyroid hormone receptor β (THRβ) mRNA expression, which was confirmed using both RT-PCR and in situ hybridization (whole mount and paraffin- section). Interestingly, exposure to the native thyroid hormone, triiodothyronine (T3) also led to similar responses: decreased THRβ mRNA expression, decreased melanin pigmentation and increased apoptosis, suggesting that 6-OH-BDE 47 may be acting as a T3 mimic. To further investigate short-term effects that may be regulated by THRβ, experiments using a morpholino gene knock down and THRβ mRNA over expression were conducted. Knock down of THRβ led to decreases in melanin pigmentation and increases in apoptotic cells in the eye of zebrafish embryos, similar to exposure to T3 and 6-OH-BDE 47, but THRβ mRNA overexpression rescued these effects. Histological analysis of eyes at 22 hpf from each group revealed that exposure to T3 or to 6-OH-BDE 47 was associated with a decrease of melanin and diminished proliferation of cells in layers of retina near the choroid. This study suggests that 6-OH-BDE 47 disrupts the activity of THRβ in early life stages of zebrafish, and warrants further studies on effects in developing humans.

*Correspondence to: Heather M Stapleton; heather.stapleton@duke.edu.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article is available online at the publisher's website.

Keywords

apoptosis; melanin pigmentation; PBDE; thyroid hormones; thyroid hormone receptor; zebrafish; whole mount in situ hybridization

Introduction

Until 2005, polybrominated diphenyl ethers (PBDEs) were widely used as fire retardants in a variety of household products, including residential furniture, textiles and electronics.[1,2] Due to their persistence and extensive use over the past few decades, they are frequently detected in the environment.[3,4] PBDEs have been shown to be metabolized in vertebrate and human tissues to their hydroxyl-metabolites (OH-BDEs), both of which are commonly detected in human serum.[5-8] PBDEs and OH-BDEs are also potential endocrine disruptors that are known to impact thyroid hormone regulation.[6,9-12] OH-BDEs in particular have chemical structures that are very similar to the endogenous thyroid hormones thyroxine (T4) and triiodothyronine (T3); and, the toxicity and possible thyroid mimicry of this compound, reported herein, could influence the activity and expression of thyroid hormone receptors (THRs), thereby perturbing natural development.[12]

In mammals, THRs regulate transcription and have both a DNA-binding domain (DBD) and a ligand binding domain (LBD).[13,14] During embryonic development, THR mRNA expression can vary considerably with age and by species. In rodents, THR mRNA is expressed in the developing middle ear and nearby brain tissue, suggesting that it is related to central nervous system development,[15,16] In addition, THRβ is also known to play a key role in pituitary and liver thyroid hormone metabolism in rats.[17] In contrast to mammals, THR mRNA has been observed, although at low levels, in teleost eggs immediately after fertilization in flounder (Pleuronectes americanus)[18] and in rockfish (Sebasticus marmoratus).[19] This suggests that thyroid hormones play key roles in early embryonic development of fish, yet the exact stages of development modulated by thyroid hormones in fish are not clear.[18,20]

In our previous study, we showed that 6-OH-BDE 47 (6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether) exposure in early zebrafish embryos increased the mRNA expression of thyroid regulating deiodinases.[9] In developing retinal epithelium cells, pigmentation is dependent upon the number of normally functioning melanocytes in photoreceptor morphogenesis.21 However, potential connections between this reduction in melanin pigmentation from exposure to 6-OH-BDE 47 and possible effects on the thyroid hormone system remain unresolved.

Interestingly, a similar decrease in skin pigmentation was reported in Xenopus tadpoles after exposure to 1000–5000 μg g^{-1} of the commercial PBDE flame retardant mixture known as DE-71.[22] Eye pigmentation is partly controlled by the thyroid system. In chickens, THR expression was observed in the retina.[23,24] And in mice, THRβ2 controlled eye pigment development, including medium, long and short wavelength sensitive opsin photopigments.[25] Additionally, Xenopus retinal development may be regulated by THRβ2, which may affect photopigments.[26,27] Zebrafish retinas have red- long wavelength sensitive,

green- medium wavelength sensitive, blue- short wavelength sensitive, and UV-wave length sensitive cones. THRβ2 expression in cone precursors is required if pure red cones are to be produced.[24] Furthermore, THR may be important in regulating apoptosis and cell proliferation in these tissues.

The goals of this study were to determine whether 6-OHBDE47 exposures caused a reduction of melanin formation in the zebrafish (Danio rerio) embryo eye and whether exposure affected THRβ expression. Accordingly, we compared phenotypes from exposures to 6-OH-BDE 47 and the thyroid hormone T3. To detect the influence of 6-OH-BDE 47 on pigmentation and THRβ expression, we measured mRNA expression using both WISH & RT-PCR and used a morpholino gene knockdown approach to determine the role of THRβ in eye pigmentation in zebrafish. Lastly, we also compared the effects of 6-OH-BDE 47 with its parent compound BDE -47.

Results

Effects of 6-OH-BDE 47, BDE 47 and T3 on pigmentation in the eye of zebrafish embryos

Under laboratory conditions, pigmented eyes were first observed in control (i.e. unexposed) zebrafish embryos at 22 hpf; and, thereafter, levels increased gradually until a maximum was reached around 96 hpf. For this study, we initially selected 30 hpf as the point of reference for measurement and comparison of melanin pigmentation, in intact embryos, because this was the earliest point when eye pigmentation appeared uniform in whole animal preparations. In later studies we compared eye structure at 22 hpf in all groups and we performed THRβ expression measurements in dissected eyes of all groups.

Both exposure to 6-OH-BDE 47 and T3 significantly decreased eye pigmentation at 30 hpf (**Fig. 1**). Relative to control, exposure to 1-, 10- or 100 nM 6-OH-BDE 47 resulted in decreased eye pigmentation by 4% ($P > 0.5$), 36% ($P < 0.05$) and 84% ($P < 0.05$), respectively (**Fig. 1A–D**). Similarly, 1– 100 nM T3 exposure also significantly decreased eye pigmentation by 17–43% (all $P < 0.05$) (**Fig. 1 F–I**). No significant change in pigmentation was detectable in the 0.01 nM T3 treatment. In contrast, 1, 10 and 50 μM BDE 47 exposure resulted in no changes in eye pigmentation at 30 hpf (see supporting information) (**Figs. S1, S2, and S3**).

Localization of THRβ mRNA by WISH

We examined localized THRβ mRNA expression in early embryonic stages of zebrafish using whole mount in situ hybridization (WISH) and in paraffin sections (*in situ* hybridization). The added resolution of the latter extended our observations to intrachoroidal eye tissue (i.e., retina). However, only at later times were we able to extend observations to specific layers (2) within the retina. From analysis of our whole mounts, THRβ mRNA expression was detectable starting at the 18 somite stage (18hpf, **Fig. 2 B and C**) and in all subsequent stages (e.g. Twenty-two hpf (**Fig. 3A**) and 24 hpf (**Fig. 2D**)). Expression was localized in the periventricular zones of mid- and forebrain at 22 – and 24 hpf. From 48 hpf, expression was also found in midbrain and eye of whole mounts (**Fig. 2E and F**). When sections were reacted for *in situ* hybridization, THRβ mRNA expression was found to be

localized in 2 specific layers, the ganglion layer and the amacrine and/or bipolar cell layer of the developing retina. It was also expressed in the periventricular zone of the brain at 30, 48 hpf and 10 dpf (**Fig. 2G–K**)

Six-OH-BDE 47, BDE 47 and T3 effects on THRβ mRNA expression in zebrafish embryonic brain

To determine whether the decrease in pigmentation in zebra-fish embryos by 6-OH-BDE 47 and T3 was associated with changes in THRβ, we measured THRβ mRNA expression in embryos after exposure to 6-OH-BDE 47 or T3 at 22 hpf. We examined THRβ mRNA expression using both WISH and quantitative reverse transcription polymerase chain reaction (qRT-PCR) approaches. Results from our WISH method demonstrated that exposure to 1-, 10- and 100 nM 6-OH-BDE 47 significantly decreased THRβ mRNA expression by 41.2%, 38.2% and 45.7% in the periventricular zone (forebrain) at 22 hpf respectively (**Fig. 3B–E**). For increased localization, qRTPCR was performed on dissected eyes rather than whole embryo homogenates. This refinement showed significantly decreased THRβ mRNA expression by 33.8%, 42.4% and 46.0% at 22 hpf, respectively (**Fig. 3F**). Moreover, THRβ mRNA expression was decreased with increasing 6-OH-BDE 47 exposure (1-, 10-and 100 nM) at 48 and 96 hpf, but statistically significant at 10 and 100 nM at 48 hpf and only at doses of 100 nM at 96 hpf (**Fig. 3F; Fig. S4**). Although exposure to 0.1–100 nM T3 resulted in a slight decrease of THRβ mRNA expression in the periventricular zone (forebrain) of 22 hpf embryos (**Fig. 4B–D**), the differences were not statistically significant using WISH (**Fig. 4E**).

