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Anthropogenic pollution and its cytotoxic effects in an Iranian river, Zayandehrood river

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Abstract

Zayandehrood River located in central Iran have been suffering from pollution by municipal, industrial and agricultural wastewater. The research investigates the occurrence of five pharmaceuticals including amoxicillin, paracetamol, metronidazole, ranitidine and diphenhydramine and two estrogenic compounds consisting estradiol and ethynilestradiol contamination in Zayandehrood River. Water samples were collected in five stations including upstream and downstream receiving sewage water treatment effluents. The concentration of compounds in the upstream was below the detection limit up to 2 ng/l and reached its highest level about 6-46 ng/l in downstream. Also, the cytotoxicity of water samples was evaluated using two human cell lines (HepG2, HEK293T) and one fish cell line (RTG2). No cytotoxic effects were observed but cell viability decreased in cells exposed to river water receiving waste waters. Residues of pharmaceutical and steroid compounds were detected in water samples of Zayandehrood river and these compounds can enter the drinking water sources and affect its quality if not properly treated.

Key words: Endocrine disrupters, Cytotoxicity, Zayandehrood River, Solid Phase Extraction.

Introduction

The occurrence and fate of emerging pollutants in the aquatic environment have become a research topic in recent decades, including a wide range of compounds from petroleum compounds to personal care products (PPCPs) and UV filters. Among them, pharmaceutically active compounds (PhACs) are a group of substances broadly used to treat or prevent diseases in humans and animals. Due to the extensive consumption of pharmaceuticals reaching bodies of water, they are one of the major inputs into the environment. Approximately 3000 pharmaceutical products are available for human use (Fent et al., 2006), and several thousands are in the process of development.

Likewise, the rate of pharmaceutical consumption in Iran is considerable; Iranian people consume about 442 units per year, further than the world standard. Iran is among the top 20 countries in the world in terms of pharmaceuticals consumption and the second in Asia only after China (WHO, 2000). On average, the highest pharmaceutical consumption per capita from 2013-2018 was in United States and Japan, and it has been four times more per Iranians (Aitken, 2016). Also, the rate of actual and potential pharmaceuticals wastage in Iran is estimated to be about 38.8% and 58.8%, respectively (Zargarzadeh, 2005) which is noticeable. Therefore, it is likely that urban and rural wastewater entering the river contain significant concentrations of these medicinal and hormonal compounds.

On the other hand, being located in a semiarid region of the Middle East, Iran has been struggling with water shortage for years. The country, frequently, suffers from severe and persistent droughts causing tremendous damage to the agriculture, forests, water resources and other sectors comprising the national economy (Saravi et al., 2015). While the river receives wastewater from agricultural activities, industrial areas and household sewage, over 99% of drinking water for approximately 5 million inhabitants across the area is supplied by this river.

This study has tried to investigate the occurrence of a number of pharmaceutical and estrogenic contaminants at different points located upstream and downstream in Zayandehrood River. Also, in order to test the toxicity of water samples for human and aquatic organisms, in vitro cytoxicity test has been developed to see if the river water and the following waste water have a potential to be acutely toxic to human and fish cell lines. Human hepatocellular carcinoma cell line (HepG2) and human embryonic kidney cells (HEK293T) along with one fish cell line, rainbow trout gonadal cells (RTG-2) were chosen to assess the potential cytotoxic effects of Zayandehrood River water.

Material and methods

Sampling

Zayandehrood River, originates from Zagros Mountains in the northern part of the Isfahan catchment area, travels from northwest to south-east, and after a distance of 350 km flows to the Gavkhouni wetland. Five locations were selected along the river including upstream parts and downstream sites up to the position that there is still water flowing through the river. Upstream samples were collected from 2 to 5 km upstream in highland mountain areas with minimal anthropogenic impacts, while downstream sites were within 100 to 200 m of wastewater effluent discharge sites.

