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Comparative Toxicological Analysis of Polycyclic Aromatic Hydrocarbon (PAH)-Rich Soot Extracts from Gasoline and LPG-fueled Taxis Using the Zebrafish Embryo Toxicity (ZFET) Test.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds which are known to be teratogenic and embryotoxic. Many fuel sources, such as liquefied petroleum gas, are currently examined as alternatives to gasoline use in automobiles to help reduce environmental pollutants, such as PAHs. In this study, a comparison of the kinds, concentrations, and toxicological potencies of PAHs from soot extracts obtained from LPG-powered and gasoline-powered taxi tailpipes was conducted using GC-MS analysis and the zebrafish embryotoxicity (ZFET) test. GC-MS results showed that both liquefied petroleum gas (LPG) and unleaded gasoline (UG) extracts contain comparable levels of environmentally relevant PAHs. In the ZFET Test, dilutions ranging from 1:0 (undiluted) to 1:4 were embryotoxic to zebrafish embryos. A dose response relationship was seen in both types of soot extracts with decreasing mortalities and developmental abnormalities observed from dilution 1:5 to 1:25, in zebrafish embryos exhibiting lethal and sublethal endpoints. The embryotoxic responses of zebrafish upon exposure to LPG and UG soot extracts were statistically comparable. The results obtained from the study could provide valuable inputs for the development of policies on the use of alternative fuels to help reduce PAH levels in the Philippines.
Keywords: LPG, gasoline, PAH, soot, zebrafish, embryotoxicity.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous semi-volatile organic compounds commonly found in the atmosphere [1], food, water, oil, coal tar, and many other sources [2]. Since PAHs are regarded as known carcinogens [3], there have been renewed interests among scientists to determine their mutagenic and teratogenic properties. One of the main contributors of PAHs in the environment is weathered crude oil [2], which is a known aquatic pollutant introduced through maritime disasters such as oil spillage. The prevalence of oil spills had been pointed out to be the main cause of declining fish populations in the area of spillage, by introducing PAHs that affect developing embryos [3,5]. Despite their relative insolubility [6], PAHs can also find their ways onto potable and wastewater. Consequently, the mechanism of teratogenicity of PAHs has been a subject of growing ecotoxicological investigations [7].

Vehicular emissions resulting from rampant utilization of fossil fuel and other petrogenic resources have increased PAH levels in the environment [8-14]. These emissions are caused mainly by the rampant use of gasoline and diesel powered engines, whose numbers increase daily [15]. Like any other country, the Philippines also experiences unabated increase in PAH levels due to the usage of the same types of automobiles. In the last seven years, PAH emissions from automobiles have contributed more air pollution in the National Capital Region, as compared to those from solid waste burning and industries. Consequently, alternative fuel sources have been sought of intensively to diminish levels of PAHs in the environment. The Department of Energy (DOE), as part of their Alternative Fuels Program (AFP), has introduced and promoted the use of liquefied petroleum gas, which is regarded to be “clean fuel” and reported to be more energy- and cost-efficient [16]. Studies have shown that levels of LPG particulate emissions suspended in the air are significantly lower than that of gasoline [17]. However, contradicting studies have also reported that PAH levels are similar and statistically comparable with that of unleaded gasoline emission levels [18-20].

Studies in the field of medicine, marine biology, and ecotoxicology are now using fish instead of mammals to model human diseases. Characteristics that make the zebrafish (*Danio rerio*) ideal for experimentation include high fecundity, transparent chorion, and quick organogenesis [21-23]. Currently, zebrafish has been preferably used as a toxicological model to explain various mechanisms of toxicity of chemicals since its embryogenesis is comparable to that of higher vertebrates, and for having analogous genes, receptors, and molecular processes of development [21]. Chemicals that have been tested for these parameters include dioxin [24], fullerene [25], and many others, including PAHs [3, 26].

PAHs utilized in previous studies had been sourced from non-edible sources such as weathered crude oil, generally composed of petrogenic PAHs [26-30]. Soot, which is composed of particulate emissions derived from the incomplete combustion of fuel, settles in tailpipes of automobiles and represents particulate matter to which humans are exposed. It has been determined that automobiles contribute to the majority of PAH emissions in the atmosphere [20, 28, 31-32] and thus exposure to particulate matter as represented by tailpipe soot can be potentially hazardous to embryonic development. Nevertheless, the determination of fuel sources which impose greater embryotoxic and teratogenic risks to embryos will be essential to alter fuel consumption of the transport sector to provide a safer environment for growth and development.