However, significant decreases were shown when qRT-PCR of homogenates of dissected eyes were conducted. In these experiments, THRβ mRNA expression of T3 exposed individuals decreased in a concentration dependent fashion, by 19.3% ($P < 0.05$), 67.0% ($P < 0.05$), and 55.3% ($P < 0.05$) at 22 hpf, respectively (**Fig. 4F**). With exposure to 6-OH-BDE 47 THRβ mRNA expression decreased with increasing exposure to (1-, 10-and 100 nM) at 48 and 96 hpf, but was only statistically signifi-cant at doses of 100 nM at 48 hpf and 1, 10 and 100 nM doses of T3 at 96 hpf (**Fig. S4**). In addition, data generated from RTPCR at 22 hpf also demonstrated decreasing expression of THRβ (**Fig. 4F**). By comparison, exposure to 1, 10 and 50 μM BDE 47 had no significant effect (only a trend) on THRβ mRNA expression relative to control embryos and no difference was found by RT-PCR (total RNA from whole embryos) at 22 hpf relative to control embryos (see supporting information) (**Figs. S5 and S6**).

Effects of THRβ morpholino knockdown and mRNA over expression on pigmentation in eyes of zebrafish

THRβ morpholino oligonucleotides (MOs) and mRNA overexpression were used to investigate phenotypes produced from repressed expression of THRβ. We observed no changes in the control-MO and in the THRβ mRNA over expression group, but the THRβ knockdown group showed significantly decreased pigmentation (37.3%) in the eyes at 30 hpf compared to control embryos (**Fig. 5A, B and D**). In addition, there was 1.7 times more TUNEL-positive cells (i.e. apoptotic cells) in the eye at 22 hpf compared with control embryos (**Fig. 5A, B and D**).

Next, we sought to determine whether overexpression of THRβ mRNA would rescue eye pigmentation after exposure to 6-OH-BDE 47. Partial rescue of these effects was demonstrated by a much smaller decrease of eye pigment (**Fig. 1K and L**). The THRβ mRNA injected group showed 82% and 58% ($P < 0.05$) more eye pigmentation when exposed to 10 nM and 100 nM 6-OH-BDE 47, respectively, than did the non-injected treatments (**Fig. 1E and K**). Likewise, THRβ mRNA injection recovered eye pigmentation by 93% and 83% ($P < 0.05$) in 10 nM and 100 nM T3 exposed groups, respectively (**Fig. 1J and L**).

Importantly, THRβ mRNA overexpression also rescued the THRβ MO effect, where no significant difference in melanin pigmentation was observed (**Fig. 5C and D**, Panel 1). Nor was there a difference in the number of apoptotic cells (**Fig. 5C and D**, Panel 2) when compared to control-MO treated embryos.

Effects of 6-OH-BDE 47 or T3 on apoptosis in developing retina of zebrafish embryos

To localize the position of apoptotic cells in the eye of zebrafish embryos at 22 hpf, we carried out TUNEL staining on tissue sections of paraffin-embedded embryos. TUNEL-positive, brown colored i.e., apoptotic cells were exclusively found in the retina (**Fig. 6A–J**, Panel 1, same below). Rare to no apoptotic cells were observed in retina of controls (**Fig. 6A**), indicating a low background level of apoptotic cells in the developing eye, at this time. In the THRβ mRNA group, the number of TUNEL-positive cells remained low (**Fig. 6F**). An increase in the number of apoptotic cells in the retina was detected in 10 and 100 nM 6-OH-BDE 47, and 10 and 100 nM T3 exposed fish (**Fig 6B-E**). The effect of 6-OH-BDE 47 and T3 was also rescued by over-expression of THRβ mRNA (**Fig 6G-J**). In rescued animals, the number of apoptotic cells decreased to a level comparable with the control (**Fig. 6A**). Pigment (melanin) was seen as thin black lines of varying length and intensity. Note that the pigmentation seen in the 10 nM 6-OH-BDE 47 is similar to that of the controls (**Fig. 6A** versus **6B**). However, when the exposure was increased to 100 nM pigment was decreased (**Fig. 6C**). In contrast the lack of resolution in WISH specimens (whole embryos described above) did not permit such findings. Similar concentration dependent differences were seen with T3 (**Fig. 6D** versus **E**). In **Fig. 6F**, the effects of THRβ mRNA rescue are shown. The similarity of **Fig. 6A** and **6F** are readily apparent. When THRβ mRNA was used, the effects of 6-OH-BDE 47 were blocked (compare **Figs. 6G and H to B and C**). Similarly, when THRβ mRNA was administered with T3, rescue was apparent (compare **Figs. 6I and J to 6D and E**).

Whole mount, TUNEL staining, of zebrafish embryos at 22hpf showed few apoptotic cells in eyes of control and 1 nM 6-OH-BDE 47 treated group (**Fig. 6**, Panel 2, same below). Significant increases in the number of apoptotic cells were detected in 10 and 100 nM 6-OH-BDE 47 exposures at levels that were 3.1 ($p < 0.05$) and 5 times ($p < 0.05$) above control, respectively (**Fig. 6G**). However, THRβ mRNA injected embryos (**Fig. 6C and F**) did not show a significant increase in apoptotic cells after exposure to 6-OH-BDE 47. When THRβ mRNA was quantitatively expressed (**Fig. 6**. G- Histogram) a relationship to apoptosis was seen. Administration of THRβ mRNA rescued the apoptotic effect.

Similar to 6-OH-BDE 47, T3 induced apoptosis in the retina of zebrafish at higher exposures. Few apoptotic cells were found in the control and 0.1–10 nM T3 treated groups (**Fig. 6A, B, and D**, Panel 3, same below); but, in the 100 nM T3 exposure, apoptosis was increased by 2.44 times ($P < 0.05$) (**Fig 6E and G**). Likewise, THRβ mRNA injection rescued these effects in the 100 nM T3 treatment (**Fig. 6F and G**). Compared to 6-OH-BDE 47 and T3, 1, 10 and 50 μM exposure to BDE 47 failed to induce any significant increase in apoptosis by TUNEL staining at 22 hpf relative to control embryos (supporting information) (**Figs. S7 and S8**).

To determine presence of alterations within the eye at 22hpf, we fixed embryos for longer durations and prepared sections at 5 μm (see Methods). Sections stained with hematoxylin and eosin revealed elon-gated nuclei of retinal cells. Control eye structure showed abundant cells within lens and at the lens margin. However, at this time of development, the definitive layering, seen later at 96 hrs is not present. **Figure 7A** shows the high cellularity of the retina and the pigment layer of the retina and surrounding chorion. The lens contains a dense collection of cells at the periphery and the centrally located cells are showing vacuolation and pyknotic change in some nuclei (**Fig. 7D**). A single cell is shown in the future ganglion cell layer that has the morphologic features of an apoptotic cell (**Fig. 7D arrow**). The nucleus is pyknotic and there is enhanced eosinophilia in the cytoplasm. It is well recognized that cells undergoing apoptotic cell death reveal a characteristic sequence of cytological alternations including membrane blebbing and nuclear and cytoplasmic condensation and that these can be seen with light microscopy in survey histo-pathology.[28] When eyes from T3 exposed embryos were examined (**Fig. 7B and E**), fewer cells were seen in the retina and the bases of cells near the pigment layer revealed spaces (red arrows). Pyknotic cells (arrow head) were more numerous in the retina. The enlarged **Figure 7E** shows vacuolation in the central lens and less cells at the periphery. Pigmentation was greatly diminished and gaps were seen within this structure. Exposure to 6-OHBDE 47 led to the most striking alterations. Pigment was greatly diminished and seen as a thin and irregular line (**Fig. 7C**) that, under oil immersion, was revealed as a collection of punctate pigment. Also, in the enlargement (**Fig. 7F**) numerous vacuoles and high amplitude swelling characterized cells of the future ganglion layer. Pyknotic cells were enhanced compared to control and T3 exposed individuals.

Discussion

Our results demonstrate that 6-OH-BDE 47 exposure in embryonic zebrafish led to reduction in pigmentation, THRβ expression, and increased apoptosis in the retina. These effects were similar to effects elicited by T3, and were rescued or partially rescued by overexpression of THRβ, suggesting that 6-OH-BDE 47 may be acting as a T3-mimic. Combined, these data suggest that 6-OH-BDE 47 may be exerting certain adverse effects through thyroid hormone dysregulation, perhaps via down-regulation of the THRs.

Thyroid hormones (THs) are very important, regulating many cellular functions.[29,30] THs are not only a trigger for vertebrate metamorphosis, they are critical to proper assembly of the central nervous system.[31-34] THRβ in the brain and sensory systems mediates both hormone-dependent and hormone-independent actions with changing TH levels during

development, providing a switch between repression and activation.[35] THRβ mRNA expression in the retina has been reported in the mouse, and is hypothesized to play a role in protecting the retinal cells from fluctuations in thyroid hormone levels. Furthermore, THRβ mRNA was expressed in the outer nuclear layer of the embryonic retina of rodents[35] where it was hypothesized that THRβmRNA caused the selective loss of M cones, disturbing the gradient of cone distribution.[35,36] Using in vitro studies of fetal human retinal cell lines, THs were shown to elicit responses similar to those of rodent retinal cells, indicating that these effects are conserved across species.[37] Alterations in layers of the zebrafish retina were also observed following exposure to DE-71, a commercial PBDE mixture.[38] Therefore, this data plus our findings appear to be a link between THR expression/activity and retinal development in various vertebrate species, including zebrafish.

In the present study, we found that THRβ mRNA was first expressed in the brain area of zebrafish embryos from the 18 somite stage (18 hpf) where it was localized in periventricular zone from 22 hpf until 48 hpf (**Fig. 2**). Expression of this gene was later found in the retina and mid-brain from 30 hpf with strong expression at 48 hpf (**Fig. 2F**). Our findings complement the report of THRβ expression in the retina of flounder (Pleuronectes americanus) up to 21 d after hatching.[39] The localized expression of THRβ further supports the role and importance of THs in retinal development. However, additional studies are needed to establish this brain to retina developmental linkage.

