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Induction of Ethoxyresorufin-o-deethylase (EROD) activity by Benzo[a]pyrene in Primary Culture of Gill Epithelial Cells from Tilapia fish

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ABSTRACT

Using in vitro techniques in ecotoxicological studies to detect and measure chemically induced distress in waterbodies is gaining momentum. In South Africa, toxicity testing is a part of managing water resources. In our study, primary gill epithelial cells established from the gills of indigenous fish (*Oreochromis mossambicus*) were used to assess the CYP1A induction, serving as an alternative to whole fish toxicity testing. Benzo[a]pyrene (B[a]P), a potent aryl hydrocarbon receptor (Aryl) agonist usually found in contaminated water, was exposed to primary cultures of gill epithelial cells and a continuous fish gill cell line RTgill-W1 cells. The primary gill epithelial cells responded to CYP1A induction, while the commercially available RTgill-W1 cell line showed no activity ($p < 0.001$). Cytotoxicity, determined by the methyl thiazole tetrazolium (MTT) assay, was not observed following a 72-h exposure in the primary gill epithelial cells and the RTgill-W1 cell line to differing B[a]P concentrations. The gill epithelial cells isolated from the gills of Tilapia fish (*Oreochromis mossambicus*) were similar in morphology to fish gills. The results showed gill filament EROD activity as a sensitive, rapid, cost-effective biomarker that detects readily metabolized Aryl agonists in polluted water.

Keywords: Cultured gill epithelial cells, *Oreochromis mossambicus*, *in vitro assay,* benzo[a]pyrene, EROD.

INTRODUCTION

Many industrial wastes and compounds occasionally move into the aquatic ecosystem, consequently causing stress to the hydrosphere (Schwarzenbach et al. 2006). In South Africa, a major challenge to the water ecosystem involves mining. Emalahleni is a Municipality in the Mpumalanga Province of South Africa that hosts most of the country's coal reserves, and supplies 83% of the total produced coal in the country (Vureen 2009). Disposal of mine wastewater into natural receiving water systems from coal mining creates an environmental problem such as acid mine drainage (de Villiers and Mkwelo 2009), which purportedly triggers fish and crocodile die-offs (Paton 2008).

Polycyclic aromatic hydrocarbons (PAHs) are commonly associated with coal production, where they seep along with other toxic metals in the environment (Wang et al. 2008). There are benchmarks set by the Department of Water Affairs and Forestry (1996) for contaminants such as metals in water compartments; however, none exists for PAHs in South African waterways. PAHs ensue due to the incomplete combustion of organic materials like coal (Chen et al. 2022). The coal application process releases

PAHs and other potent inducers of CYP1A, contributing to point source contamination (Gadagbui and Goksøyr 1996; Olajire et al. 2005; Abdel-Shafy and Mansour 2016).

PAHs, as hydrocarbons, have two or more benzene rings, are hydrophobic or lipophilic, and can bind to DNA (Kuang et al., 2011). PAHs within water compartments bind to living organisms' lipids and can bio-accumulate in the food chain (Jones and Voogt 1999; Oliva et al. 2020; Recabarren-Villalón et al. 2021). Sediments from rivers also act as reservoirs for PAHs (Fatoki et al. 2010). Further concerns with PAHs are their endocrine-disrupting ability in humans and wildlife (Harrison et al. 1995; Kavlock 1996; Sanderson 2006; Patel et al. 2015), immune impairment (Safe 1994; Burchiel 2005), and disease susceptibility properties (Leonards 1997; Lee et al. 2016). They are also considered putatively carcinogenic or mutagenic (Conney 1982; Xue and Warshawsky 2005, Munoz et al. 2011, Li et al. 2020).