MATERIALS AND METHODS

Sample Collection

Samples were collected from tailpipes of 40 taxis, 20 of which use Liquefied Petroleum Gas (LPG) as their fuel source and the remaining 20 use Unleaded Gasoline (UG). The taxis were situated at Clean Fuel Gasoline Station along Roxas Boulevard, Pasay City, Metro Manila, Philippines.

Soot Extraction and PAH Chemical Analysis

The collected soot samples were subjected to organic solvent extraction to afford the polycyclic aromatic hydrocarbon (PAH) extracts that will be utilized in the zebrafish bioassay. A total of 170.1 g of collected LPG soot extract and 31.2 g of collected UG soot extract were soaked in ethyl acetate - methanol mixture (1:1) overnight. The ethyl acetate - methanolic extract from LPG and UG were collected, filtered in

Celite and concentrated under reduced pressure using Buchi rotavapor at 45 ^{o}C resulting to 10.8 g of LPG-PAH extract and 3.18 g of UG-PAH extract. The LPG stock solution was prepared by mixing 0.0637 g of LPG-PAH extract with 10 mL 1% DMSO. The same procedure was done to prepare the UG stock solution by mixing 0.1019 g of UG-PAH extract with 10 mL 1% DMSO. To assure dissolution of both the extracts, the solutions were subjected to ultrasonication for 1 hour.

The PAH soot extracts were analyzed and chemically profiled using gas chromatography-mass spectrometry (GC-MS). A splitless mode on a Varian 400 GC-MS device was utilized, with the injector and transfer line temperature set at 280 ^{o}C, ion trap at 150 ^{o}C, and the ion source at 230 ^{o}C. The column temperature program, on the other hand, was set as follows: initial temperature at 55 ^{o}C, hold at 1 minute; ramp of 30 ^{o}C/min to 140 ^{o}C; ramp of 5 ^{o}C /min to 240 ^{o}C, hold at 5 minutes; and ramp of 8 ^{o}C/min to 300 ^{o}C, hold at 12 minutes. Using the NIST software, the presence and concentration of specific PAHs were confirmed and identified. The quantity of the PAHs was obtained using the external calibration method.

Zebrafish Embryo Toxicity (ZFET) Test

Parental Zebrafish Procurement and Maintenance

Sexually mature zebrafish (*Danio rerio*), 20 adult male and 20 adult female, about 4 to 6 months, were procured. Two (2) glass aquaria [16x8x10 inches] housed the male and the female sexually matured zebrafish separately. The tank system was maintained at the following conditions: 12-hour light/12-hour dark cycle using 18-watt daylight energy-saving light bulbs; temperatures ranging from 27 – 28° Celsius, pH (7.5 ± 0.5), dissolved oxygen (10.5 ± 0.5 mg/L O2), hardness (400 mg/L CaCo3), and conductivity (800 microSiemens/cm).

Zebrafish Spawning and Egg Collection

A separate aquarium, with dimensions of 16x8x10 inches filled with 6 gallons of dechlorinated water, served as a spawning chamber. Before zebrafish spawning, a spawning tray was placed at the bottom of the aquarium for egg collection and a 1x1 meter fishnet with a grid size of 2.5 mm was submerged. The sexually mature zebrafish, 6 males and 3 females (ratio of 2:1 according to [33]) were placed over the submerged fishnet to prevent egg predation while mating and spawning. Mating was triggered when light from the fluorescent lamp installed atop the aquarium was turned on. Spawn collection was done approximately 3-4 hours after the light was turned on. The eggs were collected randomly by suction using a micropipette. To avoid possible egg abrasion, the micropipette tips were widened in order to allow easier suction and emptying of the zebrafish eggs to Petri dish plates.

Zebrafish Exposure to Test Substance

Petri dish plates served as the primary exposure chambers containing the varying dilutions of LPG and UG PAH extracts, as well as the positive and negative control. Each petri dish plate contained 15 embryos for exposure. The varying concentrations of the LPG-PAH extracts and the UG-PAH extracts differed according to the degree of dilution with reconstituted water. Dilutions of 1:0, 1:1, 1:2, 1:3, 1:4, 1:5, 1:9, 1:13, 1:17, 1:21 and 1:25 were prepared for both PAH extract soot samples from tailpipes of taxis which use either Liquefied Petroleum Gasoline (LPG) or Unleaded Gasoline (UG) to ascertain fair comparison of results. 2% Ethanol served as the positive control while reconstituted water served as the negative control. The preparation of reconstituted water was based on the protocol set by ISO 6341.