We also investigated the impacts of 6-OH-BDE 47, BDE 47 and T3 on THRβ mRNA expression simultaneously using both WISH and RT-PCR approaches. These 2 techniques provided similar results. A significant reduction in THRβ mRNA expression at 22 hpf followed exposure to 6-OH-BDE 47 or to T3. Analyses using RT-PCR rely upon homogenates of whole embryos; and, tissue specific changes are likely diluted by this whole animal approach. Thus, in this case, WISH techniques here provided a greater resolution in identifying changes in gene expression in the eye.

Zheng et al. also reported that 6-OH-BDE 47 decreased THRβ mRNA expression in entire zebrafish larvae using RTPCR.[12] Others used transient transfection assays,[37,38] and showed that several PBDE congeners (BDE-100, BDE-153, BDE-154, BDE-209, and DE-71) but not BDE-99, BDE-47, or 6-OH-BDE 47 suppressed THR-mediated transcription in CV-1 monkey fibroblast-derived cells. Furthermore, this group suggested that PBDE congeners might disrupt normal brain development.[40,41] Liu et al. found 5 nM T3 exposure decreased THRβ mRNA expression after 24 hours, and induced TR β mRNA expression at 72 hpf and 144 hpf.[42] In one study, T3 exposure had no affected on THRβ mRNA expression during early embryogenesis.[43] This apparent discrepancy between studies may be due to use of different methods and/or tissue selections. A table summarizing the various published studies investigating effects of T3 exposure on THRβ expression is included in the Supporting Information (S9 Table 1). Our results also demonstrated that in T3 and 6-OH-BDE 47 exposed embryos, pigmentation was decreased in the eye at 30 hpf and THRβ mRNA expression was decreased in the brain at 22 hpf in the 6-OH-BDE 47 exposed embryos. Whereas the control eyes showed a well-established pigment layer (**Fig. 7A**), pigment layers in T3 and 6-OH-BDE 47 exposed individuals were thinner, revealing gaps or punctate pigment in non-connected patterns (**Fig. 7E and F**). One finding in T3 exposed

individuals (**Fig. 7E**) was the presence of large, clear apparently swollen areas at bases of elongated retinal cells and adjacent to pigment. We had previously hypothesized, and now proven, that these effects were driven by T3 mimicry by 6-OH-BDE 47, resulting in a downregulation of THRβ. However, these same effects (particularly decreases in pigmentation) could also potentially be driven by delayed development. To examine this possibility, we used eye area as a metric for developmental stage (**Fig. S6**). Interestingly, we saw no reduction in this parameter over the doses used for 6-OH-BDE 47 or T3 used in this study (**Figs. S6 and S7**). It should be noted that decreased eye area was encountered only after exposure to higher concentrations (250 nM supplemental data). Therefore, it does not appear that a general developmental delay was driving this phenotype. Further observations following sections of whole embryos and of dissected eyes, raised questions regarding developmental delay being combined with compound-induced effects of increased cell death to bring about overall changes. Higher magnification of lens and adjacent retina showed cells with pyknotic nuclei and enhanced eosinophilia of cytoplasm. These are characteristics of apoptotic cells.[28] In the control lens (**Fig. 7D**) numerous vacuoles of diameter equal to adjacent nuclei were seen. In addition, pyknotic nuclei were abundant. This is similar to the studies of Cole et al.[44] who investigated apoptosis in developing lens of zebrafish at the same, 22 hpf, developmental stage. Fewer nuclei were seen in lens of the T3 and of the 6-OH-BDE 47 exposed individuals (**Fig. 7B, E, and C, F** respectively). This could represent a delay in lens development or damage by exposure. The more striking changes were seen in the developing retina in which the T3- exposed embryos and especially the 6-OH-BDE 47 exposed individuals showed fewer cells in the outer layers (**Fig. 7E and F**). In addition, the 6-OH-BDE 47 exposed individuals revealed abundant signs of cell alteration including high amplitude swelling and more numerous apoptotic cells in the developing retina (**Fig. 7F**). To review, our findings suggest that delayed development occurred in the lens was based on the reduced proliferation of outer layers of the developing retina, decreased number of cells in the lens (problematic since loss of lens cells is a feature of developing lens[44]) while fewer lens cells in treated individuals might signify an initial delay. Damage to eye was evidenced by alterations in cells of retina and the swollen spaces between outer retinal cells and the pigment layer. These findings clearly indicate responses within the eye of exposed individuals.

To better understand the role of THRβ in producing this phenotype, we used mopholino gene knockdown to silence this expression. Our knock down reduction of melanin pigmentation in the retina of zebrafish embryos was similar to the effects of 6-OH-BDE 47, although the reduction was stronger with exposure to the metabolite. This important finding suggests that the developmental effects of 6-OH-BDE 47 are in large part mediated by down regulation of THRβ. Further studies are needed to examine the effects on THRα.

Previous studies have examined the impact of PBDE exposure in the retina of other species. When Xenopus tadpoles were exposed to C-BDE-99 (specific radioactivity 49 Ci (1813 GBq)/ mol with BDE-99), radioactivity in the melanin lining of the retina was observed after 1.5 days, indicating that transport and accumulation of BDE-99 had occurred in that region.[14] In our study, we found increased apoptosis in the developing retina following exposure to 6-OH-BDE 47, and a similar effect was observed for T3 (**Figs. 6–2 and 6–3**).

Apoptosis is a common response to disease and chemical toxicity. In this study, 10 nM to 100 nM 6-OH-BDE 47 resulted in increased apoptosis in the intrachoroidal tissue of the eye. At the same time frame, decreased THRβ mRNA expression was observed in the brain of zebrafish embryos.

Usenko et al. reported that 3 isomers of OH-BDE 47 resulted in increased cell death and oxidative stress in the tail of zebrafish larvae at 28 hpf. Embryos proved most sensitive to 6-OH-BDE 47 compared to the other 2 congeners.[45] Similarly, cell culture studies in which 6-OH-BDE 47 induced apoptosis and inhibited cell proliferation also suppressed neuronal and oligodendrocyte differentiation in adult neural stem cells.[46] BDE-47 has also been reported to induce apoptosis and DNA damage in primary cultured rat hippocampal neurons.[47] In amphibians, a gradual increase in TH concentrations helps to regulate the transformation that occurs during metamorphosis.[48,49] During metamorphosis natural apoptosis occurs as the intestine is remodeled and the tail and gills are assimilated. In this process, apoptosis was first found in the tail muscle cell at NF-stage 58 and peaked at NF-stages 61–63,[50] when endogenous T3 and T4 were also found at peak levels (5–10 nM). These observations may suggest that exposure to T3 at 30 hpf in zebrafish resulted in increased apoptosis through a similar mechanism that occurs during metamorphosis in amphibians.

These reports, together with our results, indicate that exposure to 6-OH-BDE 47 or T3 leads to an increase in apoptosis in the retina (**Figs. 6–1, 6–2 and 6–3**) and subsequently decreased melanin production. Importantly, both the effect and pathway may be common in a broad spectrum of vertebrate species. A possible mechanism by which 6-OH-BDE 47 or T3 may induce apoptosis at the brain and retina of zebrafish embryos is via generation of nitric oxide (NO) (**Fig. 8**).[51–54] THR are ligand-dependent transcription factors that regulate cell differentiation, proliferation, and apoptosis.[15,55,56] In turn, T3 and the thyroid hormone response elements regulate functions of THR.[57] In THRβ knockout mice, higher amounts of nitrated proteins and nitrotyrosine were found, and this increased nitrosative stress in turn promoted apoptosis.[50,58] The exact mechanisms producing the apoptosis in the present study are unclear, but may be related to increased oxidative stress, although other pathways may have a role in this response.[59]

In the present study, we also found that BDE 47 was consistently less toxic than its metabolite, 6-OH-BDE 47, in short-term exposures, which is consistent with the reports from Usenko.[12] In a recent study, embryo lethality in zebrafish exposed to one of 3 hydroxylated PBDEs (3-OH-BDE 47, 5-OH-BDE 47 and 6-OH-BDE 47) was examined. Of these 3 compounds, 6-OH-BDE 47 produced the greatest lethality.[45] Of interest, this congener was detected in >67% of blood samples collected from pregnant women in the US,[6] and highlights a need to better understand the developmental toxicity of this compound.

In conclusion, our study found that exposure to 6-OH-BDE 47 decreased THRβ mRNA expression, increased apoptosis and reduced melanin pigmentation in the eye, and THRβ mRNA over expression rescued these effects. Decreased THRβ expression is known to promote higher levels of NO, which can induce nitrosative stress and lead to apoptosis. Subsequently, melanin pigmentation was decreased at 30 hpf in retina of zebrafish embryos,

perhaps due to increased apoptosis in the retina. To fully understand this mechanism, future studies will be required to study effects of these exposures on additional genes, including cholinergic-related genes,[60] which are important pro-apoptotic genes and their analysis will elucidate where and how 6-OHBDE 47 induces apoptosis. Further studies may also be warranted to investigate the effects of OH-BDEs on fetal development regulated by thyroid hormones.

Materials and Methods

Fish care

Adult wild type 5-D zebrafish (Danio rerio) were maintained in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) at 28°C under a 14:10 light:dark cycle. Adult fish were fed brine shrimp and Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats, USA). Embryos were collected after natural spawning of adult zebrafish and were maintained in 30% Danieau solution in petri dishes in an incubator under the same conditions as the adults. All care and reproductive techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A053–10–03).

