

Cyto- and genotoxic potential of water samples from polluted areas in Kosovo

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Received: 8 December 2015 / Accepted: 21 June 2016 / Published online: 3 August 2016
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Abstract Reports on the state of the environment in Kosovo have emphasized that river and ground water quality is affected by pollution from untreated urban water as well as the waste water from the industry. One of the main contributors to this pollution is located in Obiliq (coal power plants). Prishtina—the capital city of Kosovo—is heavily influenced too. Furthermore, the pollutants combined together with those from heavy traffic are dissolved in Prishtina runoff water, which is discharged into the creek entering the river Sitnica together with urban waste water. The available data show the complex pollution with excessive quantities of nitrites, suspended materials, organic compounds, detergents, heavy metals, polychlorinated biphenyls, etc. In this study, the cytotoxic and genotoxic potential of water samples taken at these sites was tested in primary rat hepatocytes. The results obtained indicate that water samples collected in Prishtina and Obiliq had a significant cytotoxic potential in primary rat hepatocyte cultures even when diluted to 1 %. The increased cytotoxicity, however, was not accompanied by an increased genotoxicity as measured by the percentage of micronucleated cells. Further investigations addressing the chemical composition of the samples and the

identification of the toxicants responsible for the cytotoxic effects found will be carried out in a next step.

Keywords Cytotoxicity · Genotoxicity · Rat hepatocytes · Kosovo · Environmental pollution

Introduction

Environmental pollution in the Kosovo is one of the concerning issues in the current phase of the development of this young republic. Among the main contributors to environmental pollution are the power plants located in Kastriot/Obiliq. These power plants use coal (lignite) for energy production. Their responsibility for an increase of health problems of workers of the power plants and people living in this area was reported in 1970s of the last century (Begraca 1978) and confirmed by recent reports (USAID 2009; World Bank 2012; MESP 2013) showing that the incidence of infant fatalities, lower respiratory tract infections, lung cancer, or other respiratory diseases is much higher than in other non-exposed areas. In addition, plants cultivated in Kastriot/Obiliq showed increased numbers of chromosomal aberrations and aberrant mitoses (Savic et al. 1989), while ash (from lignite burning) and water from the vicinity of power plants in Kastriot/Obiliq caused genotoxic effects on primary rat hepatocytes (Eckl et al. 2010). Ash from lignite burning was additionally shown to increase the genetic load in *Drosophila melanogaster* (Alija et al. 2014).

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Available data show that the pollution emitted from power plants in Kastriot/Obiliq is exceeding the European standards and is very complex including pollutants such as fly ash, sulfur oxide (SO_x), nitrogen oxide (NO_x), hydrocarbons, ammonia (NH₃), and hydrogen sulfide (H₂S) (INKOS 1992; MESP 2013). In addition, the ash left after coal (lignite) burning has an influence also on the river Sitnica receiving the waste water from the power plants (MESP 2009; MESP 2010; MESP 2013). The presence of inorganic compounds, heavy metals, and radioactive elements is reported too (INKOS 1992; Dumani 1995; Dumani 2000).

Prishtina, the capital city of Kosovo, is situated only 5 km from this source of pollution (Kastriot/Obiliq). Therefore, the inhabitants are highly exposed to the emissions of the power plants, especially from particulate matter (Tahirsylaj and Latifi 2010; Ukehaxhaj et al. 2013). In addition, Prishtina is facing additional problems such as the overloaded traffic, an intensive phase of construction activities, waste water, and untreated urban water, which influence the quality of the environment (MESP 2010; MESP 2013).

The waste water from households and factories in Kosovo flows (usually untreated) directly into rivers and fields. Therefore, most of the rivers, which are of good quality at the source, change quickly as the rivers pass towns and industries downstream (MESP 2010). According to the same report (MESP 2010), the river Sitnica (receiving the waste water from Prishtina), along its stream from Ferizaj to Mitrovica, represents the most polluted river in Kosovo, and the measured physical and chemical parameters indicate a permanent river pollution with nitrites, phosphates, and sulfates together with heavy metals such as chromium (MESP 2010).

On the other hand, polluted waste water from Prishtina also influences the river Sitnica. Monitoring of this water body collecting all urban waste water discharged by the city of Prishtina at Bresje—before it enters into Sitnica river—in the period 2007–2010 showed the presence of high values of ammonia (NH₄⁺), phosphates (PO₄³⁻), total phosphorus (poly and ortho), detergents, biodegradable organic compounds, etc., and there is lack of dissolved oxygen (MESP 2010).