Four 96-well plates were used as test chambers, two for LPG-PAH extract solutions and the other two for the UG-PAH extract solutions. Prior the transfer of viable zebrafish eggs, each well were pre-saturated with 0.2 mL of the test solution of varying dilutions for 24 hours. After 2 hours of pre-exposure, a zebrafish egg was collected from the primary exposure chambers along with 0.1 mL of the test solution and was transferred to a well using a micropipette with a widened tip. For both well-plates, 10 wells of each row contained 0.3 mL of a certain dilution and the last 2 wells contain the negative and the positive controls, respectively. Each well housed one viable egg subjected to 2 hours of pre-exposure.

Qualitative Bioassay Data Evaluation

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Using Olympus™ MIC-D Digital Microscope, endpoints for evaluation of embryotoxicity were observed and recorded based on lethal endpoints at 24 hpf and 48 hpf which include non-formation of somites (24 hpf), egg coagulation (24 hpf), non-detachment of tail (24 hpf), non-development of the eyes (48 hpf) and absence of heart beat (48 hpf), and sublethal endpoints at 72 hpf which include lack of pigmentation, yolk sac edema, pericardial edema, spinal deformity and delayed hatching.

A binomial convention, where each embryo/larvae exposed would have a corresponding score for its overall effect, will be utilized and entered in an Excel template (Microsoft Excel Starter 2010). Normal zebrafish embryos/larvae will be given a score of 0, while embryos that exhibited lethal endpoints and considered dead will be given a score of 1 at each specified time points. Percent mortality for a test solution was calculated by dividing the number of dead embryos over the number of surviving and normal embryos at the beginning of exposure (n = 30).

Data Processing and Analysis

To test the normality of the data obtained for both LPG and UG, the Shapiro-Wilk Test was utilized. The statistical significance of mortality arising as an effect of treatment dilutions was analyzed using Kruskal-Wallis ANOVA among groups of LPG dilutions and among groups of UG dilutions. To compare the statistical significance between groups of LPG and UG dilutions, the Mann-Whitney U Test was used. IBM® SPSS® ver.20 statistical software was used to analyze the data. Statistical significance was accepted at 95% confidence interval ($p<0.05$) for all tests.

RESULTS

Gas Chromatographic-Mass Spectrometric (GC-MS) Analysis

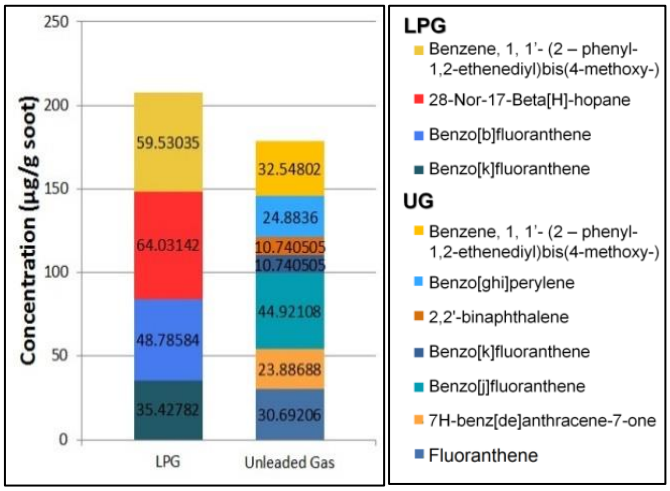

Figure 1: Concentrations of PAHs in both LPG and UG soot extracts determined through GC/MS.

Several types of PAHs have been detected in both LPG and UG soot extracts. PAHs present were predominantly high molecular weight PAHs (with more than 4 rings), which is in line with literature as products of combustion such as soot have higher proportions of high molecular weight PAHs, which are characteristic of pyrogenic PAHs (Kimbrough and Dickhut, 2006). Seven PAHs (fluoranthene, benzanthrone, benzo [j] fluoranthene, benzo [k] fluoranthene, 2,2' – binapthalene, benzo [ghi] perylene and benzene, 1,1'-(2-phenyl-1,2-ethenediyl) bis(4-methoxy-) were found in the UG soot extract whereas four PAHs (benzo [k] fluoranthene, benzo [b] fluoranthene, 28-Nor-17 –Beta [H] – hopane, and benzene, 1,1'-(2-phenyl-1,2-ethenediyl) bis(4-methoxy-) were found in the LPG soot extract.