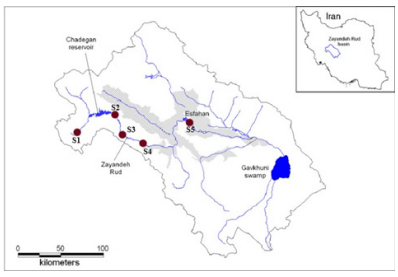

Fig. 1. Sampling sites; S1. Koohrang(headstream), S2. Hojat abad, S3. Saman, S4. Cham aseman, S5. Isfahan

Chemical analysis

Pharmaceuticals were obtained from Amin Pharmaceutical Co. (Isfahan, Iran) and estrogenic compounds were provided by Aburaihan Pharmaceutical Co. (Tehran, Iran). All the standard materials were of analytical grade, with 98 % purity or higher. All solvents used, including the HPLC grade methanol (MeOH), acetonitrile (ACN), HPLC grade water, acetic acid and phosphoric acid 98% were purchased from Merck (Darmstadt, Germany). Triethylamine ((C2H5)3N) was obtained from Sigma-Aldrich (Steinham, Germany). Nitrogen for drying was of high purity grade (>99/9%) and was supplied by

Isfahan Gas Co. (Isfahan, Iran). Chromabond C18 cartridges (6 mL, 500 mg) were supplied by Machery Nagel (MN, Germany).

Preparation of standard solutions and samples

Stock standard solutions were prepared individually in a concentration of 1 mg/ml, by dissolving 10 mg of solid reference of each standard in 10 mL of proper solvent. Amoxicillin, Paracetamol, Ranitidine, Metronidazole and Diphenhydramine were dissolved in HPLC grade water. Estradiol and Ethynilestradiol were dissolved in methanol (HPLC grade, Merck) as solvent and kept at 4 °C prior to analysis.

Natural water samples were collected in 1-L dark glass bottles along the river and were kept in 4°C. After passing through 1 µm glass-fiber filters, water samples were extracted with Chromabond C18 cartridges (500 mg, 6 cm3). The cartridges were conditioned by 10 mL of MeOH followed by 10 ml of HPLC water. After that, 1000 mL of river water samples were passed through the cartridge at a flow rate of 14 ml/min. After preconcentration of the samples, 3 mL methanol and deionized water mixture (95:5) was loaded onto the cartridges and then the analytes were eluted by passing 4 ml methanol through the cartridges with the help of gravity in a vial. Next, the extract was evaporated to dryness under a gentle stream of nitrogen at 30 °C and finally it was reconstituted with 100 µl of mobile phase. 20 µl of the reconstituted solution was injected into HPLC- MS/MS.

In order to have calibration curve for each compound, solutions with standard concentrations of 2, 5, 10, 20 and 50 ppm were prepared. Ultrapure water was used to dissolve pharmaceutical compounds and methanol was used for hormonal compounds. The solutions prepared for each compound were injected into the device (with one repetition) and by recording the results, the calibration curve and curve equation were obtained. Calibration curves were plotted by the ratio of peak area versus the corresponding concentration of each standard solution. The equation was linear in the range of the mentioned concentrations and the obtained r2 was greater than 0.99. To optimize the calibration of the device, desired standard solution of 2000 ppm were first prepared for each compound. Then, each solution was injected into the device separately and the peak area related to each compound was determined.

High-performance liquid chromatography–tandem mass spectrometry

Chromatographic separations were carried out using Knauer High Performance liquid chromatography system (1260 HPLC system), equipped with UV detector 2600 Smartline, Micro Vacuum Degasser, Binary pump 600 bar, automated injection system using a PerfectSIL ODS-3-C18 (150 mm × 4.6 mm, 3.5 µm) column with guard injection 20 µl and oven temperature was 25 °C. The optimum separation conditions were different for pharmaceutical and estrogenic compounds. The mobile phase for estrogenic compounds composed of solvent A: water acidified by adding acetic acid at 0.1% and solvent B: acetonitril acidified by adding acetic acid at 0.1% at a flow rate of 0.5 ml/min. Separation for pharmaceutical compounds was performed in isocratic mode using HPLC water (84.2%), methanol (15%), phosphoric acid and triethylamine (0.4 % each) as mobile phase. The other conditions were the same. The flow rate of the mobile phase was set at 0.5 ml/min and the volume of the injection loop was 20 µl. The column temperature was set equal to room temperature (20 °C). Measurements were performed according to Stafiej et al., 2007. The specific information required for measurement related to each compound was provided from Pharmacopoeia 2016.

