"Effect of Benzo(a)Pyrene on The Development of Zebrafish (*Danio rerio*)"

Dissertation submitted in partial fulfilment for the degree of

Master of Science in Applied Microbiology

Submitted By

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CERTIFICATE

This is to certify that the dissertation entitled "**Effect of Benzo(a)Pyrene on The Development of Zebrafish (*Danio rerio*)**" Submitted by **Hasnaa Ahmed Ahmed Elfawy** in partial fulfilment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, Kalinga Institute of Industrial Technology (KIIT), Bhubaneswar, Odisha, India bearing Roll No. **1662024** & Registration No. **16657851584** is a bonafide research work carried out by her under my guidance and supervision from **01.12.2017** to **19.05.2018**.

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This is also to certify that the above said work has not previously submitted for the award of any degree, diploma, fellowship in any Indian or foreign University.

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DECLARATION

I hereby declare that the dissertation entitled "**The Effect of Benzo(a)Pyrene on The Development of Zebrafish (*Danio rerio*)**" submitted by me, for the degree of Master of Science to Kalinga Institute of Industrial Technology (KIIT) is a record of bonafide work carried out by me under the supervision and guidance of **Dr. Srinivas Patnaik**, Associate Professor, and **Dr. Biswadeep Das**, Assistant Professor at School of Biotechnology, Kalinga Institute of Industrial Technology (KIIT), Bhubaneswar, Odisha, India.

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Abstract

Currently, toxicological screening methods rely on in vitro or in vivo based experiments to affirm biological effects. The mammalian system is considered very complicated, too tedious, and expensive to perform high-throughput chemical screening. Zebrafish is a perfect model for investigating vertebrate development because of its external fertilization, small size, cost-effective, transgenic, high fecundity rates, rapid organogenesis, and concordance with mammalian developmental phenotypes. From these advantages, the zebrafish embryo is an ideal alternative model for traditional in vivo developmental toxicity screening. Polycyclic aromatic hydrocarbons (PAHs); such as Benzo(a)pyrene (BaP), are the most common carcinogenic compounds present in the environment. PAHs are located in natural and anthropogenic sources as well. In the present study, embryonic zebrafish were incubated with Benzo(a)Pyrene for 5-days at environmental levels (0.25, 0.5, 0.6, and 0.8 μM) to investigate the potential developmental toxicity. Cytotoxicity and genotoxicity assessments were performed using experimental assays including phenotypes analysis, hatching rate, and viability rate analysis along with apoptosis analysis, and analysis of expression of key genes of bone development BMP2b, SOX9a, osteopontin, and collagen type 1 by using RT-PCR. All the experiments were performed in triplicates and repeated thrice. Our findings indicate those skeletal abnormalities phenotypes, induction of apoptosis levels, and a significant down-regulation of bone formation genes. From the environmental risk assessment perspective, Bap needs to be categorized as a skeletal toxicant.

Keywords—PAHs; Benzo(a)pyrene; Zebrafish; Apoptosis; Bone Development.

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Abbreviations

- AHR: Aryl hydrocarbon receptor
- BaP: Benzo[a]pyrene
- BPDE: Diol epoxide
- cDNA: complementary DNA
- CYP: Cytochrome P450
- DNA: Deoxyribonucleic acid
- Dpf: Days post-fertilization
- EC50: The concentration of xenobiotic that gives half-maximal response
- EH: Epoxide hydrolase
- EPA: Environmental Protection Agenc
- Hpf: Hours post-fertilization
- In vitro: Outside a living organism
- In vivo: Inside a living organism
- LC50: The lethal dose at which 50% of the population at certain time
- MSCs: Mesenchymal stem cells
- PAH: Polycyclic aromatic hydrocarbon
- RNA: Ribonucleic acid
- RT-PCR: Reverse transcriptase –Polymerase Chain Reaction
- TI: Teratogenicity index.
- Xenobiotic: Foreign compound

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1 Introduction

1.1 General background

Polycyclic aromatic hydrocarbons (PAHs) include diverse compounds that encompass of fused aromatic benzene rings as defined by the International Agency for Research on Cancer (IARC 2010). These PAHs compounds such as benzo[a]pyrene (BaP), are common ecological pollutants that present in natural and anthropogenic sources. PAHs are formed naturally due to volcanic eruptions and forest fires. at man-made sources, PAHs originated in urban effluent, coal burning, asphalt production, automobile exhaust ,and oil spills (IARC 1983), Various processes of cooking contribute into its formation for instance, heating, microwaving, frying, roasting, grilling, smoking, drying, baking, etc. (Singh, Lochan 2016). Benzo(a)pyrene presents in high concentration in cigarette smoke (Kier, Yamasaki, & Ames, 1974). PAHs are environmental contaminants that present in air, water, soil and food. Benzo[a]pyrene often exist in mixtures of PAHs which are highly stable in the environment. The major routes of human exposures to PAHs occur mostly via inhalation (lung), ingestion (GIT), topical (skin), (Nielsen, Jorgensen et al. 1996). High occupational exposure to PAHs happens in many industries and occupations. Workers exposed to diesel engine exhaust, coke oven worker and firefighters are continuously exposed to polycyclic aromatic hydrocarbons (PAHs). The occupational exposure *to* benzo[a]pyrene and other carcinogenic PAHs lead to increase the incidence of cancer and neurobehavioral dysfunctions (Stec et al. 2018).

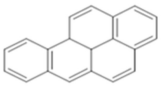
The impact of PAH on human health is widely recognized, an explosion of study has been done in discovering how human health is getting affected by benzo[a]pyrene. It's associated with increased risks of Alzheimer's disease (Wallin et al., 2017), systemic inflammation (Delfino, Staimer et al. 2010), neural tube defects (Lin et al., 2018), renal dysfunction (Farzan, Chen, Trachtman, & Trasande, 2016), cardiopulmonary mortality (Lee, Magari, & Christiani, 2011) ,lung cancer mortality (Hoshuyama, Pan et al. 2006) (Grant 2009), breast cancer (Shen et al., 2017), digestive tract cancers (Diggs et al., 2011) oxidative damage (J.J. Briede, R.W. Godschalk, M.T. Emans, et al.,2009), and many are mutagenic or carcinogenic chemicals. (Pashin & Bakhitova, 1979). The major

factors of carcinogenesis are smoking cigarettes, infection that leads to inflammation, nutrition and dietary factors. (Sugimura, 2000). *In vitro* and *in vivo* toxic effects of benzo[a]pyrene have been discovered, but the developmental effects of exposure are still to be explored.

Benzo(A)pyrene is a potent mutagen and carcinogen, nevertheless it has some commercial and industrial uses such as manufacture of pigments, coal gasification, coke production, aluminum production, iron and steel foundries, creosote and asphalt production (Fitzgerald, Robinson, & Pester, 2004). EPA has described it's industry usages as adhesives, sealant chemicals, and fuel additives (2014). PAHs are not synthesized chemically for industrial purposes. Nevertheless, there are some commercial and industrial uses for many PAHs (Casarett, Doull, & Klaassen, n.d, 2008). Bap is synthesized from pyrene and succinic anhydride (The Merck Index 2013).

1.2 Characterization of BaP

Table 1 gives an overview of the chemical and physical properties (Pschenitza et al. 2014).

Color	Pale yellow crystals
Odor	Faint aromatic odor
Molecular Weight	252.316 g/mol
Boiling Point	496°C
Melting point	178.1°C
Density (at 20°C)	1.4 g/cm³
solubility	In water , g/100ml at 20°C < 0.1 (poor) In many organic solvents, Bap is soluble(chloroform benzene, toluene, xylene
Vapour pressure at 20°C	Negligible
The formula	$C_{20}H_{12}$
Chemical diagram	
Absorption and fluorescence detection	Abs= 297 nm Fluor= 405 nm

1.3 An overview of the content of BaP found in India

One of the most common anthropogenic source of the PAHs including B(a)P in the atmosphere is burning of fossil fuels (Gupta et al. 2011) and it has had a huge usage in the total domestic energy of India since 1992 (Shukla n.d.1998). In rural and undeveloped villages in India, bio-fuels, such as cow dung cake, are still used (Padhi & Padhy, 2008) because of its cheap and available but the incomplete combustion of these bio-fuels leads to the production of PAHs compounds into the air (Bhargava et al. 2004).

Environmental pollution is a serious problem in countries, such as Egypt, China and India because they are developed countries. India ranked the second most populated country globally with more than 1.2 billion people (http://censusindia.gov.in/) and quickly developing country. Due to the large population, India needs more energy sources. The usage of low efficiency bio-fuels in rural places and the huge number of vehicles with lack of standers maintenance is getting increased day by day. These are the most significant reasons for the atmospheric pollution in India (Badami 2005). B(a)P emitted from motor bikes is 50% higher than that from cars and other vehicles are fueled by gasoline. The percentage of B(a)P in the total PAHs emissions from the exhaust of two wheelers, which utilize gasoline, account for more than the diesel vehicles, such as buses and trucks (Khillare, Balachandran, & Hoque, 2005).

Table 2 shows a general overview of the range of BaP has been found in different Indian environment (air, water, sediments, and soil) at different cities in various period by using different method of extraction (Suvarapu and Baek 2016).

Type of Sample	BaP concentration
Ambient air	0 - 129.9 ng/m^3
Soil	0.112 - 19.6 µg/g
Water	not detected level to −3.41 µg/L

1.4 Introduction to the Zebrafish

The Zebrafish, Danio rerio, is widely used in many research fields, and it is contributing major insights. Zebrafish are located in freshwater streams and slow-flow waters from South and East Asia (Pakistan, Burma, India, and Nepal). Danio lives in shallow water at temperatures ranging from 24°C to 38°C, conductivities of 10 to 271 µS and pH 6.0–8.0 (Engeszer et al. 2007). Zebrafish are available in artificial aquariums at pet stores all over the world. They can be most simply maintained in 10 gallon (45 liter) aquaria heated to 28.5ºC (above 31ºC and below 25ºC, Zebrafish probably won't produce embryos and the development will be abnormal). The name “zebrafish” refers to the description of horizontal stripes on their bodies. Recently *Danio rerio* has been appreciated as an alternative model system for many research fields, because these fish offer sufficiently important advantages to be worthy of attention over higher vertebrates. (Haffter et al., 1996).

The embryonic zebrafish is an ideal invivo model for studying mechanistic-based toxicology during development. The organogenesis of Zebrafish develops rapidly (most of the organs are fully formed in the 5th dpf), externally developed that suitable for non-invasive examination and imaging during the progression of development, their great fecundity, cost-effective, the transparency throughout organogenesis, in addition, The zebrafish and homosapien genomes are highly conserved, and many of cellular and physiological characteristics are common with vertebrates, including humans (Barbazuk 2000). The zebrafish genome is fully sequenced, desired molecular and genetic targets can be investigated. Expression of specific genes can be studied or transiently knocked down during development , and many transgenic zebrafish lines are available. The size of the zebrafish is very small that makes them adaptable to cell culture techniques (Dai et al. 2014).