Figure 1 shows the concentrations of PAHs detected through GC-MS. Individual PAH concentrations found in the LPG soot extract were generally higher than the concentrations of PAHs detected in the UG extract. Furthermore, the total PAH concentration of the LPG soot extract (207.78 µg/g soot) is higher than the

total concentration of the UG soot extract (178.41 µg/g soot). Aside from the PAHs listed in Figure 1, the GC-MS analysis also detected unknown organic compounds which were not identified by the NIST database.

Reconstituted water parameters

Throughout the zebrafish embryotoxicity test (ZFET), pH levels of reconstituted water ranged from 7 – 7.2, whereas temperature levels were fairly constant at 26 ± 1°C. These levels are within the accepted parameters set by the ZFET Standard Operation Procedure. Conductivity of the reconstituted water solution ranged and was consistent between 602 µS/cm and 607 µS/cm.

Zebrafish Embryotoxicity (ZFET) Test

In the ZFET Test, all embryos exhibited 100% mortality at 1:0, 1:1, 1:2, 1:3, and 1:4 dilutions of LPG and UG. Beginning at 1:5 dilution, survival was observable. No particular trend based on percent mortality was established for both LPG and UG dilutions of 1:5, 1:9, 1:13, 1:17, 1:21, and 1:25. However, based on statistical findings, manifestations of embryotoxicity arising as an effect of concentrations were found to be statistically different for the LPG dilutions ($p = 0.000$). Likewise, embryotoxicity arising as an effect of concentrations were statistically different for UG dilutions ($p = 0.000$).

Lethal Endpoints

Embryotoxicity was evaluated by observing the presence or absence of lethal endpoints. Coagulation at 24 hpf had a high occurrence in all dilutions for both LPG and UG as seen in Figure 3 and Figure 4. Non-detachment of tail and embryo coagulation, at 24 hpf, exhibited a dose-dependent response on lower dilutions unlike non-formation of somites (24 hpf) and absence of heartbeat (48 hpf) which occurred at different dilutions.

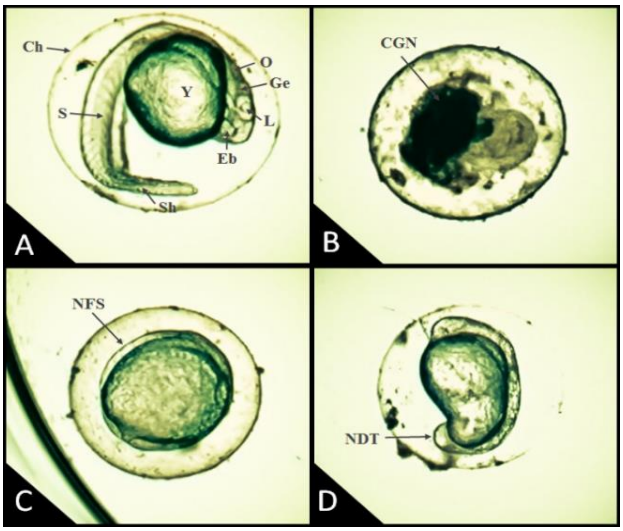

Figure 2: 24 hours post fertilization (hpf) developing control embryo (A); chorion (Ch); ear bud (O); brain (Ge); lens (L); eye buds (Eb); yolk (Y); somites (S); tail (Sh); B-D: 24 hpf test embryos showing lethal endpoints: coagulation (B) as seen in an embryo exposed to 1:5 LPG dilution; non-formation of somites (C) as seen in an embryo exposed to 1:9 UG dilution; and non-detachment of tail (D) seen in an embryo exposed to 1:5 LPG dilution.

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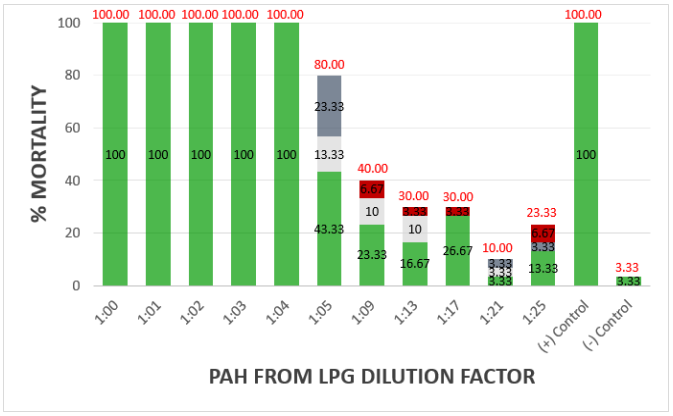

Figure 3: Response of zebrafish embryos to varying dilutions of PAH from Liquefied Petroleum Gas (LPG) soot extracts showing the contribution of four lethal endpoints, namely, coagulation (green), non-formation of somites (white), non-detachment of tail (dark blue) and absence of heartbeat (red). Values in red are the overall percent mortality per dilution.