The tandem mass analysis was performed by a 3200 QTRAP hybrid triple quadropole linear ion trap mass spectrometer (Agilent, CA, USA) equipped with a turbo Ion Spray source. The temperature of the electrospray source was 400 °C. Ion Spry Voltage (IS) was 5500 V. Curtain gas (CUR) was10 psi and ion source gas pressures (GS1) and (GS2) both of them were 50 psi. MS parameters which dependent to compounds includes declustering potential (DP), collision energy (CE) and collision cell (CXP) which were optimized by infusion of individual standard solutions of each compound at concentrations of 2 mg/l. Nitrogen was used as a drying gas for solvent evaporation. The API housing and drying gas temperatures were kept at 50 and 350°C. Protonated analyte molecules were subjected to collision induced dissociation using argon as the collision gas to yield product ions for each analyte and the IS. Mass spectrometry analysis (MS) was performed in the positive ion mode (PI). It was operated in multiple reaction monitoring (MRM) mode. Moreover, for each compound, two MRM transitions were monitored that the most abundant fragment ion was used for quantification and the other one used for identification. A dwell time of 100 ms per ion pair was used for all antibiotics. The resolution on both analyzers was unit. A summary of individual mass parameters is presented in Table 1

Compound	Therapeutic class	Ionisation mode	RT (min)	Molecular weight (g/mol)	Orifice Voltage (V)	Ring Voltage (V)	Collision energy (eV)	Precursor ion (*m/z*)	Product ion I (*m/z*)	Product ion II (*m/z*)
Amoxicilin	Antibiotic	ES+	11	365.4	11	360	29	365.8	349.0	208.2
Paracetamol	Analgesics	ES+	12	151.1	CA V.= 4000	-	16	152.0	110.0	-
Metronidazole	Antibiotic	ES+	6	171.1	36	190	19	172.1	127.8	82.0
Diphenhydramine	Antihistamine	ES+	4	255.3	CA V.=35	-	10	256.0	167.0	-
Ranitidine	H2 histamine receptor antagonist	ES+	7.5	314.4	CA V.=3	Co V.=25	25	315.1	129.9	-
Estradiol	Estrogenic compound	ES-	-	272.3	-	Cone V: 38	78	271.0	145.0	-
Ethynilestradiol	Estrogenic compound	ES-	-	296.4	Ion spray voltage (ISV): 5000	10.4	50	295.1	144.7	-

CA= Capillary Voltage, Co = Cone Voltage.

Bioassay

Cell lines culture and treatment

HepG2 and HEK293T cell lines were obtained from Pasteur institute of Tehran, Iran. Cells were grown and maintained as a monolayer culture in DMEM supplemented with 10% fetal bovine serum (Gibco, USA). The culture was maintained at 37°C in a humid atmosphere at 5% of CO2 and a half-open system. Cells passages were carried out at 80% confluence using 0.25% of trypsin. Rainbow trout gonad-2 (RTG-2) cell line was ordered from ATCC (Summit Pharmaceuticals International, Tokyo, Japan). Cells were maintained in E-RDF (KYOKUTO # 551-26500-2, pH 7.8) with 7.5% FBS (GIBCO # 26140079, inactivated), 25 mM HEPES (Sigma-Aldrich) and antibiotics (Penicillin (50 units/ mL), Streptomycin (50ug /mL), all from (Sigma-Aldrich, Czechia). The RTG-2 cell line was cultured at 18°C and were passaged after reaching the 70-80% confluency. For exposure to surface waters, cells were seeded at a rate of 2×104 cells/well in a 96-well polystyrene microplate. After the cultures reached semiconfluency, culture medium was replaced with media containing water samples collected from different stations at the river, and the cells were exposed for 24 hours, DMEM medium only was used as negative control.