Concerning health problems observed in the Kosovo and their correlation with environmental pollution, there were only few sporadic efforts to elucidate the impact of this pollution on potential long-term effects in order to take the necessary measures for minimizing or

preventing this impact. Therefore, to investigate in more detail the cytotoxic and genotoxic potential of water samples, an *in vitro* assay with primary rat hepatocytes (Eckl et al. 1987) was carried out particularly addressing water from Prishtina and Kastriot/Obiliq.

Methods and materials

Prishtina water samples were taken in the Calabria quarter (42.64430° N, 21.14600° E) from the water flow which is a collector of the urban waste water whereas in Kastriot/Obiliq, water samples were taken as grab samples from the Sitnica river near Kosovo A power plant (42.66900° N, 21.07600° E) which is downstream the place of discharge of the water flow under investigation as well as other discharges including those from the Drenica river. Water was collected and kept in freshly bought sterilized bottles. Bottles were kept in the refrigerator before transport to Salzburg. Water samples were handled according to Eckl (1995). They were transferred to the laboratory, filtered through a paper filter to remove particulate matter, and stored at -20 °C until further use. Control water consisted of double-distilled water. Powdered minimum essential medium (MEM) was added to the filtered samples and the pH brought to 7.4. In order to sterilize the samples, they were filtered through a 0.2-μm pore size cellulose nitrate filter (Sartorius).

The experiments with rat hepatocytes were performed at the Department of Cell Biology—University of Salzburg.

Female Fischer 344 rats weighing approximately 100 g were obtained from HARLAN, Winkelmann (Germany), and were kept in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Water was provided *ad libitum*. The animals were allowed to acclimate for at least 2 weeks prior to hepatocyte isolation. Hepatocytes were isolated from rats by the *in situ* two-step collagenase perfusion technique as described by Michalopoulos et al. (1982), plated at a density of 20,000 viable cells/cm² on collagen-coated 60-mm-diameter plastic culture dishes. Hepatocytes were plated in 5 ml of serum-free MEM containing 1.8 mM calcium supplemented with non-essential amino acids, pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM), and penicillin (100 U)/streptomycin (100 μg/ml) (Alija et al. 2010). The cultures were incubated at 37 °C, 5 % CO₂, and 95 % relative humidity. The

medium was exchanged for fresh MEM after an incubation period of 3 h, and the cultures were returned to the incubator.

Approximately 24 h after the initial change of medium, the prepared water samples were added to the hepatocyte cultures to yield final concentrations of 100, 10, 1, and 0.1 %; the cultures were then incubated for 3 h. The medium was then removed, the dishes washed twice with fresh medium to completely remove any toxic/genotoxic substance, and fresh medium was added. Thereafter, cells were incubated for an additional 48 h.

As described by Eckl (1995), cells were fixed after 48 h with cold methanol/glacial acetic acid (3:1) for 5 min on Petri dishes, rinsed with distilled water for 2 min, and then air dried. The fixed cells were stained with the fluorescent dye DAPI (4',6-diamidino-2-phenylindol, 0.2 µg/ml McIlvaine citric acid- Na_2HPO_4 buffer, pH = 7) for 30 min, washed with McIlvaine buffer for 2 min, briefly rinsed with distilled water, and mounted in glycerol. To determine the mitotic indices and the frequencies of apoptotic and necrotic cells, 1000 cells per dish were analyzed under a fluorescence microscope (Leitz Aristoplan). Cell densities as an indirect measure for dead cells which detached from the growth surface were evaluated by counting the number of adherent cells/visual field.

Data normal distribution was examined by the Shapiro-Wilk test. Since some of the data points showed no normal distribution, non-parametric tests were used for statistical evaluation. The Kruskal-Wallis test was used for multiple comparisons and the Mann-Whitney *U* test for pairwise comparisons. Statistical analysis was performed using the IBM SPSS Statistics software package (version 22).

Results and discussion

As shown in Table 1, water concentrations of 1, 10, and 100 % from both Prishtina and Kastriot/Obiliq samples collected in 2012 induced a statistically significant cytotoxic effect ($P < 0.05$). Water from Kastriot/Obiliq was demonstrated to be cytotoxic even at a concentration of 0.1 % ($P < 0.05$). The highest percentage of dead cells had a necrotic morphology with pycnotic nuclei whereas apoptotic cells (fragmented nuclei) were not significantly elevated compared to the control cultures.