1.5 The scope and objectives

PAHs; such as benzo[a]pyrene (BaP), are the most widely recognized cancer-causing mixes introduce in nature. Polycyclic aromatic hydrocarbons (PAHs) are found in natural and anthropogenic sources as well. In the present study, Zebrafish embryos were exposed to Benzo(a)pyrene to investigate the potential developmental toxicity, Embryonic zebrafish were incubated with benzo(a)pyrene for 5-days at environmental levels (0.25 , 0.5 , 0.6 ,and 0.8 µM). Mechanistically cytotoxicity and genotoxicity assessments were performed using experimental assays including phenotypes analysis,

hatching rate , and viability rate analysis along with apoptosis analysis ,and analysis of expression of key genes of bone metabolism.

Developmental Exposure indicates that the potential risks of PAH especially BaP exposure during development of fetus, infants, and young children. PAH metabolites and PAH-adducts were found in the urine sample of pregnant women and children (Kang, Cho et al. 2002) and have been tested in placenta, cord blood, maternal blood, and human breast milk (Perera and Herbstman 2011). Prenatal PAH exposures have been associated with reduction of the birth weight and growth retardation, with smaller head circumference and body length measurements (Tang, Li et al. 2006), long term effects on cardiovascular, function of lung, neurological , and decrease in children's intelligence and behavior problems in children and young adults (Perera, Rauh et al. 2006; Gunes, Koklu et al. 2007; Geerts, Bots et al. 2008; Edwards, Jedrychowski et al. 2010; Perera and Herbstman 2011; Geerts, Bots et al. 2012).

Waterborne PAH exposure caused mortality, toxicity and altered development of many species of fish at early life-stages (Barron et al., 2004; Hawkins et al., 2002; Incardona et al., 2004; Colavecchia et al., 2004). Fundulus heteroclitus, a model fish species utilized as a part of ecotoxicological studies, had elevated deformity lists (heart elongation, pericardial edema, tail shortening, and hemorrhaging) after exposure to binary mixtures of PAHs including BaP, β-naphthoflavone (BNF), α-naphthoflavone (ANF), fluoranthene (FL), piperonyl butoxide (PBO), and 2- aminoanthracene (AA) (Wassenberg and Di Giulio, 2004). Cardiac deformities, spinal curvatures,craniofacial defects, and altered hatching rate were also observed after BaP exposure in the absence of other PAHs (Wills et al., 2009; Seemann et al. 2015). In pink salmon (Oncorhynchus gorbuscha) and rainbow trout (O. mykiss), dissolved PAHs from crude oil caused higher mortality, alterations in time to hatch, growth reduction, spinal deformities, jaw deformities, yolk sac edema, and impaired swimming (Carls and Thedinga, 2010; Barron et al., 2004; Hawkins et al., 2002). In Sebastiscus marmoratus, a teleost of the scorpion fish family, skeletal deformities including incidence of spinal curvature and craniofacial defects were caused by BaP as well as altered expression of genes involved in bone formation (He et al., 2011; Seemann et al. 2015).

1.6 Achievement

- Zebrafish care and maintenance of the aquarium
- Health-monitoring
- Identifications male and Female
- Set up a successful breeding
- Collecting the eggs
- Observing the embryogenesis and development of the organs
- Handling the fluorescence microscope, ZEN Microscope and imaging software
- Imaging the effect of Benzo(a)Pyrene on the early stages of zebrafish
- Study the apoptosis by using acridine orange AO
- RNA extraction for RT-PCR

2 Review of Literature

2.1 Different *invivo* models

Biomedical research relies on in vitro and in vivo animal studies. Cell culture is an important tool for biological research as an in vitro model. It's used for providing predictions of drug activity, cellular and molecular mechanism. There are numerous advantages for utilizing cell lines as an in vitro experimental model such as easiness in handling and manipulating, high homogeneity, high variety of cell line are available, immediate accessibility, unlimited sub-culturing ,and reproducibility of results. Nevertheless, some limitations are counted, for instance growing in an artificial environment, high chance of contamination, lacking of in vivo state, loss of natural heterogeneity, and genomic instability. Due to these disadvantages of in vitro model, there is a great need for using more efficient models for understanding the human disease.

Utilizing animal models is a cornerstone of biomedical field to understand and investigate the pathogenesis of human disease, the toxicity of the chemical, preclinical screening, drug discovery, pharmaco-dynamic and pharmaco-kinetic profiles, cancer insights at a cellular and molecular level. Mammalian models, as the mouse, have been distinguished in modeling human diseases, basically because of the evident homology between mammalian genomes and the many similarities in aspects of anatomy, cell biology and physiology. Transgenic mouse models that accurately recapitulate the pathology of homosapien diseases are commercially available (Giacomotto and Ségalat 2010).

Experimental Biomedical Research exploits a number of multicellular models to gain insights into the basic of physiology, and mechanisms of any dysfunctions in vivo. Figure1 is a phylogenetic tree highlighting the evolutionary distances and relationship between animal models that are used in biomedical fields and the human. The nematode C. elegans and the fruit fly Drosophila melanogaster are used commonly as genetic model organisms as well as they have been used in pre-screening tools in drug discovery (Segalat, 2007).

These invertebrate models are lack of complicated systems or organs, for instance, heart, immune system, and kidneys relevant to human anatomy and physiology. Ver-

tebrate models, by contrast, usually have most of the complex tissues which are common in human systems. but, not all vertebrate model organisms are similar in the organism-based drug discovery screens. Table 3 shows some of the general advantages and limitations of the popularly used model organisms. Animal models have small size, high fecundity, external fertilization and organogenesis, low cost, and easy to be cultured under simple conditions are suitable for chemical screening.

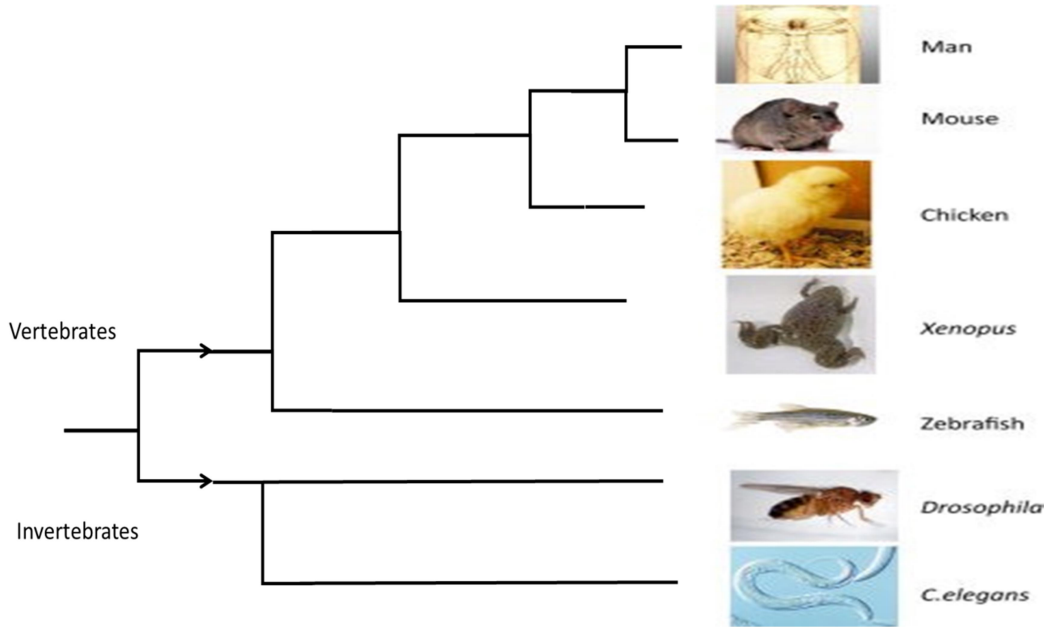


Fig.1. Phylogenetic tree showing the main models commonly used in biomedical research and their evolutionary relationship (Wheeler and Brändli 2009).

Only zebrafish and Xenopus fulfill the above-mentioned standards for chemical screening. However, Xenopus laevis embryos are bigger than zebrafish, they fit in multiwell plates (Tsang 2010) Overall, Zebrafish and Xenopus share similar experimental pros for high-throughput chemical screening. The similarity in genome sequence of the frog and the human is 80% and approximately 70% of zebrafish genome and human genome. Zebrafish and Xenopus have most organs affected by common human diseases, including a cardiovascular system, a digestive tract, kidney, a hematopoietic system, and a central nervous system CNS therefore they are ideal models to study human disease. Most drawbacks of zebrafish and Xenopus as a model for chemical screens are the limitation of screening only on the embryonic and larval stages not suitable for adult.

2.2 Zebrafish as a model

The past two decades and a half has seen a rapid increase in the publication numbers of using zebrafish as a model. The zebrafish researchers have designed heavily information and database resources (The Zebrafish Model Organism Database (ZFIN) and the Zebrafish International Resource Center web sites) that provide free exchange of materials and protocols. The pros and cons of utilizing Zebrafish as a model in various biomedical approaches are summarized in Table 4.

	C. elegans	*Drosophila*	Zebrafish	*Xenopus*	Chicken	Mouse
Broodsize	250-350	80-100	100-200	1000-5000	1	5-8
Costs per embryo	low	low	low	low	medium	high
High-throughput multiwell-format screening	good	good	good	good	poor	poor
Access to embryos	good	good	good	good	good	poor
Micromanipulation of embryos	limited	limited	fair	good	good	limited
Genome	known	known	known	known*	known	known
Genetics	good	good	good	fair*	none	good
Knockdowns (RNAi, morpholinos)	good	good	good	good	limited	limited
Transgenesis	good	good	good	good	poor	good
Evolutionary distance to man	very distant	very distant	distant	intermediate	intermediate	close

Table 3. Advantages and Disadvantages of Common invivo Models for Organism-based Chemical Screening. Color code: green, best in category; red, worst in category (Wheeler and Brändli 2009).

Studying the stages of Embryonic Development of the Zebrafish is a tool that provides accuracy in anatomical approaches, genetic approaches, and developmental biology. The stages of development which is between fertilization and hatching are generally called embryogenesis. The fertilization occurs external and involves the fusion of sperm from male and egg of female. Gamete cells begin to develop, and embryos basically are considered in this stage until they hatch or get out of the chorine. Cleavage is a stage which rapid mitotic divisions occur. The zygote is divided into many cells and at the end of the cleavage series, the blastula have formed. In the gastrula stage the rate of mitotic division becomes slow to generate the different organs. Somites are developed during the segmentation period. Pharyngula stage is described by spontaneous movements, detachment from the yolk; early pigmentation, retina pigmented, cellular degeneration of the tail end, circulation in the aortic arch visible strong circulation; single aortic arch pair, early motility, and heart beating.

The hatching period is ranged from 48 to 72 hours post fertilization (hpf). After hatching, it's defined as a Larva and in this stage Heart-beat is regular, yolk extension beginning to taper; dorsal and ventral pigmentation, segmental blood vessels detectable: thickened sacculus with two chambers visible, and foregut development. In the Juvenile phase, the most adult features have been formed in the absence of sexual productivity. Adult stage starts at 90 day post fertilization (dpf). Selected major stages of zebrafish development are given in Figs. 3 (Kimmel et al. 1995).