MTT assay

In order to evaluate cytotoxicity using the mitochondrial activity

parameter, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used as described by Mosmann (1983). After exposure, 20 µL of MTT 5 mg/mL was added, and the plates were incubated for 3 hours. After incubation, the medium was removed by pipetting, and 200 µL of dimethyl sulfoxide (DMSO) was added to each well for solubilization. The absorbance was measured at 540 nm with a microplate spectrophotometer (BioTek, USA). All assays were repeated in at least three replicates. Cell viability was evaluated based on MTT method by usage of following equation:

% of cell viability = (Atreatment – Ablank)/ (Acontrol – Ablank) × 100% (where, A = absorbance)

Statistical analysis

One-way ANOVA and the Duncan post-test were used for statistical analysis with the Statistical Package for the Social Sciences (SPSS) 15.0. The level of significance was set at $p<0.05$.

Results:

Chemical analysis

Of the seven studied compounds, all were detected in river water samples. The most frequent compound detected was Paracetamol detected in four out of five sampling points. The other compounds were detected in most parts of the river. Also, Ranitidine and Diphenhydramine were determined at significantly higher concentrations than other target compounds. In order to initially compare the basic occurrence of the target compounds, the concentrations in both upstream and effluent-dominated downstream sites are compared in Fig. 2.

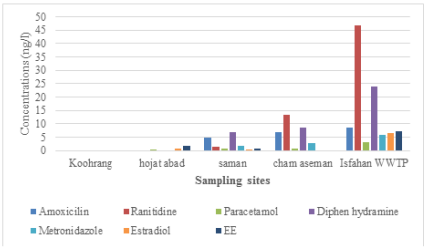

Fig.2. Concentrations of individual detected compounds in water samples in ng/l.

In Headstream parts concentration of compounds were below the detection limit. In second station (Hojat abad) three compounds including paracetamol and estrogenic hormones were detected (0.02- 1. 83 ng/l). In the third station all the tested compounds were detected and their conc. were between 0. 31 to 6. 89 ng/l. In the fourth stations the concentration of compounds was between 0 to 13. 35 ng/l. The highest numbers (5. 89-46. 70 ng/l) were detected in the last station, Isfahan. Moving from upstream to downstream, the number of detected compounds and also their cumulative concentrations in each station were increasing and reached 102 ng/l in Isfahan waste water treatment effluents (Fig.3)

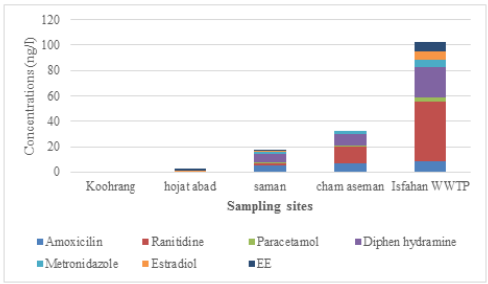

Fig.3. Cumulative concentrations of seven detected compounds in river water.

Bioassay

As shown in Fig. 4, results obtained from in vitro studies, revealed no cytotoxicity against applied cell lines. Although toxicity in cells was not significant (df= 8, F= 1.491, p= 0.228) in contact with the tested water samples, cell viability was decreased in cells exposed to some water samples. The last sample, including waste waters induced a cell viability decrease of about 40% in HEK293T cells and about 10% in Hep-G2 cells.

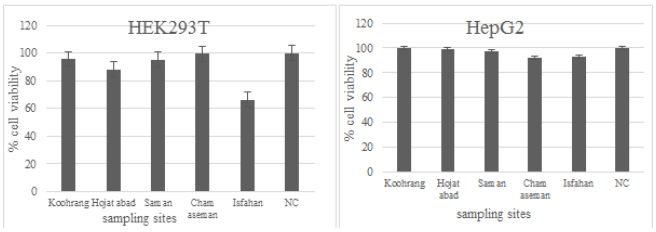

Fig 4. Viability (%) in relation to the control group in HEK293T and HepG2 cells after 24 hours of exposure to the water samples from five sites in the Zayandehrood River Basin.