The generation of CYP1A is extensively used in environmental monitoring (Bucheli and Fent 1995; Park et al. 1996). These enzymes are bio-transformed by endogenous and xenobiotic compounds which stimulate CYP1A by the aryl hydrocarbon receptor pathway (Hahn 2002; Knerr 2006). Polycyclic aromatic hydrocarbons such as benzo(*a*)pyrene (BaP) are the most recognizable PAHs (Lee and Anderson 2005; Vogel et al. 2020) known to induce CYP1A (Shimada and Guengerich 2006; Nebert et al. 2004). Rapid and cost-effectiveness advantages are presented when utilizing animal cell bioassays to screen dioxin-like compounds (Firestone 1991; Hahn 2002).

Fish function in human and ecological health studies because of their sensitivity to toxicity (Van der Oost et al. 2003; Siroka and Drastichova 2004). They have been used in risk assessment, ecotoxicity, and chemical contamination studies (Bailey et al. 1996). Tilapia (*Oreochromis mossambicus*), an indigenous fish species, is a local species found within the tropical and subtropical Southern Africa region (Skelton 1993). It has been used successfully as a test organism in biomarker studies (Hwang and Yang 1997; Li et al. 1997; Chen et al. 2001; Shailaja and D'Silva 2003).

Fish gills are the first contact of toxicants in the aquatic environment and are sensitive to water-borne contaminants (Wood 2001). The gills are vulnerable to waterborne toxicants and function to eliminate toxins (Wood 2001), making them suitable for detecting waterborne pollutants before reaching the liver (Levine and Oris 1999; Jönsson et al. 2004). Consequently, bioassays involving primary cultured gill cells in fish could be a vital means of detecting changes in water quality arising from pollutants such as B[a]P. Since primary cultures are considered more sensitive than continuous cell lines because of higher metabolic capacity (McKim et al. 1985; Lee et al. 1993) stability, (Segner 1998), and the ability to retain their CYP1A expression better than cell lines (Lee et al. 1993), they could be better suited as a replacement for whole fish testing.

Before observable adverse effects become apparent in organisms or populations, using biomarkers as early warning signals for pollutants in environmental water quality surveillance systems is necessary (Hook et al. 2014). Through laboratory-controlled toxicity testing, anthropogenic changes in aquatic ecosystems can be instituted for effective environmental assessment and monitoring. The fundamental similarity for toxicity in all vertebrates is related to the route of exposure and biotransformation of xenobiotics for contaminants such as B[a]P and other PAHs (Billiard, 2002), making fish an ideal sentinel. The metabolic outline of B[a]P in fish is comparable *in vivo* or *in vitro* (Steward et al. 1990; Nishimoto et al. 1992). The main objective of this study was to evaluate the EROD induction in primary gill cultures of Tilapia fish (*O. mossambicus*), an Indigenous African freshwater fish, as a tropical model species for B[a]P-induced waterborne toxicity.

MATERIAL AND METHODS

Study area

Ten Male Tilapia fish weighing 70 – 100g were obtained from a local fish farm (DeWildt fisheries, Brits). They were acclimatized for four weeks in a 1500 l tank containing water with constant aeration and water circulation at the Aquatic Lab, Paraclinical Sciences Department, Faculty of Veterinary Sciences, Onderstepoort. The tank was connected to an external filter system that was cleaned once a week and half the water in the tank was replaced with fresh running tap water weekly. The fish were kept at room temperature following the natural variation over the year and were fed commercial fish pellets five times a week. The pH of the running water was between 7.6 – 7.7 and the Ionic composition (mg/l): Na^{+}; 21, Cl^{-}; 27, Ca^{2+}; 23, Mg^{2+}; 13, $HCO3^{-}$; 100, Alkalinity; 100 and DO; 85%. Ethical permission for the study was obtained from the Animal Ethics Committee, University of Pretoria (**protocol number V027-12**).

Chemicals and cell culture medium

Trypsin, Leibovitz L-15 culture medium, penicillin, and streptomycin, were purchased from Gibco® (Life Technologies, USA). The Fungizone, rhodamine 123, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich® (USA). At the same time, Gentamicin (50 mg/ml) was purchased from Virbac (South Africa), and the fetal bovine serum (FBS) from Highveld Biological® (South Africa). Culture dishes (Nunculon) were obtained from Nunc® Denmark, and 7-ethoxy resorufin and dicumarol were sourced from Sigma-Aldrich®. Other chemicals were commercial chemicals of reagent grade.