Dosing

Two, 2′,4,4′ tetrabromodiphenyl ether BDE-47, and 6^{0}-hydroxy-2,2′,4,4′ tetrabromodiphenyl ether (6-OH-BDE 47) were purchased neat (>99 .5% purity) from Accustandard (BDE 047s, HBDE-4005S-CN-0.2X). Triodothyronine (T3) was purchased from Sigma-Aldrich (IRMM469–1EA)). Stock solutions of 1 mM concentration were prepared by dissolving an appropriate amount of the neat standard in dimethylsulfoxide (DMSO; Sigma; 472301–100ML). Exposure solutions were prepared via serial dilution from stock solution with fish culture system water with final concentration of DMSO < 0.1%. Zebrafish embryos were exposed to 3 concentrations of 6′-OH-BDE-47 (1, 10 and 100 nM) or T3 (0.01, 0.1, 1, 10 and 100 nM) from 4 h post fertilization (hpf) until 22 or 30 hpf.

Whole mount and section *in situ* hybridization

Upon reaching desired life stages, zebrafish embryos were fixed in 4% (w/v) paraformaldehyde (158127–500G) in phosphate saline solution (pH 7.4) overnight, dechorionated using watchmaker's forceps, and then stored at −20°C in methanol. Whole mount *in situ* hybridization (WISH) and section *in situ* hybridization were carried out according to our previously reported methods.[9,61] Embryos were hybridized with an anti-sense probe of 1161 base pairs for zebrafish thyroid hormone receptor β (THRβ). THRβ probe was cloned with the following primers: forward primer, 5′-ATGTCAGAGCAAGCAGACAAATGC-3′; reverse primer, 5′-TTCCTGGAAGTGTTTGAAGAC-3′. Following hybridization overnight at 64°C, embryos were washed with 2× SSC (300 mM NaCl(S7653– 5KG), 30 mM sodium citrate (71497–250G), pH 7.0) and then 0.2× SSC for 30 min, twice respectively. Next the embryos were blocked with 2% blocking reagent (Roche, Mannheim, Germany; 10057177103).

Bottom of Form), then incubated overnight with 3000× diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche; 11082736103) at 4°C. Lastly, the color reaction was carried out by incubation with BM-purple substrate according to manufacturer's recommendation (Roche; 11442074001).

Expression localization and quantification

THRβ mRNA expression was recorded with a Nikon Eclipse E600 light microscope, a Nikon DXM 1200 digital camera, and EclipseNet imaging software (Nikon; Capture NX 2). To best identify sites of THRβ expression indicated by WISH, locations of these sites were compared and referenced with histologic sections of zebrafish of appropriate ages using the online Zfish atlas (http://zfatlas.psu.edu/progress.php).

For quantifying intensity of THRβ expression, the following procedure was used. First the area, or tissue, of greatest staining intensity was outlined by ImageJ Analysis Software (National Institutes of Health; ImageJ) and the sum of intensity (reaction product plus background) was determined in this region of interest.[62] Next, the outline was moved to an adjacent region/tissue of the embryo that showed no reaction product and the background intensity was determined. This was then subtracted from the ROI. Using the sum intensity and dividing by the ROI pixel area, the mean intensity was calculated. A minimum of 5 images were examined per developmental stage per treatment.

To determine whether we could localize our findings to specific layers of the retina, we consulted the Zebrafish Lifespan Atlas (K.C. Cheng Laboratory, Penn State University)[63] http://zfatlas.psu.edu/ for survey histology of the developing eye including retina. Briefly our results are as follows:

At 48 hpf and examining coronal sections, the specific layers of the developing zebrafish retina are not differentiated. http://zfatlas.psu.edu/view.php?atlas=48&s=420. However, the choroid is apparent and we have referredto intrachoroidal tissue – independent of the lens- as the developing retina. See for example **Figure 2J and K**. At 72 hpf and in coronal plane, the specific layers are more apparent. http://zfatlas.psu.edu/view.php?s=30. At 96 hpf and in a coronal plane, the specific layers of the retina are differentiated. This is shown in the following section.

Based on the above, THRβ mRNA was localized (**Fig. 2K**) to 2 distinct layers on either side of the inner plexiform layer. The first, nearest the lens, is likely the ganglion cell layer and the second is within the amacrine cell and bipolar cell layers. It is entirely possible that expression – within the retinal pigmented epithelium and choroidal melanocyte layers would be obscured by presence of pigment. In a similar fashion, our experiments to rescue, with THRβ mRNA, the apoptosis caused by 6-OH-BDE 47 or T3 were in eye (whole mount- in which discrete TUNEL reactions were counted) and in sections general retina (not layer specific due to the 22 hpf age of embryos).

Total RNA extraction, cDNA generation and *TaqMan*® gene expression assays

Total RNA was extracted from dissected eye tissues of pooled embryos (n &Equals; 20) (for 6-OH-BDE 47 and T3 groups) or whole embryos (for BDE 47 group) with TRIzol®

Reagent according to manufacturer's protocol (Grand Island; 12183– 555). Quality of RNA samples was analyzed by measuring the ratio of absorptions at 260/280 nm. The purified total RNA was immediately used for cDNA synthesis. Generation of cDNA was performed with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems Inc.; 4368814) following the reverse transcription protocols of the manufacturer. The cDNA samples were kept frozen at −20°C until use.

Gene expression was measured using TaqMan® Gene Expression Assays (Applied Biosystems Inc.; 4331182). Reverse transcription reactions were performed after adding 0.2 ng cDNA mixed with TaqMan Universal PCR Master Mix, and analyzed by PCR on the 7900HT instrument using Applied Biosystems Sequence Detection System 2.0 software according to the manufacturer's instructions. Target gene expression data from samples was normalized using 18S RNA. This internal standard compensated for intrinsic variability in the amount of RNA between embryos.

MorpholinoInjections and THRβ mRNA overexpression

THRβ translation blocking (GCAGTATGTCAGAGCAAGCAGACAA, THR-MO) and 5-bp mismatch control (GgAGaATGTCtGAGCtAGCtGACAA) morpholinos (Control- MO) were designed with Gene Tools, LLC. Morpholinos were diluted in sterile dH_2O to a stock concentration of 100 μM and further diluted to 10 μM. One nL THR-MO or Control- MO morpholino was injected into the 1–2 cell stage embryo (1 hpf). Morpholino concentration was checked by standard PCR. Both Control- MO and THR-MO morpholinos were labeled with a fluorescent dye to allow examination of distribution of MO after injection with a fluorescent microscope. In subsequent analysis, only the embryos exhibiting widespread fluorescence by 4–24 hpf were kept. To test the role of increased THR-b mRNA in mediating toxicity of 6-OH-BDE-47 and T3, embryos were injected with ~3nL of corresponding mRNA in the 1–2 cell stage according to published methods[64] using a microinjection system consisting of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc.., Lewisville, TX, USA) and a Narishige IM300 Microinjector (Narishge, Japan). THRβ mRNA was synthesized with SP6 polymerase and capped using a G(5')ppp(5')A RNA cap structure analog (New England Biolabs). Injection mRNA concentration was ~265 ng/uL. Phenol red was added to RNA samples before injection (0.05% final concentration) in order to track successful injections.

Apoptosis measurement (TUNEL assay)

At 22 hours post fertilization (hpf), embryos were fixed in 4% PFA overnight, followed by terminal deoxynucleotidyl transferasemediated biotinylated UTP nick end labeling (TUNEL) staining as described previously.[65] Positive TUNEL signals were detected with ABC kit applied (Elite, Vector; PK-6100) and DAB solution added to visualize the apoptotic cells. TUNEL-positive signals in both eyes were counted for each control, THR-MO, THRβ mRNA and/or 6-OH-BDE 47 or T3 treated embryos. Numbers of discrete TUNEL positive reactions per eye of each of 10 individual embryos are shown.

Histological Methods—A total of 5 individual embryos per treatment at 22 dpf (control, or 100nM T3 or 6-OH-BDE 47) were killed by overdose in MS-222 (Sigma-Aldrich;

E10505–5G), immediately transferred to vials containing fixative, 4% paraformaldehyde (Sigma-Aldrich; P6148–500G) in phosphate-buffered saline (PBS, pH 7.2–7.4) and held at 4°C in buffer until transport to the Histology Laboratory, College of Veterinary Medicine, North Carolina State University. All subsequent steps including dehydration, clearing, orientation (left lateral recumbency), embedment, sectioning (5 μm), mounting on glass slides, and staining (Hematoxylin and eosin) were performed by staff in the Histology Laboratory). Each paraffin block contained all 5 embryos for each treatment and were serial sections throughout the entire embryos. In this way, high resolution light microscopy of eyes with emphasis on lens and developing retina enabled detailed observations on eye-specific changes. Upon receipt of the sections, 3 individuals read each of the slides and then concurred on alterations observed. Photographic analysis and recording of image data were done as described above. Images at 300 X and at 900 X magnification (oil immersion) were assembled in **Figs. 7A–F**.

