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Using Rock Dove *Columba livia* as a Sentinel Species in Biomonitoring of Benzo(*a*)Pyrene

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Abstract
Pollutants in urban environments can influence on human health and other organisms such as birds living in the same regions. Therefore, the study of these species, as a sentinel, can realized on the health status of urban environments. The present research was conducted to measure Benzo a pyrene (BaP) concentration in different tissues of Rock Dove *Columba livia* as a sentinel species in in vitro and in vivo conditions. For this purpose, five groups were exposed to different concentrations (0.1, 2.5, 5, 7.5 and 10 $mg.kg^{-1}$ bw) of BaP. Also, for the comparison of in vitro results with urban conditions, 12 body samples were captured from the Tehran megacity. Results showed different accumulation patterns among the studied groups, which can be due to different intake pathways of pollutants by these birds. According to the results, in case of bioaccumulation factor, the rapid biotransformation rate of BaP in tissues of pigeons might affect their amount. Overall, the fat tissue at first and after that, the muscle tissue of *C. livia* could serve as a suitable biomonitor for BaP in the Tehran megacity.

1. Introduction

An increase in the urban population and activities has led to generate different pollutants which some of them are hazardous and cause various environmental problems. Chemical pollution in metropolises has many side effects on human health and other organisms (Curtis *et al*. 2006). An important part of metropolises' pollution is Polycyclic Aromatic Hydrocarbons (PAHs) which are considered as carcinogenic and mutagenic. Benzo a Pyrene (BaP) is a five-membered ring compound of multi-ring light yellow crystalline PAHs' group. This lipophilic compound has little solubility in water and penetrates the environment from both natural sources such as forest fire and humane sources including incomplete combustion of fuel, burning of fossil fuel (especially wood and coal), vehicle exhaust, various combustion processes and cigarette smoke (ATSDR 1995). Based on studies, BaP affects not only the fetus health and postnatal growth of humans (Tang *et al.* 2006), but also different organs of animals such as the liver, kidneys, digestive organs, heart, vessels, and especially blood parameters. Moreover, this compound may generate cancer and tumor in different organs of human body by breathing,

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swallowing and skin contact (Kroese *et al*. 2002).

Tehran is the biggest metropolis of Iran and is among the most populated metropolises of the world. Air pollution is a most important issue of the city which is affected by different pollutants including an oil refinery, power plants, fossil fuels, road traffic, airports, as well as many large and small industries. Thus, various pollutants, especially PAHs may affect the health of its residents. Furthermore, Tehran has an ecosystem for birds such as dove, hoopoe, common buzzard, White-eared Bulbul *Pycnonotus leucotis*, parakeets, nuthatches, woodpecker, finches, tits, and myna which are also exposed by air pollution. Hence, it is necessary to study effects of air pollutants on these animals. Because these effects are not recognized by just chemical data, pollutants' bio-monitoring and using sentinel species may provide useful data (Allan *et al*. 2006; Aguirre-Rubi *et al*. 2018). By examining animal samples affected by pollution, we could predict possible hazards even for the human in order to prevent from possible dangers (O'Brien *et al*. 1993). The sentinel species used for pollutants bio-monitoring should have the capability of the pollutant accumulation and a wide distribution, its sampling should be easy, as well as its ecological and biological features should be recognized (Sicolo *et al.* 2010; Miller *et al*. 2014).

Birds are appropriate to study the stable pollutants' effects for regional research (Mo *et al*. 2013; Luzardo *et al*. 2014; Pei *et al*. 2017) because of their various ecological niches, high abundance, being everywhere and recognized biological features. Also due to their sufficient tissue, they are a good choice to show pollutants' effects as the monitoring reference of pollution distribution and effects (Bonisoli-Alquati 2014; Abbasi *et al*. 2016). Rock Dove *Columba livia* has an extensive distribution which could be find in the most urban parts. It has a common habitat with human and may be used as the sentinel species due to its high metabolic rate, breathing pollutants, and swallowing polluted particles. Notablt the Rock Dove has been used for bio monitoring of heavy metals and PAHs in different regions which side effects of pollution on this bird health are found in cities of high population and industrial activities (Cui *et al*. 2016; Pei *et al*. 2017). Regarding the side effects of PAHs and BaP on health and insufficient studies on this compound accumulation in birds of Tehran, the present study seems to be necessary. Thus to examine the BaP concentration in different tissues of *C. livia* as a sentinel species, it was exposed by pollution in laboratory (a control and five treatment groups) and then sampling was done in Tehran.