Cells isolated for primary cultures

Fish were starved for 3 days and then kept in sterile aerated tap water for 1h before the preparation of cells. After being stunned by a blow to the head, the fish were decapitated. From this point onwards, all procedures for gill cell isolation were conducted using sterile techniques in a biohazard cabinet (ESCO class 11 BSC, Labotec). The gill cell isolation protocol by Kelly *et al.*2002 was adopted with a few modifications.

Gill arches were removed into a wash solution made up of phosphate-buffered saline (pH 7.7: 136.9 mM NaCl, 8.06 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) containing the antibiotics penicillin (200 IU/ ml), streptomycin (200 µg/ml), gentamicin (12 µg/ml) and fungizone (2.5 µg/ml). Gill filaments were blotted to remove excess mucus, cut into approximately 1-3mm pieces, and washed 3 times in 10 ml of the wash solution for 10 mins with frequent manual agitation.

The washed filaments were then treated with three consecutive cycles of tryptic digestion until filaments appeared translucent using trypsin (0.05% trypsin in PBS, with 5.5 mmol/l EDTA) for 20 min/cycle at 300 rpm on a shaker (microporous quick shaker QB-9001). The tryptic digest was mechanically agitated following each cycle using a transfer pipette to release the cells which were then strained through an 80 µm cell strainer into a stop solution (10% FBS + PBS pH 7.7) using a different cell filter each time. The stop solution containing the cells was then centrifuged for 10 min (260 g at 0-4°C), leaving a pellet, which was washed in cold rinse solution (2.5% FBS + PBS pH 7.7) and centrifuged for 10 min at the same speed.

Cells were then re-suspended in cold L-15 culture medium supplemented with 5% FBS, 100 i.u./ml penicillin, 100 µg/ml streptomycin, and 20 µg/ml gentamicin into flasks or 6 well microtiter plates at a density of 1-1.25 x 10^6 cells /cm^2 and kept at 21°C in an air atmosphere incubator. After 24h, non-adherent cells were removed by changing the medium with the above-constituted L15 medium. Media change was repeated after 72 h. After a further 24-48 h in culture, assays were carried out using the 96 well plates.

Culture of RTgill-W1 cell lines

The RTgill-W1 cell line (ATCC® CRL2523™) was cultured in Leibovitz L15 medium supplemented with 5% FBS in an atmospheric air incubator at 20ºC. Cells from a sub-confluent culture were re-suspended in a cell culture

medium supplemented with 10% FBS at $0.2x10^6$ cells/ well in a 96-well plate and allowed to attach for 24 hours before assays were performed.

Ethoxy resorufin-O-deethylase (EROD) activity
This protocol was modified and adapted from Behnisch et al. 2003. RTgill-W1 cells maintained in whole culture media over 24h were plated at 0.2×10^6 cells /well in a 6-well plate. Primary gill cell cultures at days 6-8 containing an average of $1.68x10^5 \pm 0.25$ cells/well were assayed. Exhausted culture medium was removed, and cells were exposed to a serial dilution of B[a]P ($1x10^{-4}$ M to $1x10^{-12}$M) for 72h. The B[a]P was dissolved in acetone, maintaining a final concentration of 3% v/v in each treatment. Control cells contained no B[a]P but only 3% acetone. The experiment was repeated at least three times and the data represent replicates from three separate cell preparations. After 72h exposure to B[a]P, the exposure medium was removed, and cells were rinsed with PBS. HEPES Courtland (HC) was prepared at pH 7.7 using 5 mM potassium chloride, 133 mM sodium chloride, 0.9 mM magnesium sulfate, 2 mM calcium chloride, 3 mM sodium phosphate, 6 mM HEPES, and 5 mM glucose.
The HC-dicumarol buffer contained 10µM dicumarol, to which 16 µM 7-ethoxy resorufin was added. A 100 µl of HC–dicumarol + 7-ethoxy resorufin solution was added to the cells and the plates were incubated at 21°C for 2 h, after which 90 µl of the reaction mixture was transferred to a 96-well black plate (Nunc, AEC Amersham, Kelvin, South Africa) containing 100 µl methanol. Fluorescence was measured at 544 nm excitation and 590 nm emissions using a multi-well fluorescence reader (BioTek®synergy HT-BioTek instrument, Winooski, USA).