Pros	Cons
• Large numbers of embryos approximately 200-400 from a single mating pair • Transparency of embryos • Development outside of female • Rapidly organogenesis • Embryonic morphologies are predictive of adult phenotypes in most organs • Vertebrates organism including (the brain, eyes , heart, intestines , pancreas ,kidney and liver) • amenable to such genetic modifications , and transgenic approaches • Transparent adult strains (Casper) are available for invivo observation • Forward and reverse genetic are highly scalable • Tumors in Zebrafish highly resemble human tumors at the pathology, histology ,gene expression , and genomic levels • Highly homology with the human genome. • Cost-effective	• Low incidence of spontaneous tumorigenesis • Short life in comparison with humans • Simpler organs than mammalian • Some organs in mammalian are not present in zebrafish (lungs, prostate glands and mammary gland) • Skin lacks some specific cellular components present in humans. • The size of zebrafish genome is one – half the human genome size • Zebrafish prefer growing at 28.5º C (ectothermic), rather than at 37º C for human (endothermic) • It's an a aquatic organism • Egg laying is external which is different in case of mammalians • Cannot tolerate inbreeding (rapidly lose the fertility without breeding)

Table 4. The pros and cons of utilizing Zebrafish as a model in various biomedical approaches (Zhao, Huang, and Ye 2015).

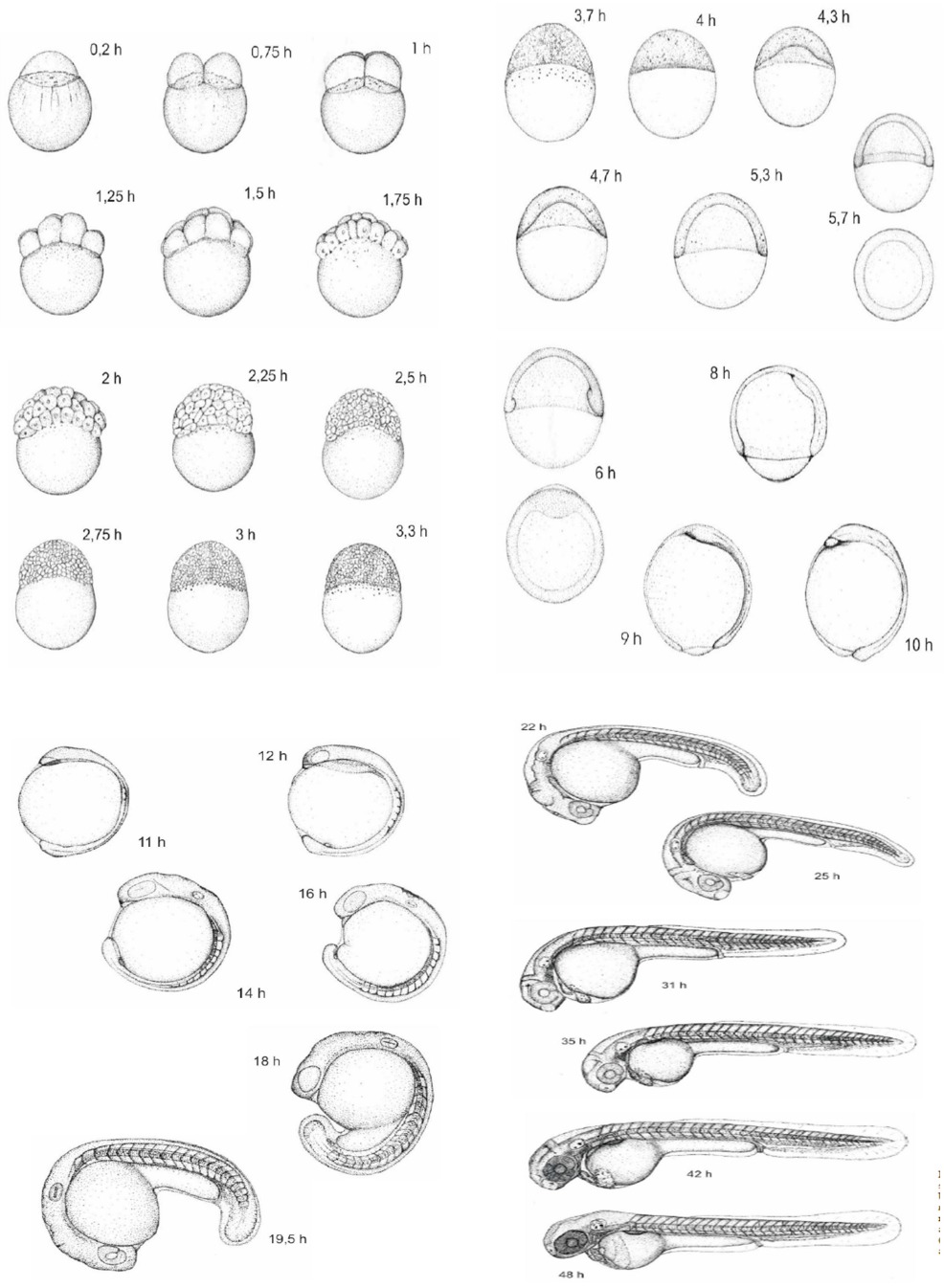


Fig.2. The major stages of zebrafish Development.

(Kimmel et al. (1995)

2.3 Bone formation

Forward genetic screens in zebrafish have followed in the identification of mutants in many developmental processes. The random way of inducing mutations via many chemicals followed by phenotypic screening and positional cloning of the affected genes has been proven to be an effective approach to identify new gene functions. Similarities of bone physiological and histological structure between zebrafish and mammalian make zebrafish as a powerful model for bone-related researches.

Osteoblast cells are responsible for the deposition of bone and proliferate and maturate into mineralized bone cells. It's originated form mesenchymal stem cells that differentiate to many types, including myocytes, chondrocytes, and adipocytes. Cartilage ossification is called endochondral. Two processes are involved in bone formation osteoblastic differentiation, and osteoclastic inhibition. We have chosen some genes that associated with bone development (Silvent et al. 2017). We have validated marker gene expression by RT-PCR to confirm the affecting osteogenesis processes by BaP. Marker genes tested in this study are pre-osteoblast markers (BMP2b, Cola1), mature osteoblast marker (OPN/SPP1), and essential markers for chondrogenesis and osteogenesis (SOX9a).

BMP2b

Bone morphogenetic protein 2 (BMP-2), is an important member of the transforming growth factor (TGF-b) superfamily and it's predominantly expressed in osteoblasts and induces osteogenesis, osteoblastic differentiation, and bone and cartilage formation. BMP-2 also plays a pivotal role in regulating vertebrate embryo development. Any dysfunction in the expression of BMP-2 can cause serious bone dysfunction and bone-related disorders (Chen, Deng, and Li 2012).

Collagen type I

Collagen type I is one of the key important proteins involved in the formation, maturation, development of skin, development of scales, and mineralization of bone. Collagen type I has a trimer chains two α1 chains and one α2 chain, encoded by COL1A1 and COL1A2 genes, respectively. In zebrafish, there are three type I collagen genes exist, col1a1a, col1a1b and col1a2 coding for α1(I), α3(I) and α2(I) chains. The dysfunction of trpye | collagen causes musculoskeletal abnormalities in a zebrafish model (Gistelinck et al. 2016).

SPP1/Opn

Osteopontin, is known as secreted phosphoprotein 1 spp1,and it is a secreted glycoprotein with the characteristics of a matricellular protein . previously It was named osteopontin because it was proposed to act as a “bone bridge” from bone cells to hydroxyapatite . It is highly expressed by bone cells but also by many other cell types, including hypertrophic chondrocytes, odontoblasts, cementoblasts, macrophages, as well as endothelial, smooth muscle, and epithelial cells. Osteopontin is involved in a diverse range of biological processes, including biomineralization, cell attachment and cell signaling, cell migration, inflammation, and leukocyte recruitment. But the highest expression is detected in mature osteoblasts at sites of bone remodelling (Topczewska et al. 2016).

SOX9a

Sox9a is a known essential transcription factor for the development of these chondrogenic elements. Sox9a is expressed in post migratory pharyngeal arch mesenchyme. It belongs to a family of DNA-binding proteins that contain a 79 amino acid long HMG (high mobility group) domain with at least 50% similarity to that of SRY, the sex determining factor on the Y chromosome (Yan et al. 2002). Sox proteins bind to a seven base pair sequence in the minor groove of DNA and bend DNA. Sox9 may also participate in transcript splicing (Ohe et al., 2002). The structure of SOX9 protein has a C-terminal transcription activation domain. it acts by regulating expression of other genes. Dysfunction of SOX9 in humans leads to skeletal disease. In zebrafish, sox9a is essential for terminal differentiation of cartilage progenitor cells in mesenchymal condensations within the ceratobranchial arches and loss of function or expression-leads to severe craniofacial defects. It has an important role in chondrogenesis or cartilage formation (Yan et al. 2005).

Current statues of BaP impacts

Developmental abnormalities, such as bone deformities, have previously been published following exposure to environmental contaminants, including PAHs. For this reason, bone abnormalities have also commonly been used as biomarkers in environmental field studies (Kingsford & Gray, 1996). Effects on bone development were observed in other species of fishes which were exposed to BaP at the molecular level. The exposure is often sublethal. Studies on fish larvae following large oil spills

suggest that craniofacial deformities induced during the larval stage can result in reduced food intake and ultimately increased mortality in the population (Carls, Rice, & Hose, 1999). Although much of the knowledge on the effects of PAHs on bone and bone metabolic processes are derived from studies on the effect of cigarette smoke on osteoporosis (Lee, Lee, Waldman, Casper, & Grynpas, 2002), studies also show that BaP exposure can have detrimental effects on bone development in fish (He et al., 2011; Seemann et al., 2015). In early life stages of fish, increased prevalence of developmental deformities such as craniofacial and spinal abnormalities are typical observations following oil exposure.

Benzo(a)pyrene B(a)P, which is a commonly potential xenobiotic used as an indicator of polycyclic aromatic hydrocarbon (PAH) environmental contamination, has a numerous number of hazardous consequences on human health. Benzo(a)pyrene (BaP) contaminate the roasting or grilling food of human being, present in cigarette smoke, contamination of aquatic environments and associated organisms consumed by man. The exposure routes of Benzo(a)pyrene are the oral route, transdermal, and via inhalation. The absorption process is the movement of BaP from its site of exposure to the systemic circulation. The effect of Benzo(a)Pyrene on zebrafish embryos has beed investigated in numbers of research articles. Developmental exposure to 4 μM B[a]P was associated with hyperactivity behaviour in normal photo cycle (Knecht et al. 2017). Mitochondrial DNA (mtDNA) copy number was dramatically elevated after exposure to BaP, Obvious morphological abnormalities including curved backbone and cardiomegaly in zebrafish were observed in the 54 hpf with more than 400 nM of benzopyrene (Kim et al. 2014). At 3 dpf, 1 uM of BaP caused malformation of head, tail, tail tip and growth retardation. The LC50 of bap is 5.1uM, the EC50 is 0.52 uM, and the teratogenicity index TI (LC50/EC50) is 9.81 (Weigt et al. 2011).