Statistical analyses

All expression data are expressed as mean ± SEM. GraphPad Prism software was used for statistical analysis. Differences between means were analyzed using one-way ANOVA followed by Dunn's test. Any p value < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PBDE	polybrominated diphenyl ether
OH-BDE	hydroxylated polybrominated diphenyl ether
DMSO	dimethyl sulfoxide
hpf	hours post fertilization
dpf	days post fertilization
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
qRT-PCR	quantitative reverse transcription polymerase chain reaction
THs	thyroid hormones
T4	thyroxine

T3	triiodothyronine
THR	thyroid receptor
WISH	*in situ* hybridization
MOs	morpholino oligonucleotides
NO	nitric oxide

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Highlights

- 6-OH-BDE47 and T3 decreased pigmentation in eye of zebra-fish embryos.
- 6-OH-BDE47 and T3 induced apoptosis in retina, and intra choroidal eye of zebrafish embryos.
- 6-OH-BDE47 and T3 decreased THRβ expression in zebra- fish embryos.
- THRβ mRNA overexpression rescued the adverse effects of 6-OH-BDE 47 in zebrafish embryos.

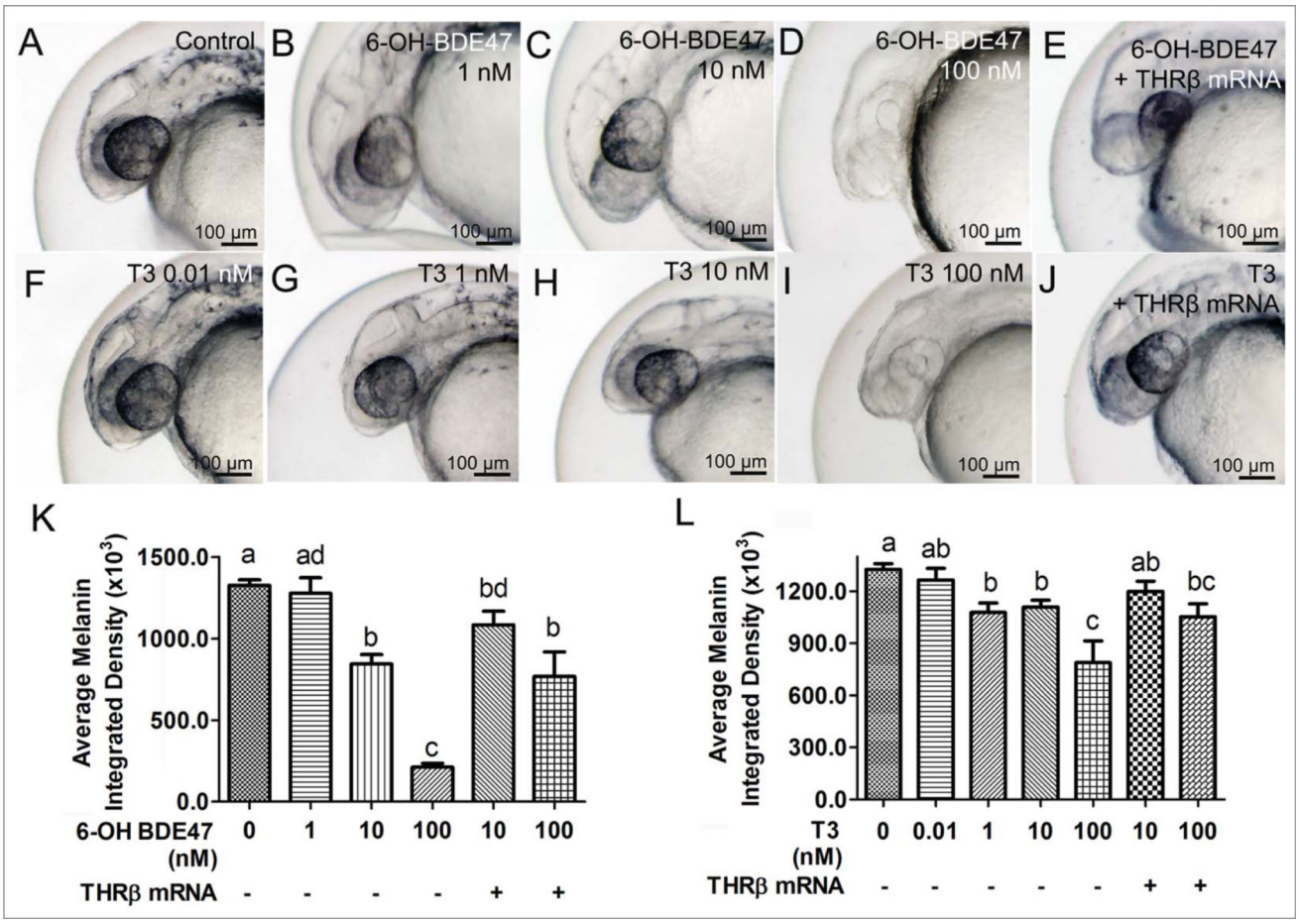


Figure 1.

Relative pigmentation intensity in eyes. Embryos, with and without injection of THRβ mRNA at 1–2 cell stage, exposed to an aqueous solution containing 6-OH-BDE 47 (1, 10 and 100 nM) or T3 (0.01–100 nM) from 4 – to 30- hpf are shown. Levels of eye pigmentation (black coloration intensity) were quantified using ImageJ as stated in the methods. (**A–J**) Various representative embryos from different exposure groups. (**K**) (6-OH-BDE-47) and (**L**) (T3) are histograms of pigment intensity (integrated density) established from quantification of 20 embryos per group. Different letters indicate signifi-cant differences ($P < 0.05$; SEM indicated by vertical bar for each graph).

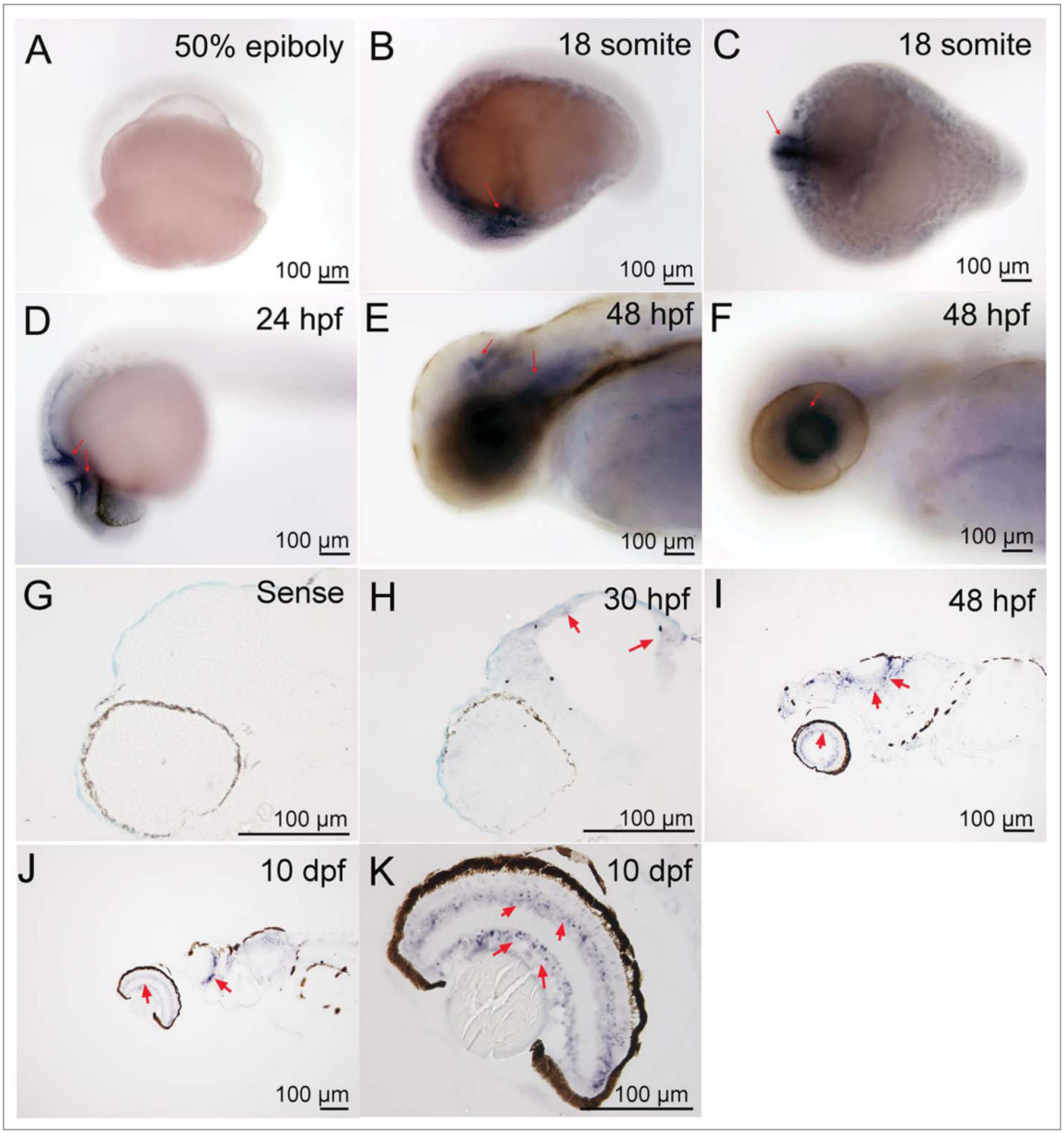


Figure 2.