Table 2 summarizes the results obtained with Prishtina and Kastriot/Obiliq water samples collected in 2013. The data obtained show a significant increase ($P < 0.05$) of the percentage of adherent apoptotic and necrotic cells at 100 % water concentration for both samples—Prishtina and Kastriot/Obiliq—compared to the control.

Compared to the effect of the samples collected in 2012, the samples from 2013 showed clear differences with respect to the percentage of apoptotic and necrotic cells and did not follow a comparable trend: while cytotoxicity at 100 % was lower in the Prishtina sample, it was higher in the Kastriot/Obiliq sample. Concerning cell densities as measure of detached dead cells, it has to be further pointed out that in 2013, the cell cultures treated with the highest water concentration (100 %) from Prishtina and Obiliq showed a decrease ($P < 0.05$) in the density indicating that cytotoxicity was even higher than the results obtained by the analysis of attached necrotic and apoptotic cells would predict. No significant difference in the density was evident in samples from 2012 (Table 1). In order to assess the genotoxic potential as measured by the induction of micronucleated cells, further experiments were carried out with rat hepatocyte primary cultures stimulated to proliferate by supplementation of EGF (40 ng/ml) after treatment with the water samples, since primary hepatocytes are proliferatively quiescent. Whenever DNA damage is introduced due to genotoxic compounds contained in the water samples, such damage will be fixed and made visible by cell division, i.e., micronucleated cells. Since proliferative stimulation may eventually influence cell death, this parameter was also evaluated in the samples from 2013 together with the proliferation rate as measured by the mitotic index (percentage of cells undergoing mitosis). The results obtained are summarized in Table 3 and indicate that the water sample from Prishtina had no effect at all on any of the parameters determined, while the sample from Obiliq induced a significant ($P < 0.05$) increase of the percentage of dead cells at a concentration of 100 %; however, no significant difference in the density was seen. This increase was accompanied by a significant decrease of the mitotic index, while there was no significant change of the percentage of micronucleated cells at concentrations of 1 and 10 %. At a concentration of 100 %, a reduction of micronucleated cells was observed. This effect, however, cannot be explained in terms of a real reduction of genotoxicity, but reflects

Table 1 Cytotoxic effects of different concentrations of water samples from Prishtina and Obiliq (samples from 2012)

Water concentrations	Adherent apoptotic cells ^a	Adherent necrotic cells ^a	Total cell death ^a	Cell density ^b
Control	0.69 ± 0.46(8)	1.81 ± 0.80(7)	2.50 ± 0.68 (7)	1.42 ± 0.33(8)
Prishtina 0.1 %	0.67 ± 0.49(4)	4.35 ± 3.51 (4)	5.02 ± 3.56 (4)	1.05 ± 0.35(4)
Prishtina 1 %	0.92 ± 1.11(4)	7.61 ± 4.27(4)*	8.53 ± 4.10 (4)*	1.03 ± 0.33(4)
Prishtina 10 %	0.71 ± 0.91(4)	11.85 ± 7.18(4)*	12.56 ± 6.66 (4)*	1.05 ± 0.30(4)
Prishtina 100%	1.48 ± 1.27 (4)	25.48 ± 27.85 (4)*	26.96 ± 27.04 (4)*	1.01 ± 0.30(4)
Obiliq 0.1 %	0.58 ± 0.52(4)	6.12 ± 4.41(4)	6.70 ± 4.08 (4)*	1.20 ± 0.30(4)
Obiliq 1 %	0.74 ± 0.75(4)	5.61 ± 3.66(4)*	6.35 ± 3.21 (4)*	1.21 ± 0.41(4)
Obiliq 10 %	0.60 ± 0.44(4)	6.74 ± 3.65(4)*	7.34 ± 3.69 (4)*	1.21 ± 0.37(4)
Obiliq 100 %	0.52 ± 0.21(4)	21.84 ± 23.37(4)*	22.36 ± 23.23 (4)*	1.21 ± 0.27(4)

Data represent the mean ± standard deviation from four independent experiments

* $P < 0.05$ compared to the control: Mann-Whitney U test

^a Values represent the mean ± SD of the corresponding percentages of adherent cells

^b Cells × 10⁴/cm²

the significantly decreased mitotic index due to the toxicity of the sample.