Phase contrast microscopy
Growth and proliferation were monitored using a phase contrast microscope (Nikon Eclipse TS100). The primary culture was observed for growth and following exposure to B[a]P for 72h, gill epithelial cells were monitored for cellular changes. Cell preparations stained with rhodamine were carried out to examine the presence of mitochondria-rich cells.

Statistical analysis
All assays were performed in three independent experiments and the results are presented as mean ± SD (standard deviation) values. The significance of mean differences at $p<0.05$ was examined by one-way analysis of variance (ANOVA), Hartley's f test for equal variance. Inter- and intra-test the appropriate Student- T-test. This data analysis was performed using Open-source calculator Version 3.

RESULTS

Cell viability of primary fish gill cells
The average yield of cells from approximately 3 g wet mass of gill filaments was $79 \pm 13 \times 10^6$ cells (mean ± S.D, *N*=12), excluding red blood cells (RBC) with about 10% (n=6) being rhodamine positive, indicating the presence of mitochondria-rich (MR) cells (Fig 1a, 1b). These MR cells were observed initially in culture but did not propagate, so they were not present at the cell confluence. The attachment efficiency in culture media, 24h after seeding was 32±5%. Several RBC and non-viable cells were removed when culture media was changed after 24 h. The single cells appeared elongated, while colonies appeared more as round cells (Fig 1b) and the cells reached confluence between 6-8 days. The attached cells 24h after seeding, were incubated with the fluorescent dye rhodamine to detect the presence of chloride cells. Fig 1c and d show bright green fluorescent cells absent at confluence.

EROD induction in cells
Following 72h exposure of the primary cultures of gill epithelial cells to B[a]P at various concentrations, CYP1A induction was evaluated as increased EROD activity. The control epithelia showed low EROD activity (200p mol/h/well). EROD induction by B[a]P was initially concentration-dependent, but as the concentration

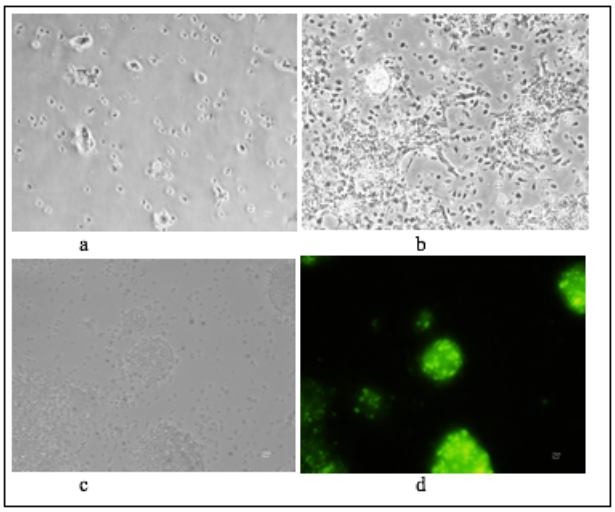

Figure 1: Photomicrographs of epithelial cells from fish gills grown in plastic culture dishes

Photomicrographs depict epithelial cells from fish gills grown in plastic culture dishes (x200) (a) cells at day 1 after seeding (b) 3 days old culture (c &d) show attached cells stained with rhodamine and fluorescing MR cells which were not present at the confluence.

of B[a]P increased from 10^{-6} M, there was a drastic decrease in EROD activity (Fig 2).