2.4 Biotransformation of BaP

PAHs such as BaP are lipophilic compounds that can pass through the membrane through passive diffusion. The second step is distribution or movement of the drug. Polycyclic aromatic hydrocarbon PAH are transformed by different kind of enzymes in serial steps, where each step forms intermediates metabolites that are either detoxified or activated to react with large cellular components like DNA and proteins. The biotransformation of BaP has different sites that are suitable for the action by the microsomal CYP450 enzymes which include over 1200 superfamily enzymes. CYP450 plays important roles in phase I of biotransformation of any xenobiotic and endoge-

nous roles such as biosynthesis of steroid hormones. The first step of enzymatic oxidation is insertion one oxygen atom into the compound through a NAD(P)H which is generated by pentose phosphate pathway (Lewis 2003). The epoxide intermediate metabolite may then be hydrolysed by epoxide hydrolase (EH) enzyme or undergo isomerization to phenols. Hence, different BP phenols and BP diols are then produced depending on the type of CYP450 enzyme and the availability of epoxide hydrolase (EH) enzyme (Conney 1982; Shou et al. 1994). Phase II is known as a conjugation process and the enzymes in this phase are UDP-glucuronosyltransferase or UGT and Sulfotransferase or SULT may further conjugate the phenols and diols. The detoxification process of the PAH occurs by increasing the polarity and enhancing the elimination and excretion of the compounds from the organism. CYP450 and GST enzymes are responsible for activation of solid phase neutral compounds in the tobacco smoke. The products of the activation process have the ability to induce molecular mutations and cellular alterations such as micronuclei induction and chromosomal aberrations. The enzymatically detoxification pathway of BaP and the formation of carcinogenic diol-epoxides are shown in figure 3.

The bioactivation of BaP includes the generation of mutagenic metabolites, like the BaP diol-epoxides (BPDE), which are strongly reactive towards DNA and proteins (Gelboin 1980). BPDE has the ability to form irreversible covalent bonds with DNA. In this pathway, there are two phases. Benzo(a)Pyrene is transformed by CYP450 to produce a BaP 7,8-epoxide. The epoxide is hydrolysed by epoxide hydrolase (EH) enzyme to form the proximate carcinogen dihydrodiols, (±) BaP 7,8 diols that may further be oxidized by P450 to form the ultimate carcinogenic compound BaP diol-epoxides (BPDE) (Levin et al. 1976). The reactive oxygen species ROS that are formed during the oxidation of BP-7,8-dione can either lead to oxidative DNA damage or produce decomposition products of lipid hydroperoxides, which are also reactive towards DNA (Quinn and Penning 2008).

The diol epoxides have different biological activities, such as adduct formation and mutagenic activity, depending on the test system used (Conney 1982). The adducts have strong conformational preferences (Karle et al. 2004). Both the structure and conformation of the BP-DNA adduct is believed to influence their interactions with the DNA repair enzymes affecting the reparability and removal rate of the BP-DNA adduct, and ultimately the risk of mutation (Suh et al. 1995). When the DNA adduct remains unrepaired, they can cause a miscoding during the replication by inducing transversion mutations involving both G and A, and hence produce a permanent mu-

tation (Dong et al. 2004). If the mutation occurs in a critical region of important genes, like oncogenes and tumor suppressor genes, a chemical induced cancer may occur (Greenblatt et al. 1994).

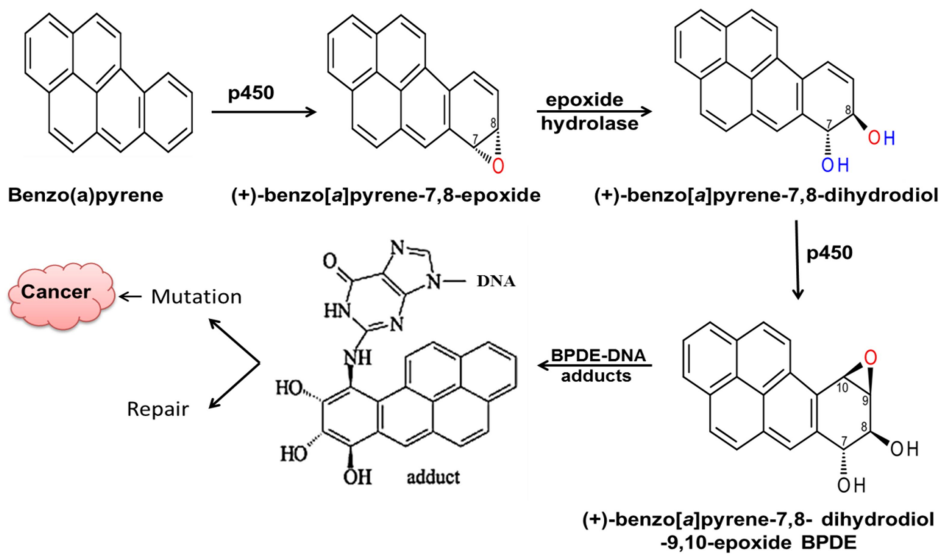


Figure 3. The Biotransformation of Benzo(a)pyrene and formation of DNA adducts.

It's shown in figure 4 the metabolic cascade or the detoxification of BaP as a xenobiotic occurs by various metabolizing enzymes in two phases. The microsomal CYP450 phase I enzymes generate a complex products of quinones, phenols, dihydrodiols, triols, pentols but also reactive epoxides (Guengerich 2000).

The phase II enzymes glutathion transferase, glucoronosyltransferase and sulfotransferase (SULT) will further work on the oxidized products which are produced in phase I step, and form conjugates of glutathione, glucoronide and sulfate.The formation of highly polar and water-soluble metabolites or decrease the lipophilicity lead to increases the excretion of BaP from the body.

Sulfate conjugation is one of the most important detoxification pathway, there are many compounds that are activated by forming electrophilic sulfuric ester metabolites (Gamage et al. 2006). There are many studies have approved that the different polymorphisms in SULT1 related to the risk of lung cancer (Lindsay et al. 2008). In addition, there are many studies have been studied the combinations of Cyp1b1 and SULT1 polymorphism lead to increase the risk of colorectal cancer (Cotterchio et al. 2008). When the sulfuric ester loses the sulfur group, it forms an extremely reactive

carbocation. This ion may then bind spontaneously with molecular or other cellular components leading to server toxic lesions and mutations.

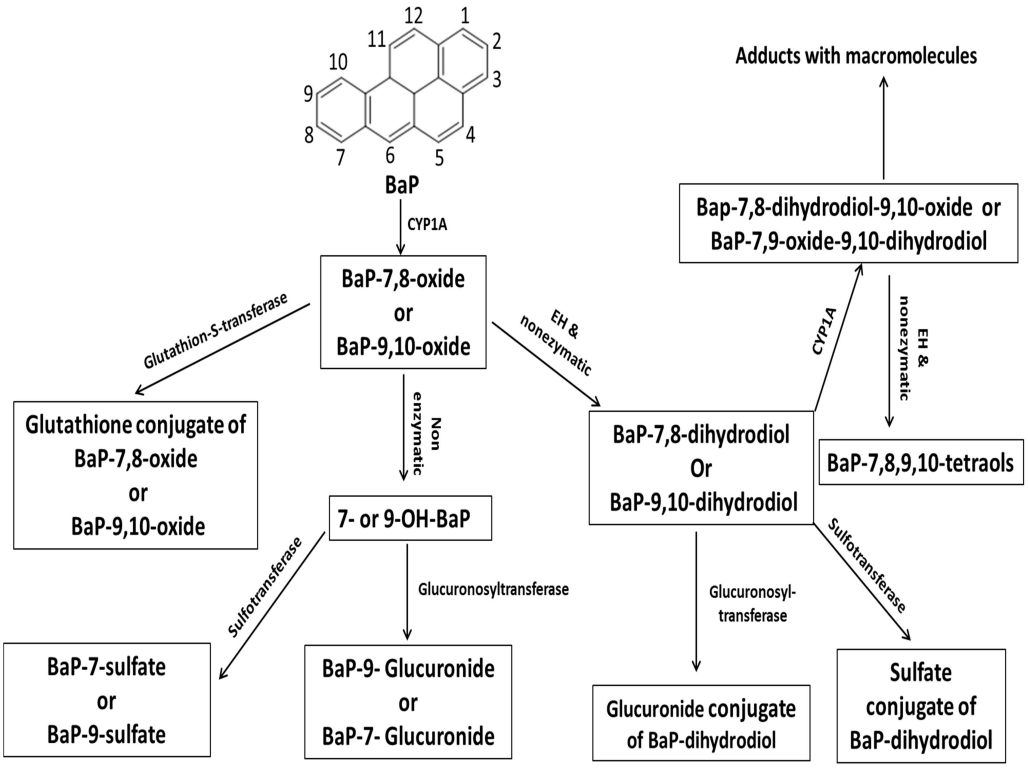


Figure 4. Metabolic cascade for benzo(a)pyrene (BaP) via the cytochrome P4501A (CYP1A) products BaP-7,8-oxide and BaP-9,10-oxide.

2.5 The signaling pathway of Ahr

The aryl hydrocarbon receptor (Ahr) is a ligand activated member of the transcription factors and is located in the cytoplasm in relation with heat shock HSP(90) and HSP90 accessory protein (Petrulis and Perdew 2002). The activation of Ahr leads to rapid transcriptional activation of numerous genes that control a wide-ranging of cellular functions. Ahr is not only involved in endogenous physiological and developmental processes but also toxicological processes and oxidative stress (Denison and Nagy 2003). BaP and other polycyclic aromatic hydrocarbon PAH are primarily activated in the body by the Ahr, which acts as a transcriptional factor of several important xenobiotic genes. in figure 5 ,BaP acts as ligand and binds to the Ahr in the cytosol. The BaP with Ahr is then translocated to the nucleus where it forms a heterodimer with the Ahr-nuclear translocator (Arnt) (Nebert 1989).

The Ahr/Arnt heterodimer recognize and bind to xenobiotic responsive element (XRE) or dioxin responsive element (DRE) sequences located in the promoter region of several genes such as cytochrome P450 Cyp1a1, Cyp1a2, Cyp1b1, glutathione S-transferases (Gst), UDP-glucoronoysyl-transferases (Ugt) and quinine oxidoreductase. The binding results in transcriptional activation of the genes and induction of phase I and phase II metabolizing enzymes (Xu, Li, and Kong 2005). The relative expression of the detoxification enzymes will determine the bioactivation degree of BaP after a given exposure.