According to the available data (INKOS 1992; MESP 2013), the air of Kastriot/Obilic which influences Prishtina runoff water contains pollutants such as SO_x, NO_x, and carbon oxide (CO_x). On the other hand, recently published reports show that the water from Prishtina entering the river Sitnica is contaminated with excessive quantities of nitrites and suspended materials, CBO₅ (biodegradable organic compounds), detergents, etc. Therefore, the water from Sitnica river has been reported to indicate permanent pollution containing the pollutants from Prishtina water as well as other

pollutants released from coal power plants and other pollutants along its stream (MESP 2013).

Apart from these chemicals, the cytotoxic effects observed in this investigation can be attributed to the presence of many chemicals especially heavy metals (Jarup 2003; Tchounwou et al. 2012). Water from Sitnica river upstream of the sampling site showed concentrations above limits for heavy metals (Hg and Cu) as well as for polychlorinated biphenyls (PCBs) (MESP 2014). According to the same report, river sediments from the vicinity of the water sampling site showed significant contamination with As, Pb, Cu, Cr, Ni, Cd, and Zn.

Table 2 Cytotoxic effects of different concentrations of water samples from Prishtina and Obiliq (samples from 2013)

Water concentrations	Adherent apoptotic cells ^a	Adherent necrotic cells ^a	Total cell death ^a	Cell density ^b
Control	0.70 ± 0.17 (4)	1.55 ± 0.55 (4)	2.25 ± 0.57 (4)	1.54 ± 0.15(4)
Prishtina 1 %	0.53 ± 0.32 (4)	1.99 ± 0.57 (4) ⁺	2.52 ± 0.82 (4) ⁺	1.29 ± 0.20(4)
Prishtina 10 %	0.74 ± 0.18 (4)	2.60 ± 1.15 (4)	3.34 ± 1.01 (4) ⁺	1.22 ± 0.23(4)
Prishtina 100 %	1.70 ± 0.52 (4)*	3.73 ± 1.22 (4) ⁺	5.43 ± 1.53 (4)* ⁺	1.16 ± 0.15(4)*
Kastriot/Obiliq 1 %	0.55 ± 0.28 (4)	2.13 ± 0.39 (4)	2.68 ± 0.64 (4)	2.90 ± 3.23(4)
Kastriot/Obiliq 10 %	0.51 ± 0.37 (4)	3.03 ± 0.89 (4)	3.54 ± 1.05 (4)	1.25 ± 0.17(4)
Kastriot/Obiliq 100 %	2.73 ± 1.82 (4)* ⁺	60.39 ± 26.07 (4)*	63.12 ± 25.57 (4)*	1.10 ± 0.08(4)*

Data represent the mean ± standard deviation from four independent experiments

* $P < 0.05$ compared to the control: Mann-Whitney U test

⁺ $P < 0.05$ compared to corresponding mean values of 2012: Mann-Whitney U test

^a Values represent the mean ± SD of the corresponding percentages of adherent cells

^b Cells × 10⁴/cm²

Table 3 Effects of different concentrations of water samples from Prishtina and Obiliq on cell death, cell proliferation, and the frequency of micronucleated primary rat hepatocytes (samples from 2013)

Water concentrations	Total cell death ^a (necrosis + apoptosis)	Mitotic cells (%)	Micronucleated cells (%)	Cell density ^b
Control	0.96 ± 0.11 (3)	1.43 ± 0.37 (3)	3.04 ± 1.25 (3)	1.25 ± 0.08(3)
Prishtina 1 %	0.78 ± 0.49 (3)	2.57 ± 1.18 (3)	4.79 ± 2.44 (3)	1.11 ± 0.05 (3)
Prishtina 10 %	1.10 ± 0.55 (3)	3.17 ± 1.03 (3)	7.15 ± 3.41 (3)	1.15 ± 0.20 (3)
Prishtina 100%	0.84 ± 0.64 (3)	3.69 ± 1.71 (3)	6.53 ± 2.76 (3)	1.25 ± 0.11 (3)
Kastriot/Obiliq 1 %	1.21 ± 0.10 (3)	3.07 ± 1.18 (3)	5.11 ± 2.61 (3)	1.10 ± 0.07 (3)
Kastriot/Obiliq 10 %	1.00 ± 0.62 (3)	2.86 ± 1.32 (3)	6.68 ± 4.12 (3)	1.18 ± 0.16 (3)
Kastriot/Obiliq 100 %	77.21 ± 2.33 (3)*	0.13 ± 0.012 (3)*	0.18 ± 0.28 (3)*	0.12 ± 0.01 (3)*