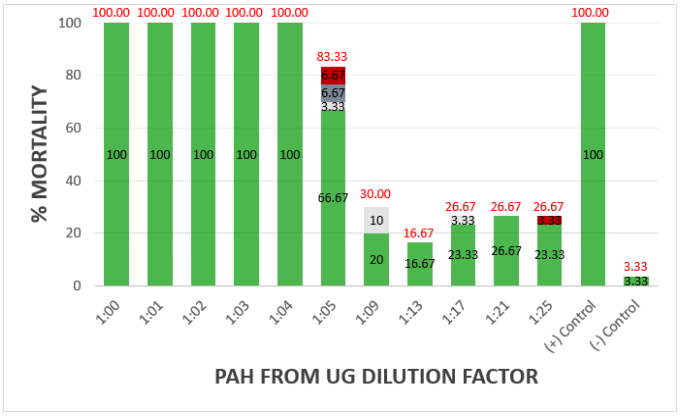

Figure 4: Response of zebrafish embryos to varying dilutions of PAH from Unleaded Gasoline (UG) soot extracts showing the contribution of four lethal endpoints, namely, coagulation (green), non-formation of somites (white), non-detachment of tail (dark blue) and absence of heartbeat (red). Values in red are the overall percent mortality per dilution.

Sublethal Endpoints

Teratogenicity was also evaluated by observing sublethal endpoints as seen in Figure 5. Yolk sac edema and pericardial edema were observed in a relatively high percentage in UG and LPG dilutions. Spinal deformity and delayed hatching were found to be dose-dependent on lower dilutions. Sublethal endpoints arising as an effect of concentrations were found to be statistically different for the LPG dilutions ($p = 0.010$) and UG dilutions ($p = 0.000$).

Comparative Assessment of Embryotoxicity between LPG and UG

In comparing the PAH mixtures in LPG and UG soot extracts, statistical analysis show that LPG and UG soot extracts are comparable in terms of embryotoxicity ($p = 0.912$).

Positive and Negative Controls

Normal development of zebrafish embryos was consistently observed in the negative control (ISO reconstituted water). The positive control, 2% ETOH, induced 100% mortality at 24 hpf.

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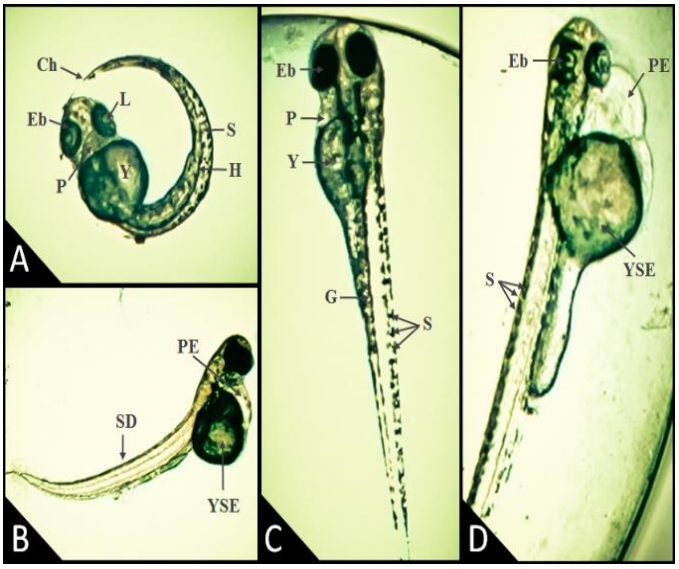

Figure 5: 48 hours post fertilization developing control embryo (A); chorion (Ch); lens (L) eye ball (Eb); pericardium (P); yolk (Y); chorda (H); somites (S); gut (G); 72 hpf developing control embryo (C); Spinal deformity (SD), a sublethal endpoint, as seen in B; Pericardial edema (PE) and Yolk sac edema (YSE), sublethal endpoints, seen in both B (exposed to 1:5 UG dilution) and D (exposed to 1:9 LPG dilution).