Expression of THRβ mRNA in different developmental stages of zebrafish embryos and larvae as shown by blue coloration in in-situ hybridization with whole mount or section. All scale bars are 100 μm. A-F shows whole mount in situ hybridization, G-K shows section in situ hybridization. (**A**) 50% epiboly; (**B**) (lateral orientation) and (**C**) (view showing rostral embryo and yolk sac) at 18-somite stage; (**D**) (24 hpf), (**E and F**) (48 hpf). (**G**) (30 hpf) control sense probe, (**H**) (30 hpf), (**I**) (48 hpf), (**J and K**) at 10 day stage. A has no expression. Red arrows (**B–F and G–K**) indicate the location of expression. Figures indicate that expression of THRβ is seen beginning at the 18 somite stage in head region (**B and C**). With further development (24hpf) definitive mRNA expression is in retina and periventricular regions of mid- and forebrain (**D and H–K**). At 48 hpf, extensive growth of

embryo is apparent and expression is localized to periventricular region of midbrain (**H–J**). (**F**) shows greater detail in eye, with central blue localization apparent. Yellow to dark brown coloration in (**E and F**) is pigment. (**G**) Sense (control) reveals pigmentation around eye likely representing pigmented epithelial layer of retina and adjacent choroid. (**H**) (Antisense) Weak reaction in brain at 30 hpf. (**I**) 48 hpf shows THRβ mRNA localized to brain. At 10 dpf, THRβ mRNA expression in brain is similar to the 48 hpf figure. (**J and K**) Expression in brain and at higher magnification, (**K**) THRβ mRNA expression is localized to 2 layers in retina.

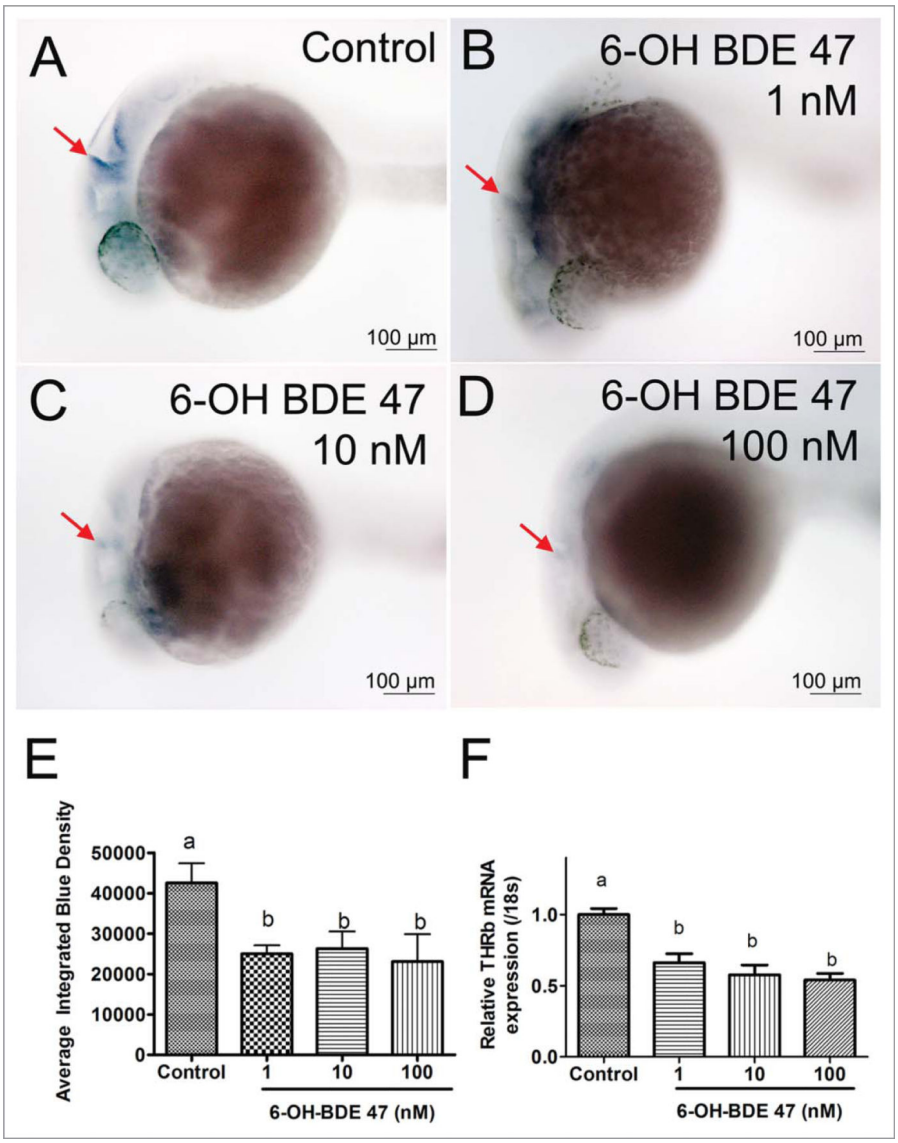


Figure 3.

Six-OH-BDE 47 decreased THRβ mRNA expression in the periventricular zone of zebrafish embryonic brain. Embryos were exposed from 4- to 22- hpf to 6-OH-BDE 47 (1, 10 and 100 nM). Note the prominent blue coloration in (**A**) (control) when compared to 6-OH-BDE exposed embryos (**B–D**). Expression was diminished at 1, 10 and 100 nM respectively. Red arrows indicate localization of expression. The average color intensity recorded in brain at 22 somite stage is shown in the histograms (**E**). (**F**) Histogram of the quantitative PCR (qRT-PCR) for THRβ at 22 somite stage. The 1, 10 and 100 nM 6-OH-BDE 47 was significantly different from the control (*, P <0.05, 3 replicates of 20 embryos each were used).

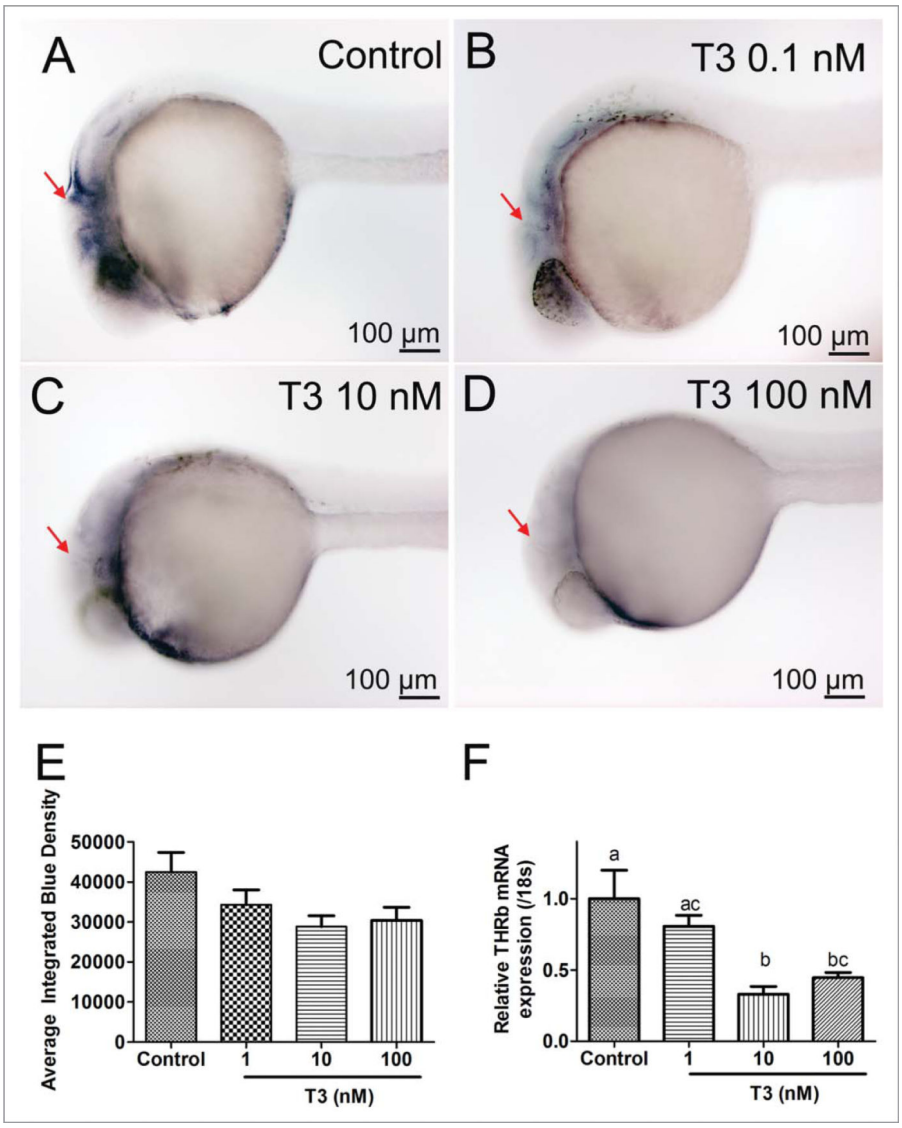


Figure 4.

Effect of T3 on THRβ mRNA expression in the periventricular zone of zebrafish embryonic brain. Embryos were exposed from 4- to 22- hpf to T3. Note the prominent blue coloration in control, (**A**) localized to fore- and midbrain regions (red arrow). By comparison, panels B,C and E show less blue coloration (red arrows) upon exposure to a range of T3 from 0.1 nM to 100 nM. The average intensity of THRβ expression recorded in brain at 22 somite stage showed dose dependent reduction (histogram, **E**). (**F**) Bar chart of THRβ expression based on quantitative PCR (qRT-PCR, total RNA from whole embryos) at 22 somite stage. The 10 and 100 nM T3 was significantly different from the control (*, $P < 0.05$, 3 replicates of 20 embryos eyes each were used). No significant differences were seen at the various concentrations.