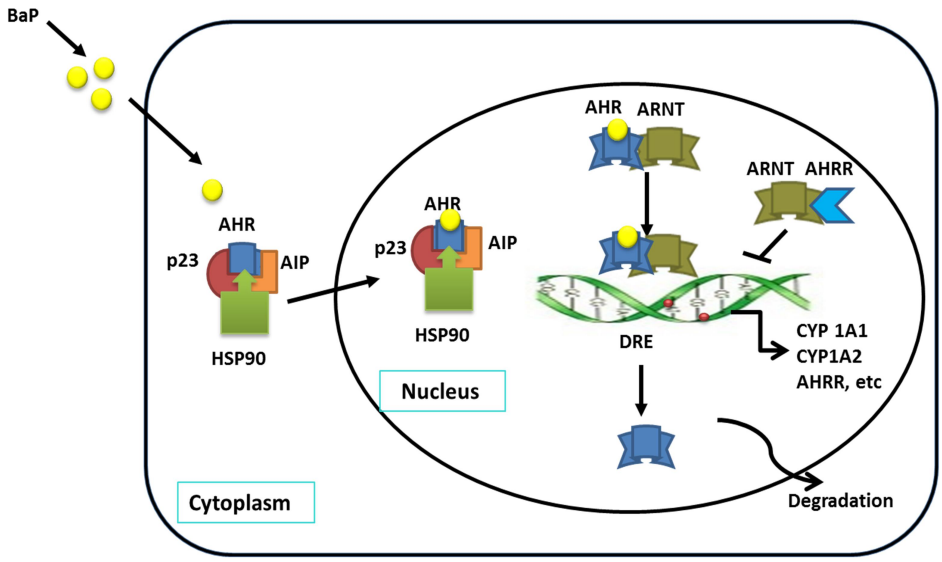


Figure 5: AHR signalling pathway model. AHR – aryl hydrocarbon receptor; AHRR – aryl hydrocarbon receptor repressor; AIP – aryl hydrocarbon interacting protein; ARNT– AHR nuclear translocator protein; CYP1A – cytochrome P450 monoxygenase; DRE – dioxin (xenobiotic) response element; HSP90 – heat shock protein 90 .

3 Aims and Objectives

The aim of this study is to gain insight into the impact of Benzo(a)Pyrene on the early stages of zebrafish.

- Handling the model, care and maintenance of the aquarium.
- Set up a successful breeding.
- Observing the embryogenesis and the normal development of the organs to be able to identify the abnormality phenotypes.
- Handling the Fluorescence microscope, ZEN Microscope and imaging soft-wares.
- Phenotypes toxicity, hatching rate, Heartbeat rate, and viability rate analysis.
- Apoptosis analysis.
- Study the expression of key genes of bone metabolism by RT-PCR.

4 Materials and Methods

4.1 Materials

Table 5 Chemicals and solutions

Chemical/solution	Concentrations	Supplier
Benzo(a)Pyrene (BaP)	0.25, 0.5, 0.6 , and 0.8 µM	Sigma-Aldrich
Dimethy sulfoxide (DMSO)	100 %	HiMedia
E3 medium for Zebrafish embryo (ddH_2O with NaCl, KCl, $CaCl_2$, $MgSO_4$)	0.6 %	
Methylene blue	0.1 % to 1 liter of E3 medium	
Acridine orange (AO)	5 µg/ml	SRL
Trizol or GENEzol™ Reagent		Geneaid
Chloroform		
Isopropanol		
Ethanol	75 %	Merck mili-pore
Verso cDNA synthesis Kit (F-470)		Thermo sci-entific ™
2X PCR Master Mix kit		Thermo sci-entific ™

Table 6. list of Primers. F: forward primers, and R: reverse primers

Gene	Primer Sequence
β-actin	F: ACACAGCCATGGATGAGGAAATCG
	R: TCACTCCCTGATGTCTGGGTCGT
Spp1/opn	F: GCCTCCATCATCATCGTA
	R: AATCACCAAGCACCAGTA
Col1a1a	F: CTTGCTTAGACCTGCGCTTC
	R: GCATTTGGTTTCGCTCTTTC
Bmp2b	F: TCTCACGGTGCTGTTGCTCG
	R: GATTTGCTTGGGGTGGGTTT
Sox9a	F: CGTCCGCTCAGCATCAAC
	R: CGTTCTGGTGACCGTTCG

Table 7. Instruments

Instrument	Provider
Stand alone aquarium system	Aquaneering
Inverted bright field microscope	(EVOS - Thermo Scientific)
Light microscope	ZEISS ZEN
Centrifuge	Eppendorf physiocare
Nano-Drop spectrophotometer	Titertek-Berthold
Gene Amp PCR system	Veriti® Thermal Cycler Thermo Scientific
Measuring weight balance	
Gel docking	ImageQuant LAS 500
Vortex	

Table 7. Softwares

Software
ZEISS ZEN Microscope Software 2.3
ImageJ
Graphpad prism 7.04
Prime 3
SnapGene viewer

4.2 Methods

4.2.1 Zebrafish

Laboratory handling and conditions

All animal procedures were approved by the relevant guidelines of Institutional Animal Ethics Committee (IAEC) of KIIT University and all the experiments were performed according to this practice guidelines and regulations of IAEC. Wild-type AB strain Zebrafish were raised up in local aquarium and were maintained in a stand-alone aquarium setup (Aquaneering, USA). This system employs multi-filtration stages to provide the safest and cleanest re-circulated water for the fishes. The first filtration step is a Dacron pad particulate filter for trapping the debris and the particles which are greater than 10 microns. It's not attached to any part of the system so it can be easily removed to wash or replace by a new one. The biological conversion of harmful ammonia occurs in the second filter which is fluidized bed biological filter. The third filter is submerged in chamber beside the biological filter, and it's known as a dual

carbon filter. The role of this one is to remove the water pollutants such as chlorine and volatile organic compounds (VOCs). The fourth step is UV sterilization lamp. It's the main part of the system because it kills any waterborne pathogens. UV sterilization keeps the system water without any toxic residuals which may be harmful to the health of zebrafish. Digital conductivity meter, pH meter, and thermometer are connected to the system. The temperature is adjusted at 28.5 ºC (±1ºC) and a pH of 7.5 (±0.3). The light cycle of the room is 14 hours of light and 10 hours of dark. The adult fishes in the system were fed with wild micro bits (enriched with protein) and freeze dried blood worms twice daily.

Breeding zebrafish

The gender identification of zebrafish has been done by the naked eyes. The typical body shape of an adult male is very slim compared to that of the female which has a protruding belly. Males tend to be more active than the females. They also have notable yellow or gold strips than females on the ventral side. The adults of zebrafish were fed before separating them. At the afternoon before mating, the sexually mature six-month-old D.rerio were separated in the breeding tank by a mesh, with a male to female ratio of 1:2. At 8:30 in the morning, the divider between the males and females was removed and the fishes were transferred to a new tank. After 30 minutes, Embryos were collected and rinsed several times in petridishes filled with E3 medium which made of NaCl, KCl, $CaCl_2$, Na_2HPO_4, KH_2PO_4, and $MgCl_2$. sodium bicarbonate $NaHCO_3$ was used to adjust the pH at ~ 7.4. Methylene Blue was added 0.1% on the E3 medium to prevent fungal growth. The early developmental pictures have been documented. After 3–24 hpf, the fertilized embryos were separated from unfertilized eggs. Normal proliferated embryos have been transformed to 6-wells plates, with 20 embryos 3hpf in each well. At the 5 dpf , we start feed the larvae by paramecium.

Experimental setup and bap exposure

BaP (purity > 99%) was purchased from Sigma (Sigma-Aldrich). A BaP stock solution (1mM) was provided by Cancer Biology lab at KIIT school of Biotechnology, and it was dissolved in 100% DMSO. The solution was stored at -20 ºC. The working concentrations 0.25, 0.5, 0.6, and 0.8 µM were freshly prepared in E3 medium. The control group was incubated in only E3 medium, and the negative control 0.1%DMSO (1ul of DMSO per 1ml E3 medium). Before exposure ,The Embryo groups were rinsed twice in glass Petri dishes with fish medium. Within 2 h post fertilization (hpf), fertilized eggs (from 4- to 64-cell blastomeres) were selected under Zen light microscope

(Zeiss). After 3hpf, the incubation was started by addition of the fertilized fish eggs to the test substance in 6 well plates. The embryos were exposed individually to different concentrations of BaP as in figure 6. The experimental setup was incubated at 28 ± 1 °C with a 12:12-h light/dark cycle. The well plates were sealed with self-adhesive foil (MicroAmp® optical adhesive film, Applied Biosystems) to prevent evaporation.

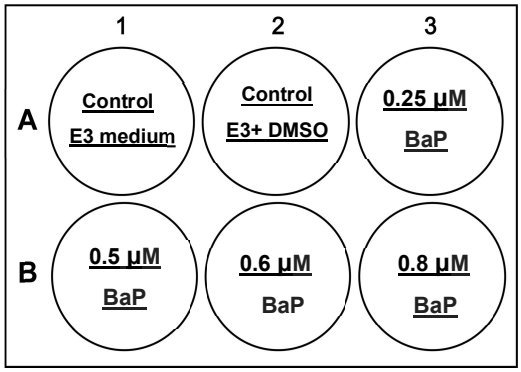


Figure 6: General layout of BaP exposure in wells, starting with control E3 medium , 0.1% DMSO with the E3 medium as a negative control and followed by the lowest concentration to the highest concentration of BaP.

4.2.2 Development toxicity analysis in Zebrafish

Microscopic observation was done by using inverted bright field and fluorescence microscope (EVOS, ThermoScientific) to visualize the morphological and developmental changes, as well as tracking the fluorescence of BaP and the bioaccumulation sites. Hatching rate was determined as a number of embryos hatched by 72 hpf as compared to untreated group. Heartbeat rate were counted to estimate the physiological abnormalities due to exposure of BaP, Mortality rate was expressed as a number of dead embryos after exposure as compared to control. Apoptosis assay was done at 4dpf larvae. All the experiments were repeated thrice. Larvae at 14 days post-hatching (dph) from both the control and BaP treated groups were sampled for gene expression analysis.

4.2.2.1 Apoptosis analysis by Acridine orange staining

Analysis of apoptotic cells in Zebrafish larvae was detected with the help of Acridine orange staining (AO).control and treated groups of zebrafish larvae were washed twice with filtered E3 medium after 96 hpf treatment and exposed to 5 µg/ml AO dissolved in E3 medium for 20 min in dark. larvae were washed with E3 medium twice after staining to remove extra stains. Images were taken in green channel of EVOS

inverted fluorescent microscope (ThermoScientific, USA) to compare the apoptosis occurred in Zebrafish larvae due to exposure of BaP at different concentration.

