“HEXAVALENT CHROMIUM IN DRINKING WATER; EFFECTS ON HUMAN HEALTH”: DESIGN OF AN EPIDEMIOLOGICAL STUDY

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ABSTRACT

Hexavalent chromium is a well-documented carcinogen via the inhalation route. However, data for potential health effects due to oral Cr\textsuperscript{6+} exposure is limited and controversial. The WHO and the European Union have established a drinking water provisional guideline of 50 μg·L\textsuperscript{-1} for total chromium, while the California Environmental Protection Agency proposed a Public Health Goal in drinking water of 0.02 μg·L\textsuperscript{-1} Cr\textsuperscript{6+}. Long-term health effects from consuming water containing Cr\textsuperscript{6+} near the provisional guideline have not been investigated.

An epidemiological study was designed to investigate health effects associated with long-term (about 30 years) consumption of drinking water containing Cr\textsuperscript{6+} in the range of 10 to >50 μg·L\textsuperscript{-1}. Based on previous laboratory and experimental studies, haematological and biochemical alterations, inflammatory responses, neurological motor dysfunction and differentiation of chromium concentrations in biological samples (blood and hair) of the exposed individuals, were determined to be investigated.

Twenty-five villages or cities located around the Asopos bed will constitute the study area. Environmental samples (water, soil and crops) will be collected and concentrations of total and hexavalent Cr will be determined. Results from previous studies will also be included in our database. The study area will be classified according to the levels of Cr\textsuperscript{6+} in drinking water. About 300 volunteers will be randomly selected as the study population. The inclusion criteria were defined as i) age range of 30-65 years old and ii) inhabitation in the study area for the last consecutive 7 years.

A questionnaire was developed to record socio-demographics, lifestyle, drinking water intake, occupational and medical history data. All participants will undergo haematological and biochemical blood examination. A general practitioner will conduct a physical examination and a neurological test (Unified Parkinson’s Disease Rating Scale-motor examination) to the individuals. Total chromium will be determined in blood and hair samples of the study subjects. Inflammatory response (cytokines) will be examined in the blood samples. Correlations and multivariate models will be applied to investigate associations between biomarkers, neurological outcomes and Cr exposure.

The main strength of the present research is that it is the first epidemiological study on the health effects that can be induced through long-term low level ingestion of Cr\textsuperscript{6+} with an individual-based design. Moreover, our results will provide the first evidence concerning associations between Cr exposure and neurological outcomes on humans. It is expected that the results will constitute a “tool” on establishing “safe” guidelines for drinking water. The main limitations of the study arise from its cross-sectional nature and the lack of records for the Cr\textsuperscript{6+} levels in all sites and for the whole 30 years time period.

Keywords: hexavalent chromium, oral exposure, human health, epidemiology.

1. INTRODUCTION

Chromium is a heavy metal that occurs throughout the environment mainly in two forms: the trivalent (Cr\textsuperscript{3+}) and the hexavalent (Cr\textsuperscript{6+}). The two forms are inter-convertible but have totally different behavioral and toxicological properties. Cr\textsuperscript{3+} is an essential nutrient
required for energy metabolism so it is relatively non-toxic, in contrast to Cr\textsuperscript{6+} which is toxic and centers all public health concerns.

**Sources.** While the primary source of Cr\textsuperscript{3+} (chromite) is of geological origin, Cr\textsuperscript{6+} is most commonly produced by industrial processes (chemical industry, production of dyes, wood preservation, leather tanning, chrome plating, manufacturing of various alloys, etc.). Cr\textsuperscript{6+} can also result from the oxidation of naturally occurring Cr\textsuperscript{3+} by Mn-oxides containing minerals such as birnessite, ultramafic rocks and serpentinites of ophiolite complexes (Oze *et al.* 2007).

**Environmental Chemistry.** Cr\textsuperscript{3+} compounds are generally insoluble in water, while Cr\textsuperscript{6+} compounds are readily soluble in water at neutral pH. The speciation of Cr in the aquatic environment is controlled by several factors, including the presence and concentrations of chromium species and oxidizing or reducing agents, the pH and the redox potential. In groundwater, Cr\textsuperscript{6+} can be reduced to Cr\textsuperscript{3+} at low pH and under reducing conditions (presence of Fe\textsuperscript{2+} and organic matter), while oxidation of Cr\textsuperscript{3+} to Cr\textsuperscript{6+} is favoured in oxidizing environments and in the presence of solid MnO\textsubscript{2} (Richard and Bourg 1991). The residence times of total chromium in lake water range from 4.6 to 18 years, with the majority of the chromium in lakes and rivers ultimately deposited in the sediments (ATSDR 2008).