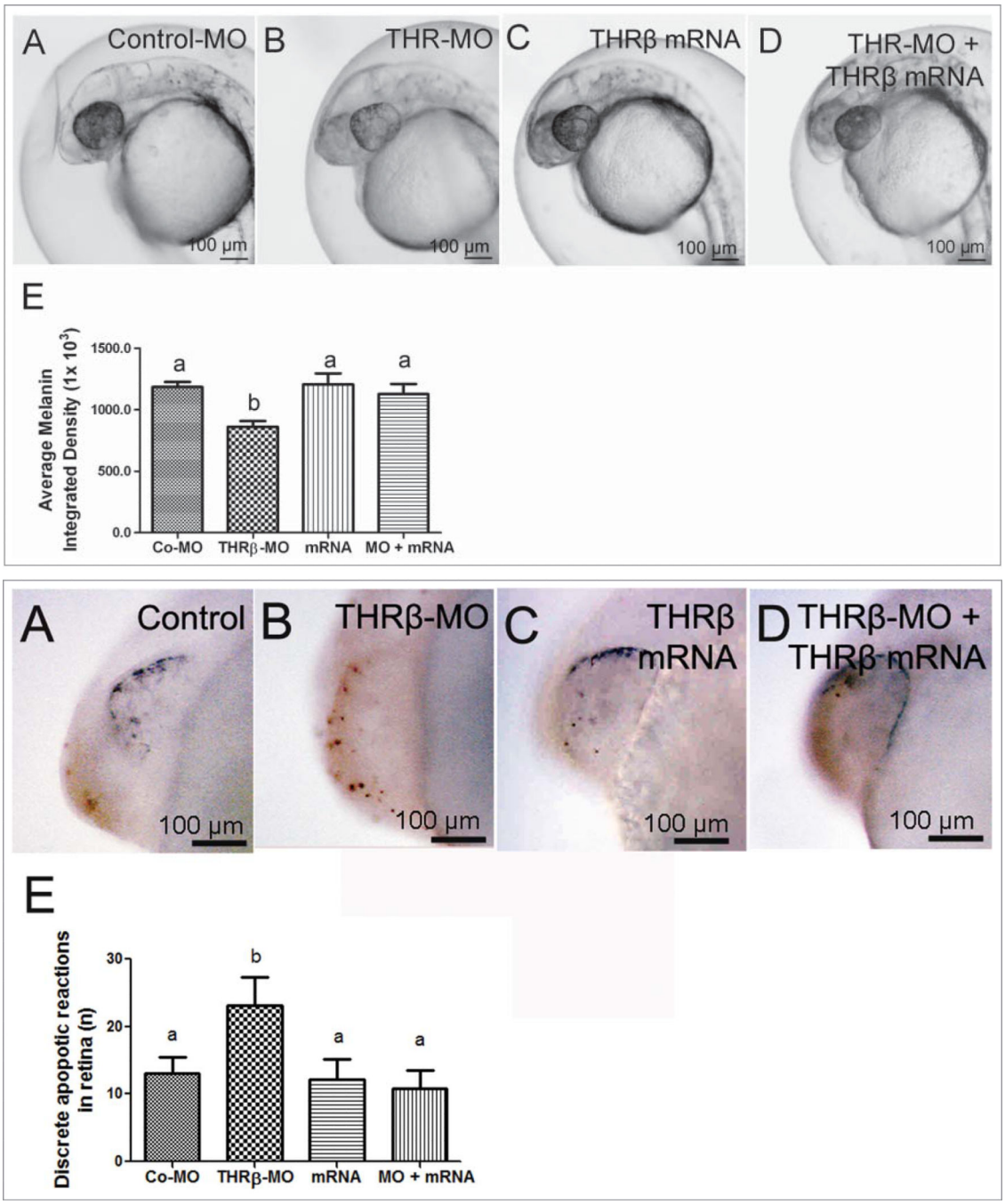
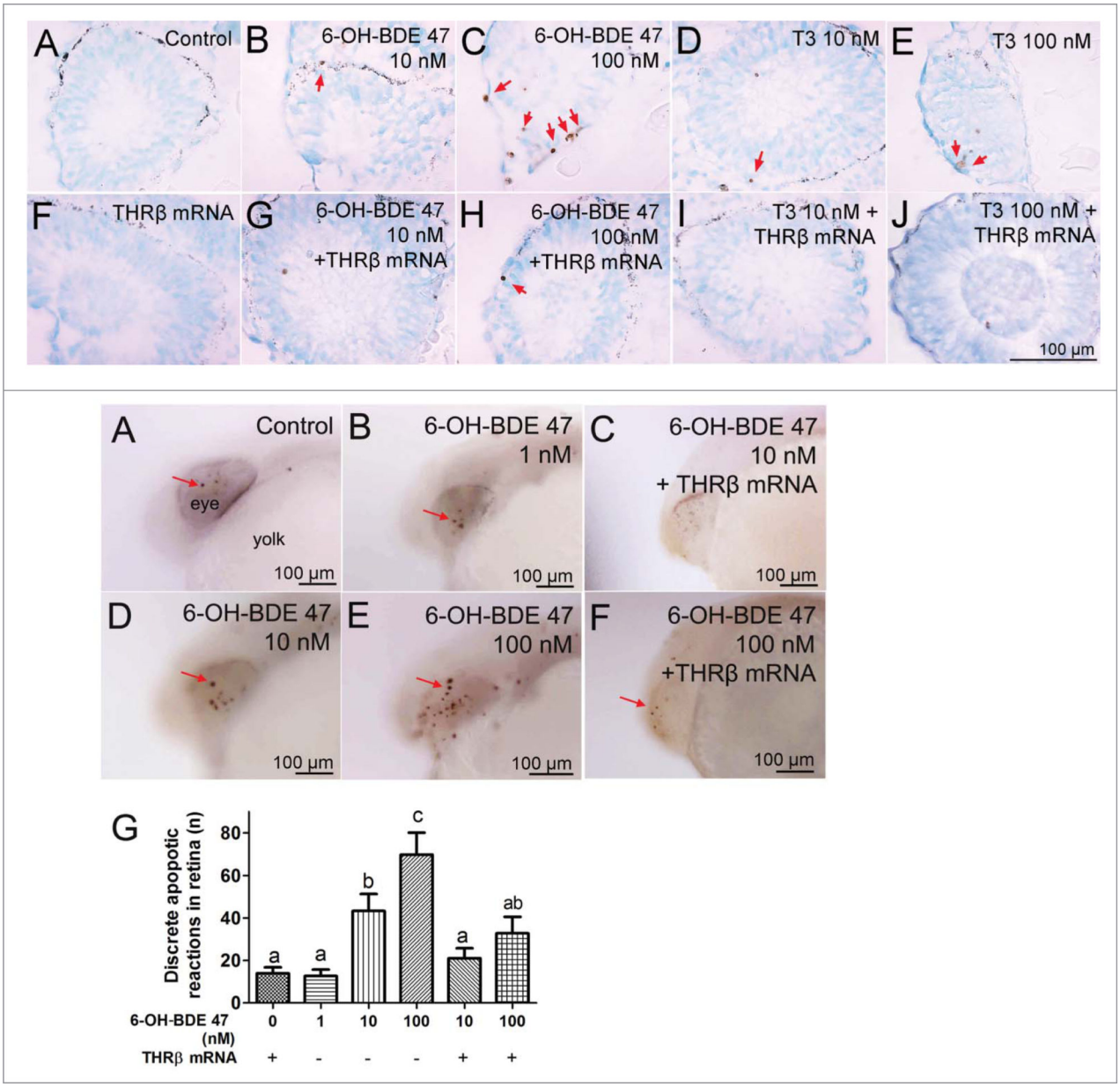


Figure 5.

Panel 1. Exposure of a THRβ morpholino to zebrafish embryos decreased eye pigmentation and THRβ mRNA rescued the decreased pigmentation in eye. After injection of morpholino and/or THRβ mRNA into the 1–2 cell stage embryos (n = 20 control and exposed) at 30 hpf and eye pigment was quantified using ImageJ. The effect of the morpholino was rescued by coexposure to THRβ mRNA. Letters in the bar graph indicate significant differences ($P < 0.05$). (**Panel 2**) Exposure of a THRβ morpholino to zebrafish increased discrete TUNEL positive reactions (apoptosis) in the retina of zebrafish embryos. THR Morpholino and/or

THRβ mRNA was injected into the 1–2 cell stage embryos (n &Equals; 20 control and exposed embryos), at 22 hpf discrete apoptotic bodies were quantified using light microscopy manually. Different letters in the bar graph indicate significant differences ($P <$ 0). Clearly, THRβ mRNA rescued the phenotype.