DISCUSSION

Over the last two decades, PAHs have been a potent environmental toxicant produced from the incomplete combustion of fuel [34]. Urban sprawl, industrial and transportation activities, and increase in the use of automobiles have greatly contributed to the yield of toxicological potencies of PAHs in the form of soot [35-36]. Gas-phase soot, which contain pertinent PAH mixtures, contributes to the hazardous atmospheric pollution. In the Philippines, 5000 reported premature deaths are attributed to atmospheric pollution [37], suggesting that the public is generally at risk due to atmospheric particulate matter including PAH-rich soot from exhaust gas emissions. The ZFET test, a developmental toxicity assay utilized in this study, was able to evaluate the embryotoxicity and teratogenicity of soot extracts containing PAHs under the premise of dose-dependent response.

Dose-dependent Response to PAHs

Lethal and sublethal endpoints were found to arise as an effect of concentration resulting to a significant difference between the dilutions within groups. This is attributed to the amount of dissolved PAHs that has diffused through the zebrafish chorion or vitelline envelope that is primarily composed of glycoprotein extracellular matrix [38]. Similar to previous studies demonstrating dose-dependent toxicity [36, 38-39], dependence in concentration was also demonstrated in this study whereby lethal endpoints, mainly coagulation, exhibiting embryotoxicity was higher in concentrations with higher dissolved PAH levels. Moreover, a dose-dependent teratogenic response as observed in the sublethal endpoints was observed in this study. Spinal deformities and delayed hatching appeared to be dependent on high PAH concentrations while pericardial edema and yolk sac edema were more prevalent at lower concentrations [40]. Moreover, Matson and co-workers [41] also determined that, under hypoxic conditions, exposure to a higher concentration (500 µg L-1) of the PAH fluoranthene resulted in a higher percentage of zebrafish embryos exhibiting lordosis, in comparison to those exposed to a lower concentration of Fl (100 µgL-1), further proving the occurrence of a dose-dependent response to PAHs.

Mechanisms of PAH Embryotoxicity

Numerous studies have already elucidated the mechanisms of teratogenicity of various types of PAHs. The LPG-PAH and UG-PAH samples have demonstrated a predominance of high molecular weight PAHs (with more than 4 rings) which have been observed to exhibit dioxin-like embryonic cardiovascular toxicities in the

sensitive early life stages (ELS) of fish models. Due to this, some PAHs have been referred to as dioxin-like compounds (DLCs), which are determined to target embryonic fish cardiovasculature [1].

Polycyclic aromatic hydrocarbons employ a variety of mechanisms to elicit embryotoxic responses. One of the most well-studied mechanisms of embryotoxicity is the AHR-mediated toxicity. As an AHR agonist interacts with AHR through ligand-activated binding, a dissociation of the AHR from its cytoplasmic chaperone proteins to translocate in the nucleus would occur. Dimerization of the receptor with the aryl hydrocarbon receptor nuclear translocator would allow it to interact with xenobiotic response elements, causing the upregulation of numerous enzymes, such as phase I and II metabolic enzymes (i.e. the cytochrome P450 (CYP1) gene family, Glutathione-S-transferases, NADP(H) oxidoreductase) [35]. In zebrafish, the AHR2 ortholog is the one responsible for eliciting dioxin-like toxicity, just like in other teleosts [42].

Recent studies have also determined the role of CYP1A activity in the developmental toxicity in zebrafish embryos, as it has been determined to be one of the effects caused by AHR binding of PAHs. The absence of CYP1A/ cytochrome P450 activity, which was made possible by knocking down CYP1A protein expression, significantly enhances toxicity of PAHs [42]. The interaction of AHR activity and CYP1A activity makes the mechanism of PAH toxicity distinct from the mechanism elicited by planar halogenated aromatic hydrocarbons (PHAHs), such as dioxin. This study expounds on the mechanism of teratogenicity of individual PAHs found in the LPG-PAH and UG-PAH extracts used in the ZFET.