**Exposure.** The general population is exposed to chromium (Cr\textsuperscript{3+} or Cr\textsuperscript{6+}) by inhaling air, ingesting food and drinking water containing chromium (ATSDR 2008). Potential health effects from chromium exposure are dependent on many factors, such as the chemical form, the amount, the exposure duration and the exposure route (ingestion, inhalation, skin absorption). Metabolic reactions and their potential effects are highly dependent on characteristics such as age, sex, weight, and status of the immune system of the individual.

**Toxicokinetics of Cr\textsuperscript{6+}.** Once ingested, Cr\textsuperscript{6+} meets the highly acidic gastric fluid, and it is reduced to Cr\textsuperscript{3+}. De Flora (2000) estimated that saliva has the capacity to reduce 0.7 to 2.1 mg Cr\textsuperscript{6+} per day and gastric juices have the ability to reduce at least 80.3 to 84 mg Cr\textsuperscript{6+} per day. These investigators indicate that the reaction is complete within 10-20 minutes, with at least half accomplished within one minute. But other researchers argue that if this reduction was to occur completely, then Cr\textsuperscript{6+} administration would be expected to behave (with respect to absorption, distribution and toxic effects), as if Cr\textsuperscript{3+} had been administered. However, studies in animals and humans have revealed that orally administered Cr\textsuperscript{6+} results in differences in blood/plasma and tissue total chromium levels and increased urinary half-life compared to Cr\textsuperscript{3+}. Increased toxicity following oral exposure to Cr\textsuperscript{6+} (compared to Cr\textsuperscript{3+}) also suggests that Cr\textsuperscript{6+} is not completely converted to Cr\textsuperscript{3+} in the stomach (OEHHA 2011a).

The amount of Cr\textsuperscript{6+} that escapes reduction will be absorbed and afterwards, the fate of chromium will depend on the oxidation state. Cr\textsuperscript{6+} can easily penetrate biological membranes, because its structure mimics sulfate and phosphate anions. Once inside the cell, Cr\textsuperscript{6+} is ultimately reduced by reductants such as ascorbic acid, glutathione, and cysteine, to Cr\textsuperscript{3+}, which, in turn, reacts with cellular macromolecules (proteins to produce toxicity and DNA to potentially cause cancer) (Costa and Klein 2006). Cr\textsuperscript{6+} displays no ability to damage DNA directly and requires reductive activation to be genotoxic. On the other hand, Cr\textsuperscript{3+} has an octahedral structure and cannot penetrate the biological membrane of the cells (Costa and Klein 2006), so the extracellular reduction of Cr\textsuperscript{6+} to Cr\textsuperscript{3+} constitutes a detoxification process (IARC 2012).

**Evidence on health effects.** There is sufficient evidence on the carcinogenicity and the genotoxicity of Cr\textsuperscript{6+} in occupational exposure via the inhalation route, so it has been
classified as carcinogenic to humans by the inhalation route by the WHO-International Agency for Research on Cancer (IARC 2012). However, the health effects of Cr\textsuperscript{6+} via the oral route remain controversial and uncertain in the scientific community. There are a few animal studies showing that Cr\textsuperscript{6+} may be carcinogenic via the ingestion route (NTP 2008, Borneff et al. 1968). Skin tumors were reported in mice after drinking water containing as low concentrations as 500 µg·L\textsuperscript{-1} Cr\textsuperscript{6+} with simultaneous exposure to UV-irradiation (Davidson et al. 2004). However epidemiological studies evaluating the association between ingested Cr\textsuperscript{6+} and adverse health effects in humans are scarce. Only two ecological studies have been conducted in China (Zhang and Li 1997, 1987) and in Greece (Oinofyta municipality) (Linos et al. 2011). Both studies estimate the cancer mortality (lung-, stomach-, etc) considered to be caused by the prolonged oral consumption of water contaminated with high levels of Cr\textsuperscript{6+} (in the order of mg·L\textsuperscript{-1}). Both studies have been criticized for some limitations, the more serious being lack of exposure data (OEHHA 2011a, Kerger et al. 2009). Zhang and Li (1987) study provides sparse information regarding the magnitude and length of Cr exposure, as well as for the two unexposed populations being used as controls. Moreover a lot of controversy has been arisen because of a following research article (1997) of Zhang and other researchers that withdrew the original association between increase in cancer incidence and high Cr\textsuperscript{6+} levels in drinking water and which was eventually retracted by the editor of the journal (OEHHA 2011b, Brandt-Rauf 2006). As for Linos et al. (2011) study, it has been criticized about the water sampling methods (whether they were representative of the water supply system or was worst-case sampling), the method of deaths identification and the lack of significant association between stomach cancer and chromium exposure (OEHHA 2011a). Moreover, Craun (2012) has questioned that Linos et al finding supports the hypothesis of Cr\textsuperscript{6+} carcinogenicity via the oral ingestion pathway of exposure because of poorly defined Cr\textsuperscript{6+} ingestion exposures, lack of data about the chemical quality of drinking water, confusion about the actual exposure and concludes that exposure misclassification bias is possible.