Data represent the means ± standard deviation from three independent experiments

**P* < 0.05 compared to the control: Mann-Whitney *U* test

^a Values represent the mean ± SD of the corresponding percentages of adherent cells

^b Cells × 10⁴ /cm²

In addition, polycyclic aromatic hydrocarbons (PAHs) were found to be exceeding the limits in the groundwater near the sampling place in Sitnica river (MESP 2014). Consequently, it is expected that they are present in Sitnica river water too. Although PAHs are considered as lead substances for complex environmental mixtures (White 2002; Tang et al. 2008; Geng et al. 2014; Pergal et al. 2014; Yao et al. 2014), and capable of promoting redox reactions and the formation of reactive oxygen species (Farmer et al. 2003), and thus eventual toxicity, it is not very likely that PAHs are responsible for the effects found because they will enter water bodies primarily bound to particulate matter. Since the water samples were filtered, sterilized particulate matter larger than 0.2 μm is not contained in the applied sample. Still fine particles could be contained in a substantial amount when considering the size distributions found in urban air (i.e., Whitby et al. 1972). Since PAHs are chemically very stable (Ravindra et al. 2008), one can further expect that testing with primary hepatocytes should result in comparable results in the individual experiments in case PAHs are responsible for the cytotoxic effects observed. As a common trend in our experiments, the activity of the samples, however, decreases with increasing storage times, an effect already described for other river water samples (Eckl 1995).

In Prishtina water, among the chemicals detected by a qualitative GC/MS analysis, the following ones were found to be present in >5 % of the total amount of analyzed compounds: methylene chloride, 2,6-dimethyl-7-octen-2-ol, 3,7-dimethyl-1,6-octadien-3-ol, 1-

methyl-4-(1-methylethyl) cyclohexanol, and octacosane. In the water samples from Sitnica river, the chemicals exceeding 5 % were the following: 5-aminoisoxazole, sulfurous acid, and hexatriacontane. It has to be emphasized that the hexatriacontane level in the sample was approximately 47 %. The common chemicals for both sampling sites were as follows: methylene chloride, hexamethylcyclotrisiloxane, and dibutyl phthalate. Among them, methylene chloride (Mizutani et al. 1988; Graves et al. 1995; Green 1997) and dibutyl phthalate (Marsman 1995; Kim et al. 2002) are reported to exert toxic effects thus contributing to the observed cytotoxicity.

Micronucleated cells were evaluated as measure of genotoxicity and revealed a clear but not significant reduction of micronucleated cells together with a significant reduction of the mitotic index and a significant increase of cell death (Table 3) for the 100 % Obiliq sample. Since formation of micronuclei requires cell division, any effect induced by the sample will be masked when cells do not divide. Fewer divisions therefore mean less micronucleus formation. As an approximation, one can standardize the percentage of cells with micronuclei to a mitotic index of 1. When doing so for this sample and the control, a percentage of 1.38 is obtained compared to 2.12 for the control. Therefore, it appears unlikely that the sample had a genotoxic potential. Still, there also appears to be a trend towards higher percentages of micronucleated cells at lower concentrations tested. These, however, are not significant due to the high variation between the results of the individual experiments.

Conclusions

Water samples from two polluted localities in the Kosovo (Prishtina and Kastriot/Obiliq) show a cytotoxic potential in primary rat hepatocyte cultures depending on the concentrations applied while there is no indication for a genotoxic potential as evidenced by the analysis of micronucleus formation. The different levels of cytotoxicity of samples collected in 2012 and 2013 most likely reflect differing contamination by urban and industrial effluents, and point to the necessity of continuous monitoring and risk assessment of the water in these areas. Further investigations addressing the chemical composition of the samples and the identification of the toxicants responsible for the cytotoxic effects found will be carried out in a next step.

Acknowledgments This study was supported by the Europe Aid project “Environmental pollution in Kosova: potential genotoxic effects and related human health risks” (no. 2012/297-252). We would like to thank Avni Hajdari for excellent technical assistance.

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