Also, RTG2 cells viability did not significantly decrease after exposure to the water samples.

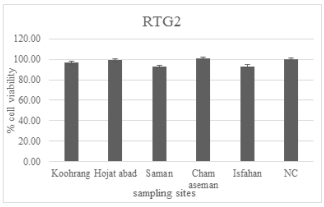

Fig.5. Viability (%) of RTG2 cell line after 24-hour exposure to environmental river water samples from Zayandehrood River.

Discussion

In this study, the occurrence of seven pharmaceutical compounds including five non-estrogenic compounds; paracetamol, amoxicillin, diphenhydramine, metronidazole and ranitidine and two estrogenic compounds containing estradiol and ethinylestradiol was investigated in surface waters of Zayandehrood River at different locations following to Isfahan, Iran. All seven compounds were detected in river waters. The concentrations varied from below detection limit in upstream to 47 ng/l at downstream parts.

A few similar studies have reported the presence of pharmaceutical contaminations in Iranian water resources. Eslami et al., measured the concentrations of ibuprofen, naproxen, diclofenac and indomethacin in surface waters and influent and effluents related to municipal and hospital waste water treatment plants in Tehran and reported the concentrations about 1.05, 0.43, 0.23 and 0.11 µg/l in waste waters and low ng/l in rivers. Also, Mirzaei et al. identified a number of high-consumption antibiotics in water and wastewater resources of Tehran province in 2018. There have also been reports of the detection of steroid hormones (estradiol and ethinyl estradiol) in the surface waters of the Karun River (Hassani et al., 2016) and in Hamedan drinking water sources and groundwaters (Jafari et al., 2009). Reported concentrations for 15 pharmaceutical and personal care products in surface waters of Somes River in Romania varied from 30 ng/l to 10 µg/l (Moldovan, 2006).

The concentration of pharmaceutical compounds had a cumulative slope through the river and highest concentrations were detected in downstream, especially in Isfahan that were about two to three times more. The increase in concentrations can be due to the influx of sewage and waste water effluents from surrounding towns and villages into the river, as well as industrial and agricultural areas, cattle farms, municipal waste water

treatment plants that their runoff strongly affect water quality. Similarly, Jarosova et al., 2012 measured the concentrations of polar organic contaminants in seven municipal waste water treatment plants situated on small rivers in which concentrations of estrogen equivalents in extracts from all downstream locations were up to 14 times greater than those at upstream locations.

In general, municipal wastewaters are considered as an important source of organic contaminants discharging into the aquatic environment, since many micro pollutants which are persistent in the environment are not readily biodegraded due to poor wastewater management practices (Wepener et al., 2011). On the other hand, the occurrence of drought and subsequent changes in hydrological conditions had a great impact on the quality of water in Zayandehrood River. In downstream parts of the rivers, especially during low-flow conditions and in densely populated areas, wastewater effluents can account for a significant fraction of the overall flow in some surface waters for distances up to several hundred kilometers (Fono et al., 2006). This happens for runoff in Isfahan in which waste water effluents consist the most part of the water flowing through the river. Moreover, considerable alterations in water flow throughout the river in recent years, which has mostly caused parts of the river to dry completely had adverse effects on river ecosystem (Pirali et al., 2015).