2. Material and Methods

2.1. Sampling from the urban environment and preparation of laboratory samples

Samples in urban areas were collected from doves recently hunted, as they are used by some people as food by legal hunters. A dozen of doves were collected from Jomhuri and Shush streets (Fig. 1), which have high population and heavy traffic and biometry was done. For experimental tests, 36 doves consisted of 18 males and 18 females of the same age and size and weight between 260–280 g were sampled from a single population of a village around the city of Urmia. Laboratory samples were randomly divided into six equal groups, each group of which had three couples of male and female. A group was used as control and other ones for being exposed by different concentrations of BaP. Before exposing process, doves were adapted to laboratory conditions.

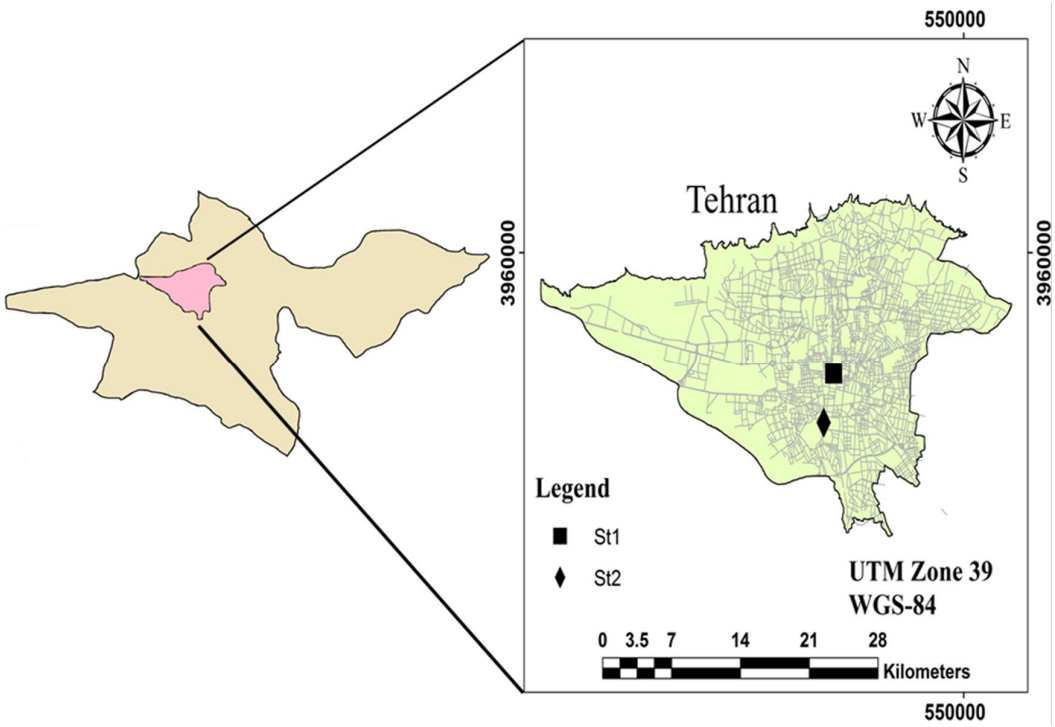

Fig. 1. Sampling localities of Rock Dove *Columba livia* in Tehran.

2.2. Preparation of stock concentration and being under exposure

BaP was dissolved in corn oil to obtain initial stock (Ou & Ramos 1992). As there was not any similar research, the concentration of 0.1 mg/kg was selected based on BaP concentration measured in the sampled dove of Ahmadabad, India (Dhananjayan 2013). The concentration of 10 mg/kg was selected according to Ou & Ramos (1992) which BaP was injected in quail breast tissue to examine its effects on aortic cells' growth. Moreover, Design Expert software (Ver. 5, Stat-Ease Inc) was used to design the test. Thus, concentrations of 0.1, 2.5, 5, 7.5, and 10 mg/kg of the body weight for groups 1 to 5, respectively, were prepared by Response Surface Methodology (RSM). Then, they were blended with 20 g of poultry commercial concentrated pellet. Prepared concentrations were blended with grain and placed for birds individually. Also, the control group was nourished with a similar food free of BaP. This process was continued for 40 days. Finally, birds were anesthetized with high concentration (near the saturation) of chloroform and died for tissue preparation painlessly.