4.2.2.2 RT-PCR analysis of bone relevant gene expression

RNA Extraction

Eight 14 dpf larvae were used for RNA extraction from the treated group 0.8 µM BaP and the control group. Autoclaved Egg water was used to wash the larvae twice. To attain a sufficient amount of RNA from 8 zebrafish larvae were transferred to 1.5 ml microfuge tube, and water was removed as much as possible with a pipette. Immediately we have added 100 µl of TRIzol reagent to the microfuge tube containing the larvae. Because of the phenol content in the TRIzol reagent , we have used it under a fume hood. Lyse and homogenize the larvae with a pellet pestle were done in the ice by approximately 25 strokes until the tissue is sufficiently disrupted. 23 gauge needle was used for taking the lysate and the disrupted cells through it 20 times . the lysate solution was taken through a 27 gauge needle 20-50 time until the solution has appeared uniform due to the sufficient homogenization. 100 µl TRIzol reagent was added to equal a total volume of 200 µl. The two solutions of control and treated group were incubated at RT for 5 minutes for complete dissociation of nucleoprotein complexes. 40 µl of chloroform was added and the tube was shaked for 15 seconds vigorously. Incubation of the samples for 2 minutes at room temperature was done and then centrifugation at 12,000 x g for 15 minutes at 4°C. The mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase which contained the RNA. The upper layer was transfered into a new microfuge tube using a pipette carefully. Isopropanol was added in equal volume of aqueous phase for precipitation the RNA. The sample were incubated 10 minutes on ice and then the sample was centrifuged at 12,000 g for 30 minutes at 4°C. RNA formed gel-like pellet on the bottom of the tube. We have removed the supernatant with a pipette and washed the pellet in 200 µl of 75% ethanol. The samples were mixed by gentle inversion and centrifuge at 7,500 g for 5 minutes at 4°C. After centrifugation, the ethanol was removed with a pipette and air dry process for 10 minutes. the pellet was resuspend by adding 20-25 µl of DEPC-treated Water. We checked the samples quantity using a NanoDrop (ND-1000 spectrophotometer).

cDNA Synthesis

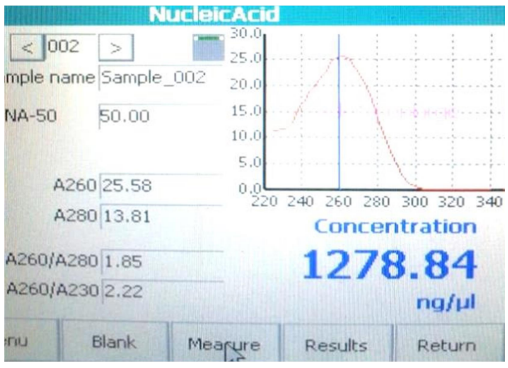
The readings of RNA in NanoDrop spectrophotometer for control and treated sample were 42.46 ng/µl and 88.44 ng/µl respectively RNA is not stable and can be degraded easily by to RNase, cDNA is developed for using in gene expression protocols be-

cause it's more stable than RNA and it has been directly synthesized from the RNA. We have used Verso cDNA synthesis kit from Thermo Scientific. Before beginning the procedure we have mixed and given each component short spin. RNA/primer mixture was mixed in a sterile 0.2 ml microfuge tube as outlined in Table 7.

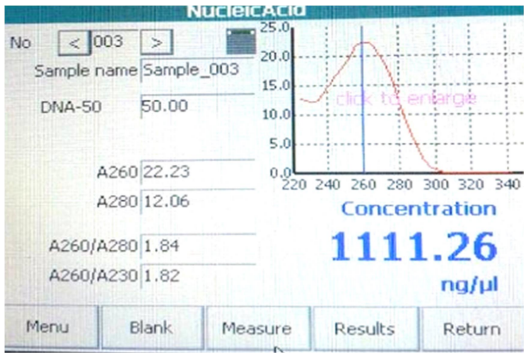
Components	**volume**
5x cDNA synthesis buffer	4 µl
dNTP	2 µl
Mix oligo(dt) (0.5µg/µl)	1 µl
RT enhancer	1 µl
Verso enzyme Mix	1 µl
Template(RNA)	Control : 200 ng or 4.71 µl
	Treated : 200 ng or 2.27 µl
Water, nuclease-free	Control : 6.3 µl
	Treated : 8.73 µl
Total volume	20 µl

Table 9: ingredients of Reverse Transcriptase PCR

The cycling program of RT-PCR is one cycle of cDNA synthesis at 42°C for 30 minutes, followed by inactivation cycle at 95°C for 2 minutes. After the PCR steps were done , the samples were mixed short spin. Spectrophotometric analysis was used to assess the quality of the cDNA product (Figure 4). The ratio A_{260}/A_{280} was more than 1.8 in the both samples. Then the samples were stored at - 20°C until use.



A: Control sample



B: Treated sample with 0.8µM of BaP

Figure 7: A NanoDrop spectrum of a cDNA sample. Spectrophotometric analysis with a NanoDrop Colibri was used to assess the quantity and quality of the cDNA product. The A_{260}/A_{280} ratio was more than 1.8.

PCR master mix for regular PCR

PCR Master Mix is a 2X concentrated solution of Taq DNA Polymerase, dNTPs, and all of the components required for PCR, except DNA template and primers. This pre-mixed components have the specific genes β-actin, Bmp2b, Sox9a, Spp1/opn, and Col1a1a, and Sox9a at different annealing temperatures 55°C, 55°C,53°C,47°C,and 50 respectively. The PCR products were loaded onto Ethidium Bromide-stained 1.5% agarose gel. A 1 kbp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was used to compare the bands of PCR products with known size bands.

Components	**volume**
PCR MSTER MIX	10 µl
F PRIMER	2 µl
Reverse primer	1 µl
Template DNA	1 µl
Verso enzyme Mix	1 µl
Water, nuclease-free	Control : 7 µl
	Treated : 7µl
Total volume	20 µl

Table 10: components of regular PCR.

The samples were incubated at 95°C for 2 mins to initiate the initial denaturation at 1 cycle. The next 40 cycles are for denaturation 95°C, annealing, and extension 72°C for 30 sec for each step. The final single cycle extension step was done at 72°C. We have used 4 different annealing temperatures according to our specific genes of interest. The annealing temperatures of Spp1/Opn, cola1, sox9a, and BMP2b are 47, 50, 53, 55°C respectively.

5 Results

5.1 Zebrafish developmental staging series

For assigning any toxic abnormalities of zebrafish developmental, observing the normal stages was necessary. To provide such a staging series, we provided serial stages of the development from 0 hpf till it becomes larva in Figure (8) . The timeline was also observed to compare it with the reference data and that was normally with no any delay in the development proliferations. The typical hatching of embryos occurs at 48-72 hpf.

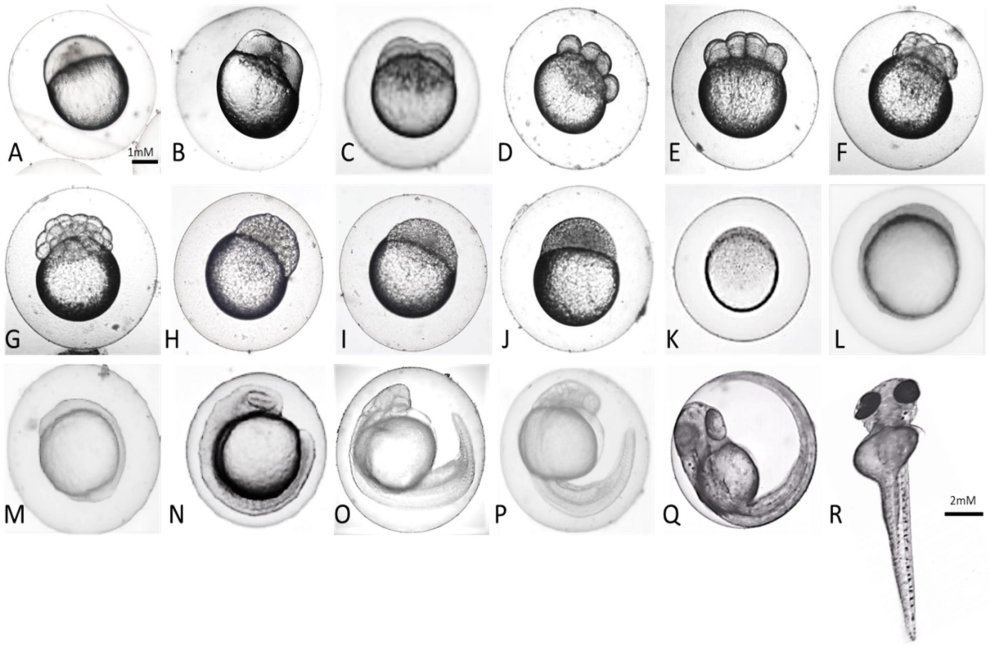


Figure 8: Bright field images of Zebrafish Developmental Staging Series

Embryos shown to the same scale, indicated by the scale bar (=1mM in **A**). Larvae shown to the same scale, indicated by the scale bar (=2mM in **R**).

A.1-Cell	B. 2-Cell	C. 4-Cell	D. 8-Cell	E. 16-Cell
E. 16-Cell	F.~ 32-Cell	G.~ 64-Cell	H.~ 128-Cell	I. ~ 265-Cell
J. ~512-Cell	K. ~1K-cell	L. Epiboly	M. Somite	N. Segmented
O. Prime-6	P. Prime-22	Q. High pec	R. Hatching 48-72 hpf	

5.2 Morphological abnormalities of zebrafish after exposure to BaP

Different deformities were clearly induced in the treated larvae directly after hatching. At 72 hpf, embryos incubated in 0.5, 0.6 , and 0.8 µM Bap showed from mild to se-

vere spinal curvature. In comparison of egg water EW as normal condition for growth and 1% DMSO as a vehicle control, Dorsal curvatures or tail malformations (TM) were more severe at the highest concentration of BaP. Notably, craniofacial defects (CD), pericardial edema (PE) and yolk sac edema (YSE) were observed at the highest concentration as shown in Figure (9).