Benzo[k]fluoranthene as a strong AhR agonist and CYP1A inducer

The presence of benzo[k]fluoranthene in both UG-PAH and LPG-PAH samples account for the prevalence of pericardial edema in zebrafish embryos 72 hpf. Barron and co-workers [43] mentioned that BkF is a very potent CYP1A inducer inferred to cause dioxin-like cardiotoxicity. Moreover, among the 74 polycyclic aromatic hydrocarbons tested for AHR affinity, benzo[k]fluoranthene was determined to be the most potent aryl hydrocarbon receptor (AHR) agonist, with a fish potency factor of 0.00128 relative to the potency of dioxin. Moreover, it is determined to be an AHR agonist as the knockdown of AHR2 in *Fundulus heteroclitus* protected it from the cardiotoxicity caused by the said PAH [42]. Incardona and co-workers [11] differentiated the cardiotoxic effects among the PAHs benzo[k]fluoranthene, benzo[a]pyrene, and benzo[e]pyrene, with BkF being the more potent PAH to cause pericardial edema in embryonic zebrafish. Pericardial edema is said to be exhibited after angiogenesis is inhibited, as well as when there is reduced blood flow as a result of circulatory failure [44].

Benz[e]acephenanthrylene (benzo[b]fluoranthene) and benzo[j]fluoranthene are weak AHR agonists

Benz[e]acephenanthrylene (benzo[b]fluoranthene) was determined by Billiard et al. [45] as an AhR agonist. However, the relative affinity of BbF is only 1/25 of the affinity of BkF to AHR of PLHC-1 cells [45], thus its potency is significantly less than that of BkF. Another study by Billiard and co-workers [5] determined that the CYP1A induction potency of benzo[b]fluoranthene is less than that of benzo[k]fluoranthene, as determined by liver ethoxyresorufin-O-deethylase (EROD) in juvenile rainbow trout (*Onchorhyncus mykiss*). Like benz[e]phenanthrylene, benzo[j]fluoranthene is also an AHR agonist but its potency is much less than that of benzo[k]fluoranthene. B[b]F and B[j]F were also among the PAHs whose fish potency factors were determined by Barron and co-workers [43] and were determined to be only one-eighth (0.000166) and one-half (0.000646), respectively, of the fish potency factor of benzo[k]fluoranthene (0.00128). In this case, these PAHs have little contribution in the development of cardiotoxicity in embryonic zebrafish.

Fluoranthene as a CYP1A enzyme inhibitor

Fluoranthene (FL), which was found only in the UG-PAH sample, is said to be a CYP1A enzyme inhibitor. Matson et al. (2008) determined that Fl causes severe pericardial edema under hypoxic conditions, eliminating the role of benzo[a]pyrene, a known AHR agonist, in the development of pericardial effusion. Many AHR agonists are also known to be CYP1A inducers, and it is likely for fluoranthene to have an extremely low potency for AHR binding (0.000000002) relative to dioxin, as determined by Barron and co-workers [43], given its nature as a CYP1A inhibitor.

Other PAHs

Numerous studies by Incardona and co-workers [28, 46] tested the toxicities of various types of individual PAHs. Naphthalene, a two-ringed PAH, and its derivative, 2,2'-binaphthalene, chrysene, a four-ringed PAH and a CYP1A inducer, and 28-Nor-17 –Beta[H]–hopane (3-ethyl-5a,5b,8,8,11a,13b-hexamethylicosahydro-1H-cyclopenta [a]chrysene), a chrysene derivative, are among the few compounds which have been determined to lack any overt embryotoxicity [28]. Moreover, Barron and co-workers [43] also determined that naphthalene, along with 2- and 3-ring unsubstituted PAHs, does not respond to AHR binding, and therefore does not contribute to cardiotoxicity.

Evidences of embryotoxicity of the PAHs benzo[ghi]perylene, benz[de]anthracen-7-one, and benzene, 1,1'-(2-phenyl-1,2-ethenediyl) bis(4-methoxy- in zebrafish embryos have not yet been documented in any study. Investigations involving the said PAHs would be essential in determining the overall mechanism of toxicity of the sample, given that they are found to have significant concentrations in the soot extracts.

Embryotoxicity of PAH Mixtures as opposed to Individual PAHs

Many studies have focused on the toxic effects and the mechanisms of single PAHs, comparing the toxicity of one PAH against the other. In the actual environment, however, PAHs typically persist within complex mixtures comprising of several PAHs and other chemicals such as heavy metals and halogenated hydrocarbons [47].