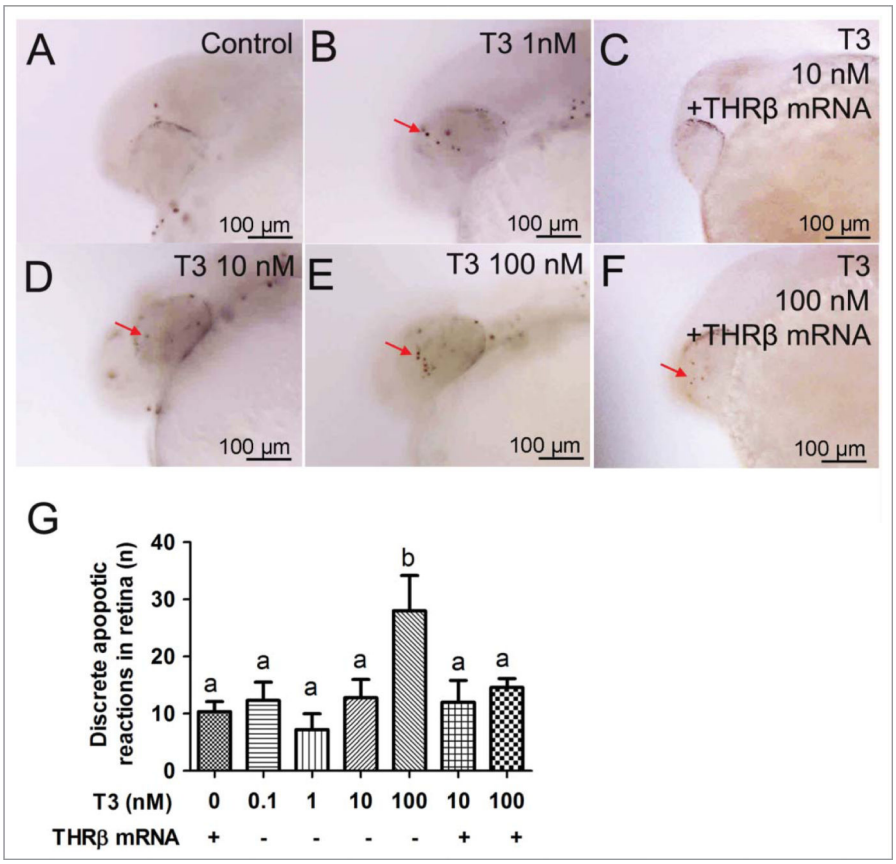


Figure 6.

Panel 1. (**A**) TUNEL staining in eyes of paraffin-embedded and sectioned embryos at 22 hpf. Embryos, with and without injection of THRβ mRNA at 1–2 cell stage, were exposed to 6-OH-BDE 47 (10 and 100 nM) or T3 (10 and 100 nM) from 4 – to 22- hpf. The location of apoptosis (brown color) was observed by light microscopy on sections. TUNEL-positive, brown colored cells (i.e., apoptotic cells) were exclusively found in the retina (**6A– J**). (**A–J**) Apoptotic cells (TUNEL analysis) in eye (red arrows). The effect of 6-OH-BDE 47 and T3 was rescued by overexpression of THRβ mRNA (**G–J**). In rescued animals, the number of apoptotic cells decreased to a level comparable with the control (**A**). Pigment (melanin) was seen as thin black lines of varying length and intensity. **Panel 2**. THRβ mRNA rescued the increased discrete apoptosis in the retina of zebrafish embryos caused by exposure to 6-OH-BDE 47. THRβ mRNA was injected into the 1–2 cell stage embryos (n &Equals; 20 control and exposed). When embryos were exposed to aqueous 6-OH-BDE 47 (1, 10 and 100 nM) from 4- to 22- hpf discrete apoptosis in eye was seen. (**A–F**) show apoptotic cells in eyes. By comparison of (**B, D, and E**), a concentration effect was seen, and THRβ mRNA rescued 6-OH-BDE 47 induced apoptosis in eye. (**G**) Histogram of the number of individual apoptotic cells in eye. Numbers of discrete TUNEL positive reactions per eye of each of 10 individual embryos are shown. Injected THRβ mRNA and/or exposure to 10 and or 100 nM concentrations resulted in quantitative differences (increase) in apoptosis that were different from values for the low concentration, control, and THRβ mRNA treated group and different from each other (different letters over histograms; $P < 0.05$). **Panel 3.** THRβ mRNA rescued

T3 induced apoptosis in the retina of zebrafish embryos. THRβ mRNA was injected into the 1–2 cell stage embryos (n &Equals; 20 control and exposed embryos), secondly, embryos were exposed by aqueous route to T3 (1, 10 and 100 nM) from 4- to 22- hpf. (**A–F**) TUNEL positive reactions, some likely involving more than one cell (apoptosis) in the eye. Comparing **Figures 6B, D, and E** to **6A, C, or F**, a general increase is suggested. (**C and F**) Show THRβ mRNA rescue of T3- induced apoptosis. (**G**) A histogram based on the number of TUNEL positive reactions in retina (n &Equals; 10 individual embryos per concentration) showed that only the 100nM concentration produced apoptotic cell numbers different from those of controls, lower concentrations and THRβ treated groups. ($p < 0.05$).

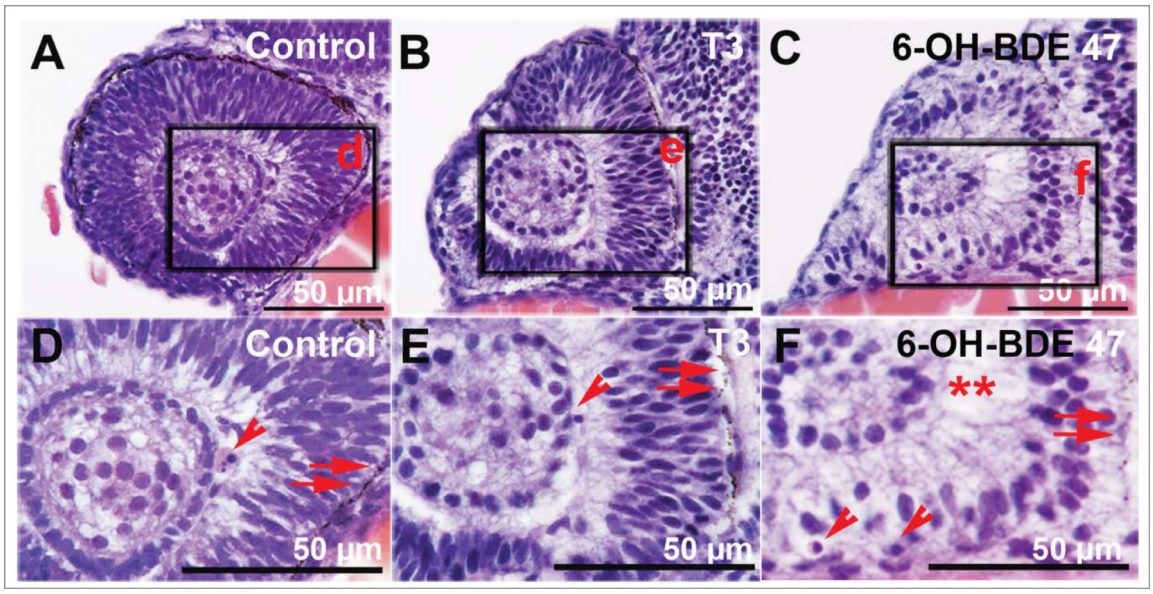


Figure 7.

H&E stains of paraffin sectioned eyes at 22 hpf. Controls are shown in (**A and D**). Eyes from T3- exposed individuals are shown in (**B and E**) and the effects of 6-OH-BDE 47 are shown in (**C and F**). Note enhanced cellularity of retina in controls vs. T3 and/or 6-OH-BDE 47 exposed individuals. The effect of exposure on pigment at bases of future retinal pigmented epithelial cells is shown. Both T3 and 6-OH-BDE 47 exposures reduced the thickness of the pigmented layer and higher magnification revealed gaps in this structure. Double arrows indicate pigmented layer. Single arrows point to pyknotic cells.

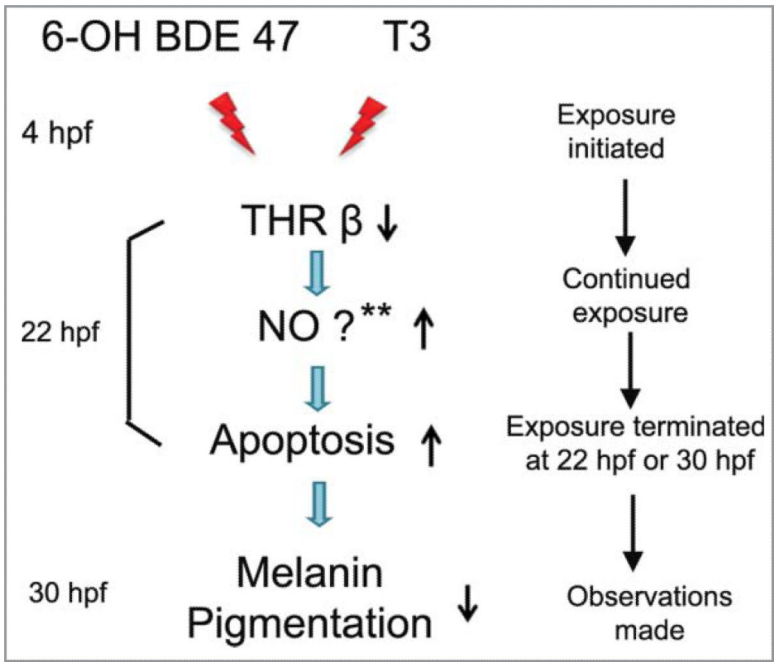


Figure 8.

Flow diagram of hypothesized mechanism for 6-OH-BDE 47 and T3 induction of apoptosis and decreased eye pigmentation of zebra-fish embryos. Time frame is shown in left panel. Responses are in center panel. Right panel shows exposure initiation and duration. Six-OH-BDE 47 or T3 combined with THRβ and decreased its expression. This may promote formation of NO [data not from this paper but from Hu et al. (2009)[51] and Santos et al. (2006).[54] Increased NO may induce active oxygen stress which in turn leads to apoptosis. Subsequently, pigmentation was decreased at 30 hpf in eye of zebrafish embryos.