In primary gill cultures exposed to B[a]P**,** the peak EROD activity observed was at $1x10^{-7}$ M which was about 4 times higher than observed for the control. The RTgill-W1 cell lines, on the other hand, did not show EROD induction or loss at the tested concentration range. The acetone vehicle that was used in both cell lines did not interfere with EROD activity induction. A significant difference in EROD activity was observed when comparing both cell types ($p < 0.001$).

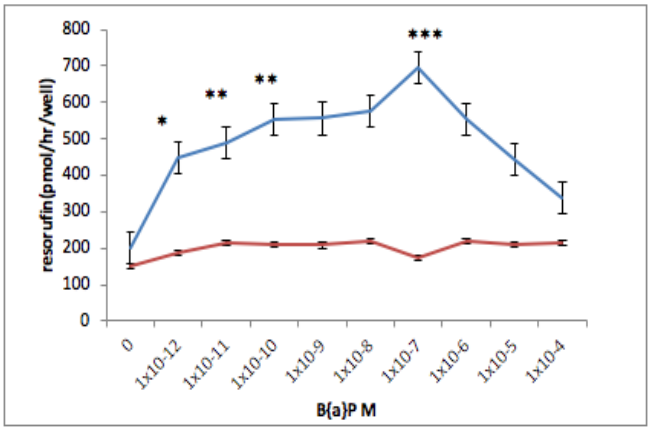

Figure 2: EROD activity of primary gill epithelial cells and the RTgill-W1 following BaP exposure

Dose-dependent effect of B[a]P primary gill epithelial cells (blue line) and the RTgill-W1 cell line EROD activity (red line) after a 72h exposure period. Each data point represents the mean of three independent cultures ±SD. Only the gill epithelial cells induced EROD activity and maximum activity was observed at 10^{-7}M B[a]P. Significant difference established at (P<0.05)

The MTT assay technique employed following a 72h exposure of the primary gill epithelial cells and the RTgill-W1 cell line to different concentrations of B[a]P did not depict cytotoxicity, as cell viability was maintained at ≥ 80% (Table 1).

Table 1: Summary of cell viability (%) of primary gill epithelial cells and RTgill-W1 exposed to B[a]P

	$1x10^{-11}$M B[a}P	$1x10^{-10}$M B[a}P	$1x10^{-9}$M B[a}P	$1x10^{-8}$M B[a}P	$1x10^{-7}$M B[a}P	$1x10^{-6}$M B[a}P	$1x10^{-5}$M B[a}P	$1x10^{-4}$M B[a}P
Mean ± SD (PGE)	83±0.6	81±0.3	82±0.7	81±0.7	82±0.8	83±0.6	80±0.6	82±0.4
Mean ± SD RTG	86±0.5	85±0.9	83±0.7	84±0.7	84±0.3	85±1	86±0.4	86±0.5

Dose-dependent effects of B[a]P on primary gill epithelial cells (PGE) and the RTgill-W1 cell lines (RTG) viability after a 72-h exposure period using the MTT assay. Each data point represents the mean of triplicate experiments ± SD.

DISCUSSION

The practicability and properties of fish cell culture assays justify their use in research (Castano 2003). Fish gills traditionally receive much more attention because they are the first barrier for waterborne toxicants (Levine and Oris 1999). Globally, fish are used for waste effluent testing and bio-monitoring, and in the US alone, about 3 million fish are sacrificed annually (Tanneberger et al. 2013). Thus, a need to reduce, replace, and refine these studies using alternatives to whole animal testing in the form of primary gill culture systems from fish gills. B[a]P, a potent CYP450-inducer found in contaminated water, can be identified using a gill-based EROD assay as a vertebrate-based biomarker in water toxicity assessment. This allows for large numbers of chemicals to be screened quickly, with fewer test substances and waste generated.