AHR agonists and CYP1A inhibitors usually co-occur in PAH mixtures in the environment [1]. Cardiac abnormalities may be induced by exposure to an individual AHR agonist (BkF) or CYP1A inhibitor (FL), however, co-exposure to B[k]F and Fl resulted to synergistic toxicity in the form of severe pericardial edema [1, 35, 48]. This provides evidence to the relatively high occurrence of pericardial edema in the UG soot extract in this study. Despite a low BkF concentration, a pronounced teratogenic effect, in the form or pericardial edema, was still observed due to the significant concentration of fluoranthene. Billiard and co- workers [1] examined the cardiotoxic interactions between AHR agonists and CYP1A inhibitors which commonly constitutes a PAH mixture. A non-additive, interactive effect in PAH mixtures accounted for the developmental cardiotoxicity [1, 40, 48-49]. A similar study by Rainieri and co-workers compared the embryotoxicity of individual PAHs were compared against the multi-component PAH mixture of benzo(a)-anthracene, chrysene, benzo(b)-fluoranthene, benzo(k)-fluoranthene and benzo(a)-pyrene. The multi-component PAH mixture resulted to an increased expression of the marker gene CYP1A that is 60-fold higher in over-expression by individual PAHs [49].

These previous studies provide evidence that the mixture of PAHs could potentially be more embryotoxic as compared to individual PAHs. The embryotoxicity of PAH mixtures, however, would depend on the interplay of mechanisms including the possible synergistic and antagonistic effects of the individual PAHs that comprise the PAH mixture as a whole. Currently, there is no study that would provide the exact molecular mechanisms and cell signalling pathways involved in the embryotoxicity and teratogenicity from PAH mixtures [35].

Comparative Toxicological Assessment of LPG and UG-PAH Soot Extracts

In comparing the embryotoxicity of PAHs from LPG and UG soot extracts, results of this study depicted that the embryotoxicity of PAHs from LPG and UG soot extracts are comparable despite the higher number of PAHs found in UG soot extracts. This finding is supported by previous studies [18-20] where PAHs from exhaust emissions of LPG-fueled vehicles are similar with that of the unleaded gasoline-fueled vehicles. Environmentally prevalent PAHs, such as benzo(b)-fluoranthene, naphthalene, fluorene, phenanthrene, anthracene, pyrene, chrysene, and benzo(a)-anthracene, were reported by Lim and co-workers [20] to be present in both LPG and UG exhaust emissions with varying PAH concentrations.

Chemical analysis of the soot extracts from LPG and UG showed that the total PAH concentration of the LPG soot extract was generally higher than the total PAH concentration of the UG soot extract. This finding provides a plausible explanation as to why there is no significant difference between the embryotoxicity of LPG and UG soot extracts containing several PAHs. Despite the higher number of PAHs found in the UG soot

extract, the higher total PAH concentration of the LPG soot extracts compensates for the embryotoxic effects, thus, resulting to comparable embryotoxicity between PAH mixtures from LPG and UG soot extracts.

Both LPG and UG soot extracts contain PAH mixtures that are embryotoxic, as well as teratogenic, as observed in this study. The synergistic and antagonistic effects of PAHs that comprise the entirety of the PAH mixtures and the interplay of mechanisms account for the embryotoxicity and teratogenicity observed in LPG and UG soot extracts. PAH mixtures from both soot extracts are comprised of high molecular weight PAHs, usually 4-6 rings. Higher molecular weight PAHs are not usually present in fuels, yet, these were observed in the soot extracts. Pyrosynthesis, presumably, had transpired during combustion resulting to high molecular weight PAHs in the soot [20].

CONCLUSION

This study has identified the various types of PAHs which can be found in LPG and UG soot extracts and has determined PAHs relevant to the environment. PAHs namely: benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[j]fluoranthene, and fluoranthene are known to cause cardiotoxic effects being AHR agonists (for BkF, BbF, and BjF) and CYP1A inhibitors (for FL). Embryotoxic responses of embryonic zebrafish upon exposure to liquefied petroleum gas (LPG) and unleaded gasoline (UG) soot extracts from taxi tailpipes are determined to be statistically comparable. The complex mechanisms of embryotoxicity exhibited by the interplay of PAHs have proved to alter the relative potencies of PAH mixtures, thus the determination of the embryotoxic potency of the component PAH and their possible synergistic and antagonistic relationships are essential to assess the embryotoxic potency of the PAH mixtures. The higher total PAH concentration found in the LPG soot extract in comparison to the total PAH concentration of the UG soot extract, as well as the statistically similar embryotoxic responses of zebrafish embryos exposed to both LPG and UG soot extracts debunk the claim that liquefied petroleum gas (LPG) is a 'cleaner and safer' alternative fuel source compared to unleaded gasoline. Given that the zebrafish is a representative organism for humans, this study reflects the possible risks that these environmental pollutants impose to human health.

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