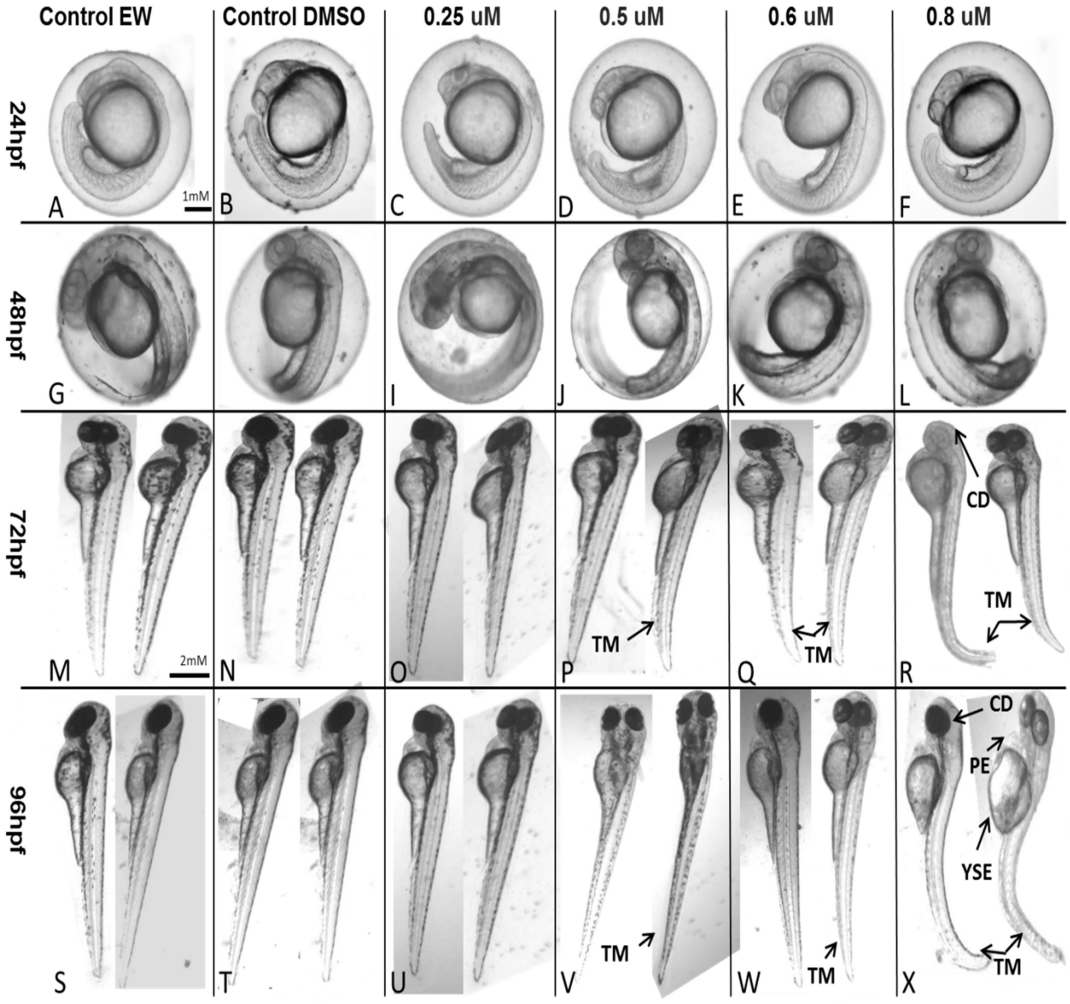
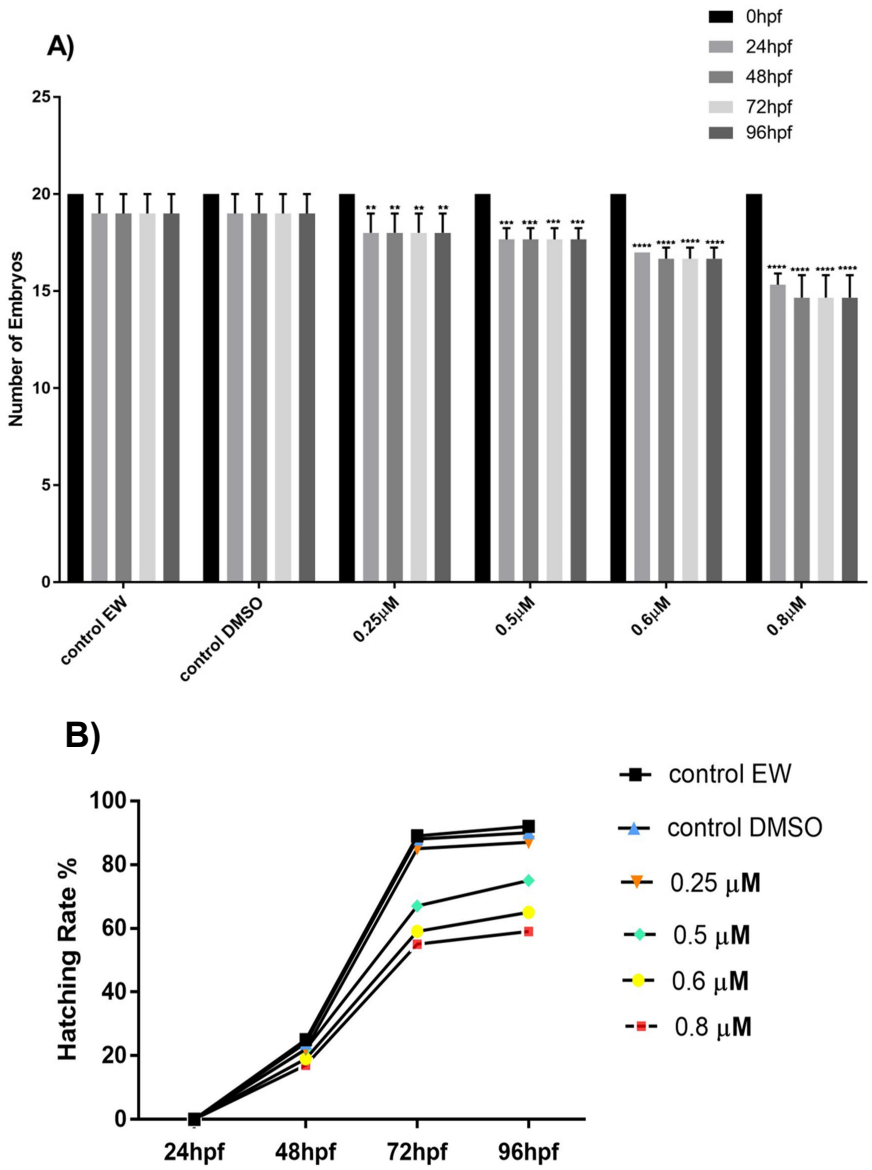


Figure 9: Brightfield images of morphological analysis of Zebrafish early stages exposed to different concentration of Benzo(a)Pyrene. TM: Tail Malformation; YSE: Yolk Sac Edema; CD: Craniofacial Defect; PE: Pericardial Edema. Embryos shown to the same scale, indicated by the scale bar (=1mM in A) 4x objective. Larvae shown to the same scale, indicated by the scale bar (=2mM in M) 2x objective.

5.3 The viability rate, Hatching rate, and Heartbeats rate analysis

The viability rate was measured in a group of 20. As shown in Figure 10A. The viability rate was defined to be reduced with an increase in concentration of BaP. Hatching rate was also calculated as the percentage of hatched embryos in the group of total exposed embryos. Figures 10B shows the hatching rate of untreated and treated embryos with BaP. It was found to be dependent on concentration. Hatching rate was delayed in case of embryos exposed to highest concentration of BaP as compared to control ones. The heartbeats rate was also determined to estimate the physiological counting the beats per minute thrice. It's visible the reduction of heartbeats rate in the



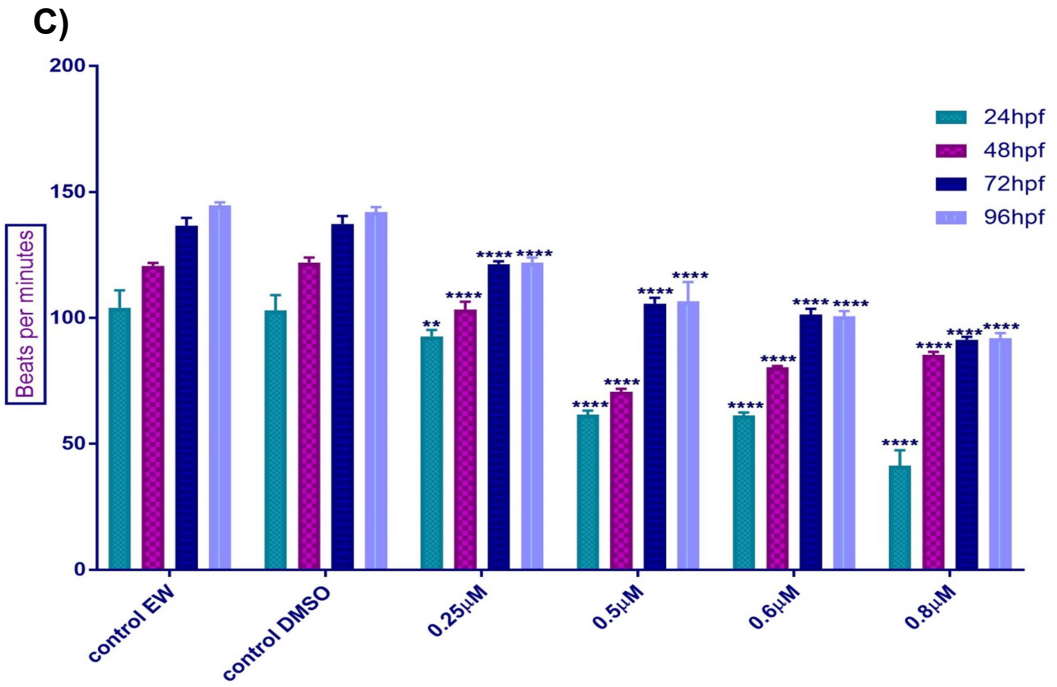


Figure 10: (**A**) Viability rate **$p < .005$, *** $p < .001$, **** $p < .0001$ (B) Hatching rate, and (C) Heartbeats rate of Zebrafish embryos exposed to Bap at different hours of post fertilization (hpf). All the measurements were taken in triplicate and the values were presented as mean ± SD of three independent experiments. **$p < .005$, ****P < 0.0001 denotes significant change from untreated embryos respectively as obtained from ANOVA analysis. Number of *presents the degree of significance.

5.4 Apoptosis analysis in Zebrafish embryos exposed to BaP

To determine the effect of Benzo(a)Pyrene in Zebrafish model at the cellular level, the embryos have been incubated in different concentrations of BaP. At the 4th day after exposure, induction of apoptosis was analyzed by using fluorescent microscopy after 20 mins of labeling time. As shown in Figure 12, Apoptotic cells were clearly identified in the head, trunk and tail region of Zebrafish larvae exposed to highest concentration of Bap. Moreover, The fluorescent intensity and the patches were analysed through the program ImageJ, highlighting areas of tissue with living cells and tissue areas with permeable cell. The use of the image analysis program ImageJ allowed to estimate the fluorescence intensity in different regions of the larvae, under control and treated conditions.