2.3. Preparation

To measure BaP concentration, samples of liver, kidney, muscle and skin tissues of doves sampled in Tehran, due to their little fat, were freeze dried about 72–96 h. Moreover, liver, kidney and muscle tissues and fat of the birds exposed in laboratory were sampled and freeze-dried for the same time interval. Then, dried tissues were powdered by mortar and added to an Erlenmeyer (250 ml), after adding surrogate and combined standard matrix including naphthalene-d_8, anthracene-d_{10}, chrysene-d_{12} and perylene-d_{12}. For saponifying fats, methanol potassium hydroxide (Me-KOH, 50 ml, 3 M, 9:1 Me/H_2O) was added and heated in oven at 60°C for 3 h. Organic matters were extracted by n-hexane (50 ml) for 2h by an orbital shaker. The mixture was added to a separator funnel (250 ml), 30 ml of a Me-H_2O mixture (4:1) was stirred and added to the funnel. The liquid phase was transferred to another 250 ml separator funnel and stirred. After the separation of two phases, the mixture was washed by n-hexane (30 ml). The volume of all extracted solutions was reduced to 2 ml by nitrogen gas and rotary device and then transferred to chromatography column to separate PAH compounds. Silica gel (5%) deactivated by water was poured into a column

(inner diameter 0.9 cm) up to 9 cm height for final purification. Also, n-hexane was added above the column to prevent it from drying. After that, samples of reduced volume have been loaded on the column by Pasteur pipette and were washed with a hexane-dichloromethane (3:1) mixture (20 ml). The volume of samples was to 2–3 ml by nitrogen gas and become ready for the second step in which the fully activated silica gel was used, the inner diameter of the chromatography column was 4.7 mm, and the height of silica gel was 18 cm. After loading samples and washing the column, the solvent was removed and samples were collected in special vials by adding 100 µl of *P*-terphenyl-d14 injected into the Gas Chromatography-Mass Spectrometry (GC- MS) (Bakhtiari *et al*. 2009).

BaP analysis was done by Mass Spectrometry instrument (Model 5975C, Agilent Technologies, USA) and Gas Chromatography (Model 7890A, Agilent Technologies, USA). The column height and inner diameter were 30 m and 0.25 mm, respectively. Static phase of 100 µm diameter and helium gas of 99.999% purity as the carrier gas were used.

2.4. In vitro Bioaccumulation Factor (BAF) of BaP

At the end of exposing period, BaP bioaccumulation of the dove's tissues (liver, kidney, muscle and fat tissues) in in vitro condition were calculated as below. To determine bioaccumulation accurately, BaP concentration was calculated in terms of each treatment body mass which was 0.1, 2.5, 5, 7.5, and 10 (mg.kg^{-1} of body weight) for treatments' body mass 283, 276, 276, 293, and 272, respectively. Multiplying by 40 days, we obtained total concentration over this period. For each tissue, BAF was obtained by dividing BaP concentration of each tissue by total BaP concentration over 40 days.

$$\mathrm{BAF} = \frac{Ci}{C0}$$

Where C_i is the BaP concentration of tissue after the exposing period and C_0 is the BaP concentration added to food over 40 days (Walker 1990; Gobas *et al.* 2016).

2.5. Statistical Analysis

Results were analyzed by SPSS ver.17 (SPSS Inc., Chicago, IL, USA). Q-Q plots and Shapiro-Wilks tests were used to examine the normality of data distribution. Moreover, the significant difference between categories and groups were examined by *t*-test and One Way Anova and Tukey *post-hoc* test. Finally, results were shown in average± standard deviation. Significance level considered for all tests was $P<0.05$.

2.6. Ethics and Safety

All steps of the present study from sampling to experimental tests were conducted in accordance with HSE committee of Natural Resource and Marine Science Faculty of Tarbiat Modares University. Also, the license of national code of ethics in research was requested.

Table 1. BaP concentration (mg.g^{-1}) comparison between field and laboratory sampled tissues

Tissues				
Groups	Liver	Kidney	Muscle	Lipid
0.1 mg.kg^{-1} bw	8.40 ± 3.16 ^{a}	5.75 ± 3.25 ^{a}	5.84 ± 0.94 ^{a}	20.49 ± 7.76 ^{b}
2.5 mg.kg^{-1} bw	17.26 ± 6.20 ^{a}	7.72 ± 2.49 ^{a}	9.59 ± 1.93 ^{a}	41.56 ± 30.59 ^{b}
5 mg.kg^{-1} bw	23.41 ± 15.78^{a}	15.05 ± 7.54^{a}	15.68 ± 5.87^{a}	216.79 ± 15.98 ^{b}
7.5 mg.kg^{-1} bw	41.98 ± 23.20^{a}	12.79 ± 5.41^{a}	19.21 ± 3.54^{a}	930.99 ± 776.73^{b}
10 mg.kg^{-1} bw	57.49 ± 18.45^{a}	18.31 ± 9.72^{a}	27.09 ± 8.17^{a}	1515.80 ± 888.76^{b}
Tehran samples	0.79 ± 0.64 ^{a}	0.74 ± 0.59 ^{a}	2.20 ± 1.51^{b}	1.53±1.26^{ab} (skin)

Latin letters indicate the significant difference of different tissues resulted from Tukey *post-hoc* test.