Table 2. Comparison of concentrations detected in current study and other studies around the world

	Conc. in upstream (ng/l)	Conc. in downstream (ng/l)	References
pharmaceuticals	0-2	10-28	Current study
	100-200	4000	Konig et al. (2016)
	12	59	Metcalfe et al. (2003)
	<10	159	Ashton et al. (2004)
Hormones	0-2.8	6.5-8.1	Current study
	0-0.5	0-4.8	Jarosova et al. (2012)
	-	14.8	Hassani et al. (2016)
	0.01	3	Jobling et al. (2006)

Concentrations of estrogenic compounds including estradiol and ethinyl estradiol in this study were identified in the surface waters of Zayandehrud River. The concentration range of these compounds reached about 1-2 ng/l in upstream and about 6-8 ng/l in downstream. By sampling 7 different rivers in the Czech Republic in 2012, Jarosova et al. reported that in the upstream areas, the concentration of estrogen compounds varied from less than the detection limit (LOD) to 0.5 ng per sampler while in the lower regions differed from less than the detection limit to 4.8 ng per sampler device. Yao et al. also studied the concentration of estrogenic compounds in river surface water in China in 2018 and reported that the concentration of these compounds in all samples was in the range of nanograms per liter and approximately 54.5% of steroid hormones was obtained from raw wastewater directly flowing in to water sources. In general, natural sex hormones, including 17-β-estradiol (E2) and its metabolites, including estrone (E1) and estriol (E3), as well as synthetic steroids such as ethinyl estradiol (EE), are mostly excreted in mammalian urine. Ethinyl estradiol, a 17β-estradiol derivative, is a bioactive oral estrogen that is used in almost all new oral contraceptive formulations and is widely found in freshwater ecosystems in many areas (Vos et al., 2000). According to European Water Framework Directive in 2018, estradiol and ethinyl estradiol are included in the list of prioritized contaminants to be considered for environmental studies in Europe due to their potential environmental hazards, making it important to be evaluated in internal water resources around the world especially in developing countries like Iran in Asia.

The results obtained from 24-hour exposure of cell lines to water samples from five sampling sites in river and drinking water sample did not show any cytotoxic effects. Nevertheless, waste water effluents decreased cell viability about 40% in HEK293T cells and 10% in Hep-G2 cells. Žegura, in 2006 analyzed mitochondrial activity in Hep-G2 cell line in water from different sources and found elevated toxicity in two river water samples. According to him, MTT assay was sensitive for the detection of cytotoxic changes in water and the mixture percentage may be successfully used. Lovecka et al., in 2015 tested the cytotoxic effects of benzonitrile herbicides and their microbial metabolites using two human cell lines, Hep G2 and HEK293T and observed high toxic effects of tested compounds on both cell lines.

Cell lines were selected in such a way to include organs which represent the effects of xenobiotics and hormones more clear and faster than others. HepG2 cells, derived from human hepatocellular carcinoma, represent the liver which has a prominent role in the metabolism of xenobiotics being particularly susceptible to exposure by chemicals and biotransformation and detoxification of many endogenous and exogenous compounds (Kamiński et al., 2007). They are a suitable in vitro model system for the study of human hepatocytes reaction to xenobiotics. HEK293T, is also widely used as in vitro system for cytotoxicity testing (Patel, 2011). Kidney is the primary route of excretion of xenobiotic compounds from the body and possesses most of the common xenobiotic metabolizing enzymes (Lock et al., 1998). On the other hand, fish cell lines are relevant and economically important in fish research, ecotoxicology or more general fish health studies. They are already used to assess mixture toxicity of chemicals such as PAHs, pharmaceuticals and personal care products (Schnell et al., 2009), but also waste water effluent samples (Dayeh et al., 2002).

Although, our results confirm that the concentrations are not toxic for living organisms, it can be due to dilution of the river water by waste water or the treatment process that is done before discharging sewage through the river. But regular monitoring should be performed and in order to prevent subsequent problems the application of this water should be restricted.

Conclusion

This study provides basic new information regarding pharmaceutical and hormonal contamination in an important river in central region of Iran. Although cities are equipped with wastewater treatment systems and rural sewage does not enter the river directly, the impact of sewage flowing to the river can be seen. This contamination might have detrimental long-term effects on aquatic life as well as human health, thus it is recommended to continuously monitor the release of pollutants into the river ecosystem and level of contamination in river.

Acknowledgements

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The authors have declared no conflict of interest.

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