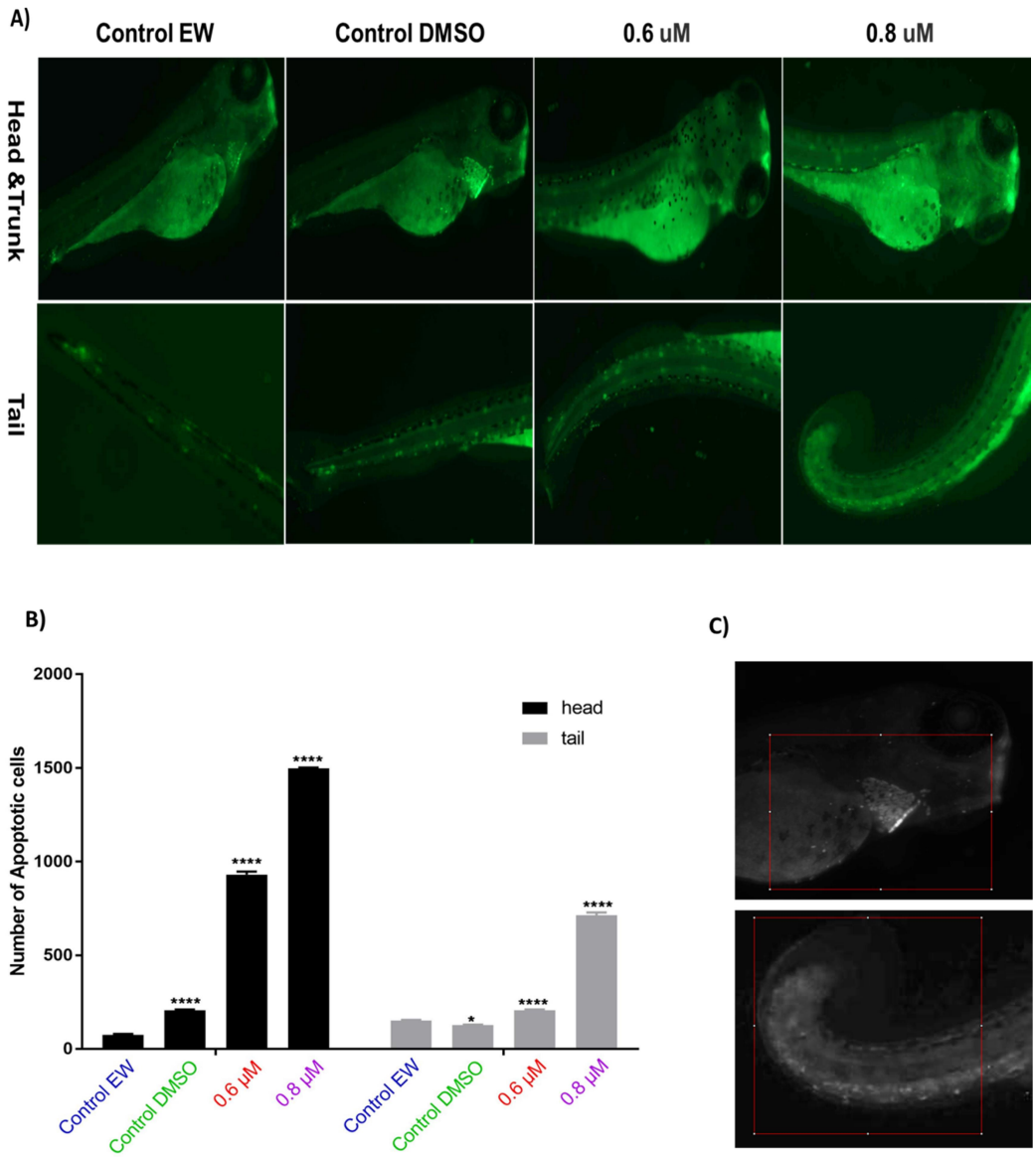


Figure 12: A) The fluorescence of AO inside the dead cells in treated with BaP and untreated sample. B) The rate of apoptosis level was calculated manually thrice by using ImageJ and the values were presented as mean ± SD of three independent experiments. *P < 0.05, and ****P < 0.0001 denotes significant change from un-treated embryos respectively as obtained from ANOVA analysis. Number of * indicates the degree of significance. C) ImageJ 32-bit images processed.

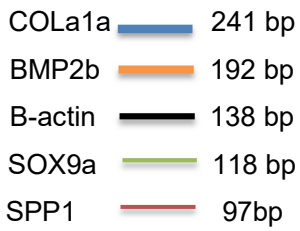
5.5 Change in expression of bone-relevant genes

RT-PCR was done to investigate differential expression of some key genes in zebrafish bone metabolism. Potentially incomplete formation of the bones found in the 14 dpf larvae which were exposed to 0.8 µM BaP. The amplicon images (PCR bands)

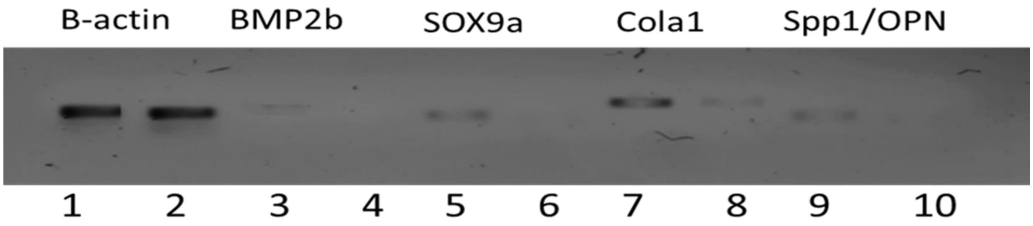
in the gel were captured by using transilluminator under ultraviolet (UV) light and by using a Doc gel documentation system. All the parameters and experimental conditions used were kept constant throughout the study. The image was saved on a computer for digital image analysis using ImageJ software version 1.5.j8. The expression of BMP2b, SOX9a , SPP1/OPN , and COLa1a was significantly down-regulated relative to controls in BaP treated sample.

A)

The gene	The PCR Product size
B-actin	138 bp
BMP2b	192 bp
SOX9a	118 bp
COLa1a	241 bp
SPP1/OPN	97 bp



B)



C)

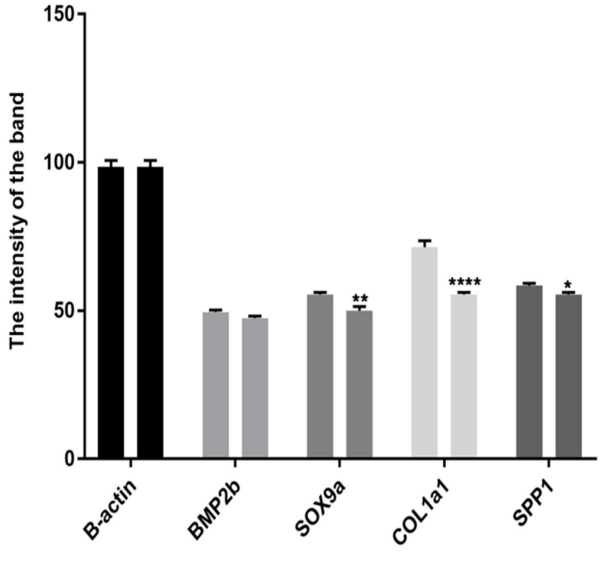


Figure 13:

A) The product size of each gene.

B) Agarose gel electrophoresis of RT-PCR product loaded on 1.5 % agarose gel. The control samples are present for each gene (1, 3, 5, 7, and 9) and 0.8 µM bap treated samples (second well of each gene 2, 4, 6, 8, and 10).

C) Semi-quantitive analysis by using imageJ software.

*P < 0.05, **P < 0.01 and ****P < 0.0001 denotes significant change from untreated embryos respectively as obtained from ANOVA analysis

6 Discussion

With the increase of the polycyclic aromatic hydrocarbons (PAHs) in the environment, their exposures to the ecosystem and human society have also extended which may lead to the adverse and lethal effects. BaP, a typical PAHs that is present in the environment. It's available commercially with high purity for the research purpose. We have dissolved it in organic solvent DMSO and we have used the same solvent with the same concentration as a vehicle control. The toxicity of BaP can be defined as the effective change in developmental, physiological and molecular level in Zebrafish model due to exposure. We have utilized Zebrafish as an in vivo model because of its advantages such as the generation of transparent embryos, external fertilization of the embryos, high fecundity, and the conservation of vertebrate organs.

In this study our objective was to study the effect of Benzo(a)Pyrene on the early development of zebrafish. BaP impact on Zebrafish embryos and adults were assessed in many studies. The present study demonstrates, for the first time, the major deformities of BaP on different kind of fishes in one sample such as Yolk sac edema, pericardial edema craniofacial defects and spinal curvature deformity. The phenotype deformities were seen in the highest concentration of BaP 0.6 and 0.8 µM. The larvae showed swimming impairment and this might be the reason beyond their death after week of the exposure, as they are not able to swim properly they lacked to reach to the paramecium while they are lacked of yolk sac as a nutrient source at this age. larvae can die without an apparent reason. Internal errors, sickness or mold, bad Water quality are the probably the main reason of death.

The viability, hatching and heartbeats rates of embryos were investigated and were significantly varied in between the control and the treated sample of BaP. The delayed in hatching rate of embryos was recorded can be attributed to the less development of chorion at higher concentration of Bap or affecting the hatching gland by BaP. Heart rate is a crucial measurement reflecting cardiac developmental toxicity. It was decreased with increase in the concentration of BaP. There are numerous of studies reported the developmental cardiac toxicity in multiple fish species due to PAH exposure. Cardiac function was significantly affected by BaP exposure (Huang et al. 2012).

Cytotoxicity of the BaP was demonstrated the cellular physiological death.induction of apoptosis level has been regarded as one of the important regulatory factors of cytotoxicity analysis. BaP have been reported to induce P53 and caspases enzymes in mice and zebrafish models (Lin et al. 2018). Using Acridine Orange staing is one of the simplest method to study the apoptotic cells .Our results indicated that BaP increases the cell damage. When AO is associated with DNA, it presented an excitation maximum at 502 nm and emission at 525 nm, in the green. When associated to RNA derived maximum excitation at 460 nm in the blue and the maximum emission at 650 nm in the red (Ribble *et al*., 2005).

The down-regulation of key genes of bone development was investigated by RT-PCR and semi-quantified by using imageJ software. The detected BMP-2b, SOX9a, OPN , and COLa1a dysfunction may be interpreted as molecular level of the impact of BaP on Zebrafish at early stages. BMP2b plays an important role in regulating vertebrate embryo development (Liao et al. 2014). SOX9a has a pivotal role in chondrocyte differentiation (He et al. 2016). OPN and COL1a play an essential role in the osteogenesis process. Any defect in collagen I will lead to reduction of bone mineralization and bone fragility (Asharani et al. 2012). The given results in this study may also give information about the effect of BaP on Bone deformities in human.

7 Conclusion

This study has provided clear evidence that Benzo(a)Pyrene has a toxic effect on zebrafish at the morphological , physiological , and molecular level. For the first time,

We have found the all expected PAHs phenotypic deformations in one sample such as tail malformations , yolk sac edema, craniofacial defect , and pericardial edema. The bioaccumulation of BaP intercellular was inside the yolk sac tissue .The apoptosis level was induced due to the BaP exposure. The down-regulation effect of BaP on the expression of BMP-2b, SOX9a, OPN, and COLa1a genes was investigated for the first time. The cellular and molecular findings obtained in this study suggest that BaP may have effect on the skeletal diseases in mammals.

8 Future work

Further investigation is necessary in order to address all the limitations of this study which are:

- The results are lacking of any measurements of the deformities.
- The bioaccumulation of BaP inside Zebrafish was not been studied.
- Using RT-PCR rather than qRT-PCR which will provide exact quantity of the gene expression.
- Studying the effect of Banzo(a)Pyrene on the protein level.
- confirm the role of AhR in mediating the effects on the osteogenesis.

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