Table 2. In vitro Examination of the BAF factor in the *C. livia* each tissue by different BaP concentrations .

Tissues				
Groups	Liver	Kidney	Muscle	Lipid
0.1 mg.kg^{-1} bw	0.0075	0.0051	0.0052	0.019
2.5 mg.kg^{-1} bw	0.00063	0.00027	0.00035	0.0016
5 mg.kg^{-1} bw	0.00043	0.00028	0.00029	0.0040
7.5 mg.kg^{-1} bw	0.00048	0.00015	0.00022	0.011
10 mg.kg^{-1} bw	0.00053	0.00017	0.00025	0.014

Table 3. BaP concentration (ng. g^{-1} dw) in tissues sampled in Tehran and their comparison with ones sampled in other regions.

Species	Liver	kidney	muscle	skin	region	Reference
C. livia domestica	0.04	-	-	0	Guangzhou, China	Pei et al., 2017
C. livia domestica	4.80	-	-	-	Beijing, China	Liu et al., 2010
C. livia domestica	0.90	-	-	-	Chengdu, China	Liu et al., 2010
C. livia	57.80	-	-	-	Ahmedabad, India	Dhananjayan, 2013
C. livia	0.79 ± 0.64	0.74 ± 0.59	2.20 ± 1.51	1.53±1.26	Tehran, Iran	Current study

3. Results and Discussion

Results are present in Table 1. There was a significant difference between BaP concentration of tissues sampled in Tehran where BaP was accumulated in the muscle more than other tissues and in the laboratory it was higher in the liver and kidney less than other tissues (P<0.05). Accumulated BaP, from maximum to minimum amount was in muscle, skin, kidney and liver, respectively. PAH compounds may be degraded and disposed in different tissues of birds at different levels. Therefore, this difference may be caused by the detoxification rate difference of tissues. For example, detoxification in the liver was at the most rate in relation to other tissues. Active epoxies were formed by microsome oxygenase system as the first step of PAH metabolism. During biotransformation, non-polar hydrophobic compounds are transformed into hydrophilic ones and become disposable (Boon *et al.* 1998). As the organisms' liver has a high amount of cytochrome P-450 enzymes, it has a high potential for PAH compound metabolism (Crowell *et al.* 2014; Head *et al.* 2015). These enzymes transform PAH compounds to hydroxyl compounds by their oxidation which increases their solubility in water and their disposal of tissues. Hence, liver detoxification mechanism reduces BaP accumulated compared to muscle. Zheng *et al.* (2016) showed that PAH compounds accumulated in the Common Quail's *Coturnix coturnix* liver were metabolized by liver microsomes.

There was not any significant difference of BaP concentration among liver, kidney and muscle (P>0.05), while BaP accumulation significantly increased in the fat tissue (P<0.05). The fat tissue is the most important organ in terms of accumulation and storage of organic compounds that BaP accumulated could be transferred to other tissues based on variety of age, habitat, bird activities and intake (Hela *et al.* 2006; Taniguchi *et al.* 2009; Pei *et al.* 2017). Therefore, it could be concluded that the increase in BaP concentration in fat tissue indicate that it is lipophilic.

The difference of BaP accumulation between doves sampled in Tehran and laboratory may be due to different amounts and ways of intake such as breathing. Pei *et al.* (2017) showed that the maximum amount of BaP is absorbed by urban doves' breathing that increases the concentration of two-ring and three-ring light PAH compounds in the lung about heavier PAH compounds. Based on results, the *C. livia* dove fat tissue could be used for biomonitoring of BaP compounds. If there is insufficient fat tissue, it could be replaced by the muscle tissue.

For the laboratory samples, BaP accumulation trend of laboratory samples was as follows: 10 $mg.kg^{-1}$ > 7.5 $mg.kg^{-1}$ > 5 $mg.kg^{-1}$≥ 2.5 $mg.kg^{-1}$ ≥ 0.1 $mg.kg^{-1}$ which was similar for all tissues of liver, kidney, muscle, and fat tissue (P<0.05). Based on this trend, BaP accumulation increased in tissues by its concentration increase in the food.