Structurally, the primary gill cultures from Tilapia fish are comparable to those observed in the epithelium from intact Tilapia fish gills (Kelly and Wood 2007). The primary cultures retain the three-dimensional structure of the tissue and maintain cell-to-cell interaction, like gills *in vivo* (Levine and Oris 1999; Kelly and Wood 2007). They contain MR or chloride cells (about 10% of the total cell population) which did not propagate in the culture conditions, possibly due to crucial factors necessary for their survival lacking, as MR cells were absent at confluence.

Toxic and biochemical responses arising from PAHs and dioxin-like compounds are facilitated through AhR (Gies 2002; Hahn 2002, Vogel et al. 2022). The study evaluated EROD induction in cultured gill epithelial cells at environmentally relevant concentrations for B[a]P. A pattern in which higher EROD induction was observed at lower concentrations and activity lowered at higher concentrations was noticed, possibly linked to the deactivation of the CYP1A enzyme at higher concentrations. Substrate competition of B[a]P and Ethoxy resorufin at the catalytic site of the CYP1A enzyme was described (Smeets et al. 1999; Risso de Faverney, 2001). Naicker 2007, indicated a similar observation in Indian catfish (*Heteropneustes fossili*) and primary hepatocytes of the African sharp-tooth catfish.

Studies have correlated contaminant-induced changes with CYP1A expression in fish tissues (Stegeman et al. 2001; Woodin et al. 1997). Cytochrome P450 activity was reported in the gills of the gulf toadfish following exposure to B[a]P in water (Kennedy and Walsh 1994) and a similar demonstration was described in the gills of other fish (Miller et al. 2004; Stegeman et al. 2001). Mdegela et al. 2010 and Zhou et al. 2005, using African Sharp-tooth catfish reported a dose-dependent CYP1A activity following waterborne exposure to B[a]P in the gill filaments and the hepatic microsomes. The importance of CYP genes in tissues as pollution biomarkers has been highlighted by Cortés-Miranda et al. 2024 and the sites of CYP1A induction following exposure to B[a]P in water identified in the epithelial gill and pillar cells in the topminnow (*Poeciliopsis spp*.) (Smolowitz et al. 1992).

Cytotoxic reaction to xenobiotics is influenced by temperature (Babich et al. 1991), adopting a protocol for primary gill cell cultures arising from fish locally sourced to determine its suitability for toxicity testing of xenobiotics such as PAH, is crucial. CYP1A induction in gills has proven to be a simple and direct biomarker for waterborne, dioxin-like pollutants. (Jönsson et al. 2004; Levine and Oris. 1999). The relationship between EROD activity and the detrimental effects on primary fish gill cultures from locally available fish may be a predictive tool for contaminant risk assessment. The measured biomarker responses may be used to assess a contaminated site (Van der Oost et al. 2003).

Biomarkers help to detect exposure to pollutants before adverse outcomes occur in affected organisms (Lionetto. et al., 2019). The successful documentation of measured EROD activity in local fish could serve as a sensitive index for pollution in contaminated sites. (Stegeman and Lech 1991; Bucheli and Fent 1995; Van der Oost et al. 2003) and provide information based on firsthand observations. (Brack et al. 2005). Similar to previous findings, the RTgill-W1 cell lines did not display CYP1A enzyme activity (Bury et al. 2014).

Conclusion

This study has shown the potential complementary use of primary gills epithelial in

evaluating exposure to B[a]P. A sensitive biomarker of exposure is the induction of CYP1A, which is used extensively in environmental monitoring. The findings underscored the importance of CYP1A-inducing compounds like B[a]P within the use of pollution biomarkers. The CYP1A-inducing compounds like B[a]P can be monitored using the primary gill epithelial cells of tropical fish *O. mossambicus*. Overall, our study suggests the existence of an interaction between B[a]P CYP1A-inducing compounds and primary cultured fish gills, which, when taken into account, is useful for biomonitoring in polluted aquatic environments. Since the impacts of environmental pollution, especially PAHs, on human diseases continue to receive increased attention, a straightforward, relatively less cumbersome technique reported in this study could be incorporated as a suitable biomarker.

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