There was not any significant difference of BaP accumulation between males and females (t-test, P<0.05) because the doves were probably not in the laying conditions. Pei *et al.* (2017) did not find any significant difference between male and female doves in terms of PAH compounds accumulated in lung, liver and fat tissues, while Shore & Weinborg (2004) believed that the less pollutant concentration in tissues of females than that of males has resulted from the laying process which organic pollutants are partially removed during the laying process. In the present study, results were integrated as there was not any concentration difference between male and female.

Although bioaccumulation factor is usually calculated for aquatic organisms, pollutants are accumulated in land organism by breathing and feeding which should be investigated (Gobas *et al.* 2016). Generally, bioaccumulation is described as a process by which chemicals are taken up by a plant or animal either directly from exposure to a contaminated medium (soil, sediment, water) or by eating food containing the chemical (US EPA 2010).

As shown in Table 2, based on calculations, the bioaccumulation factor of BaP of fat tissue was maximum compared to other tissues which could be concluded that the fat tissue may be a more appropriate index compared with other tissues. Generally, the amount of bioaccumulation of all tissues was low which may be due to BaP metabolism in tissues. Troisi *et al.* (2006) and Zheng *et al.* (2016) showed that PAH compounds are rapidly biodegradable compared to other stable organic pollutants such as PCBs and has a high rate of decomposition which may affect pollutants' bioaccumulation in the bird tissues. The instability of initial concentration causes a decrease in less accumulation in the tissue. Based on Naf *et al.* (1992), 84% of PAH compounds' concentration injected to *Gallus domesticus* was metabolized after 14 days. Moreover, Pei *et al.* (2017) showed that PAHs' concentration in lung, liver, and fat tissues of Rock Dove is less compared with PCBs, and believed that it is due to PAHs' biodegradation. Furthermore, BaP breaking and transformation to other metabolites may decrease its concentration in dove's tissues. Triosi *et al.* (2006) stated that PAHs is metabolized in the form of hydroxyl metabolites in the bird liver.

The effect of pollutant toxicity depends on the received concentration. The dove's muscle tissue may be used as human food. Thus, BaP concentration which was measured in the dove muscle was compared with the standard amount. As there was no certain limit for this tissue, the result was compared with the allowed limit defined by EPA after calculating the concentration based on wet weight. The allowed limit of benzopyrene intake by meat specially smoked meat for human is 2 $\mu g.kg^{-1}$ of wet tissue which applicable by EPA from 2014/9/1(Commission Regulation (No 835/2011 EU)). Regarding that, each gram of dry muscle was obtained from 3-gram wet muscle which BaP concentration in muscle was 0.74 $\mu g.kg^{-1}$ wet weight. Thus it does not threat consumers as is less than the allowed limit for daily intake.

To use *C. livia* for biomonitoring of BaP concentration in Tehran, results were compared with other studies (Table 3). There were few studies on organic compounds accumulated in birds and landbirds. Results were partially similar to other studies which differences among these studies may due to various ways of pollutant compounds' intake such as type of food, breathing polluted air, drinking polluted water and cleaning feathers by birds (Pei *et al.* 2016). Pei *et al.* (2016) and Liu *et al.* (2010) found the minimum concentration of 4-ring and 5-ring PAH compounds such as BaP, BKF (benzo[k]-fluoranthene), BbF (benzo(b)fluoranthene), BaA (benz[a]anthracene), DahA (dibenz(a,h)anthracene) in comparison with other PAH compounds in *C. livia domestica* tissues while Dhanajayan (2013) reported more than 78% 5-ring PAH compounds' concentration in *Columba livia* tissues. Pei *et al.* (2017) believed that the difference in concentration is due to various ways of intake in different regions. They stated that little concentration of these organic compounds is caused by the food free of pollution and intake by breathing as they hunted samples from dovecots in which doves were fed manually.

Observations of the Bird's Behavior during Experiments

All laboratory birds sampled in a clean natural environment became anxious relative to white color of lab uniform during the compatibility period. Thus, the blue color was selected to reduce their anxiety. Moreover, from the day 35 until the end of the experiment period, other behavior such as restlessness, euphemism, thirst and nervous were seen in all samples of the 10 $mg.kg^{-1}$ treatment which may be due to higher concentration in relation to other treatments.

Conclusion

Considering measurement difficulties and the high price of experiments, it is suggested to use only the fat tissue of Rock Dove for BaP biomonitoring. If there is not enough fat tissue, then the muscle tissue is the second choice. As the dove may intake BaP by different ways, its accumulation in tissues differs from tissues of

laboratory samples. Also, little bioaccumulation of BaP compounds in tissues of the bird may be due to the possibility of its biodegradation. Based on the present study and other similar studies, the Rock Dove can be an appropriate choice to monitor PAH compounds and the health of organisms in the metropolis ecosystem.

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