**Biomarkers of exposure and effect.** Chromium in body fluids (e.g., blood and urine) is the biomarker of choice used to identify or quantify exposure to chromium. Increased plasma levels of chromium may indicate exposure to both Cr\textsuperscript{6+} and Cr\textsuperscript{3+}, whereas increased chromium in erythrocytes indicates exposure to Cr\textsuperscript{6+}, since Cr\textsuperscript{3+} is not taken up by erythrocytes (ATSDR 2008).

**Haematological effects.** Several haematological effects have been reported in humans following Cr\textsuperscript{6+} ingestion: decreased haemoglobin content and haematocrit, increased total white blood cell counts and inhibition of blood coagulation. In addition, decreased erythrocyte levels, mean cell volume, platelet concentrations, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were found in animals (rats and mice) (ATSDR 2008).

**Hepatic effects.** Alterations in liver enzymes activities and serum components have been reported in humans after oral exposure to Cr\textsuperscript{6+}: Increased bilirubin, serum lactic dehydrogenase (LDH) and high levels of liver transaminases (ATSDR 2008). In experimental rats, there were also found significant increases in serum triglyceride and glucose levels (Chopra et al. 1996), and changes in the distribution and activity of alkaline phosphatase, acid phosphatase, glucose-6-phosphatase, cholinesterase (Kumar et al. 1985).

**Inflammation effects.** The possibility of using an immune-function assay as a potential biomarker for humans exposed to chromium has been examined (Snyder et al. 1996). A significant 36% decrease in the levels of interleukin (IL)-6 in monocytes in the chromium exposed group was found. The production of IL-1 and tumor necrosis factor (TNF)-α cytokines were reduced in rats exposed to potassium chromate (Cohen et al. 1998).

**Neurological effects.** One human study (Duckett 1986) described three cases with unrelated encephalopathic diseases which showed evidence of Central Nervous System chromium neurotoxicity at autopsy. Chromium was thought to enter the brain through
pathological pallidal blood vessels that showed vascular siderosis and possible breakdown of the blood-brain barrier. This appears to be the only report of pure chromium neurotoxicity in the human literature. A decrease in motor activity and balance was reported in rats given 98 mg Cr\(^{+6}\)-kg\(^{-1}\)·day\(^{-1}\) as sodium chromate in drinking water for 28 days (Diaz-Mayans et al. 1986). No other data were found regarding neurological effects in humans or animals from exposure to Cr\(^{+6}\), because “none study conducted more sensitive neurological, neurochemical, or neurobehavioral tests” (ATSDR 2008).

**Current legislation.** The WHO and the European Union have established a provisional guideline in drinking water of 50 μg·L\(^{-1}\) for total chromium. However, the California Environmental Protection Agency has recently proposed a Public Health Goal of 0.02 μg·L\(^{-1}\) Cr\(^{+6}\) in drinking water. To our knowledge, health effects from long-term consumption of water containing near or more than 50 μg·L\(^{-1}\) of Cr\(^{+6}\) have not been investigated.

2. **DESIGN OF THE EPIDEMIOLOGICAL STUDY**

**Study area.** Asopos River in Greece (fig. 1) has been found recently to be contaminated with Cr\(^{+6}\) at levels as high as 150 μg·L\(^{-1}\). Asopos rises in Viotia and discharges into the South Euboean Gulf, about 60 km away north of Athens. Since 1969 Asopos has been proclaimed a “processed industrial waste receiver” and the industrialization of the area has begun. The river runs through areas with almost 20% of Greece’s total industrial production and receives wastes from nearby industries. Chromium concentrations in drinking water are highly varied in the extended area. There are sites with non-detectable levels (<5 μg·L\(^{-1}\)), and sites where chromium concentrations exceed the current regulation for human consumption (maximum 150 μg·L\(^{-1}\)).

The water needs for all the residents in this region were covered by drills in the groundwater aquifer until 2–3 years ago. Since then, the main water supply, at least in the highly-contaminated villages, diverted to receive surface water from Mornos lake, which provides potable water to Athens city as well.

Three municipalities from the Prefecture of Voiotia (Aliartos, Thiva and Tanagra) and one municipality from the Prefecture of East Attiki (Oropos) are chosen to constitute the study area. It is estimated that about 20 – 30 towns or villages located around Asopos bed will participate. The suggested study area is depicted with red line in fig. 1.

![Fig. 1. Map of the study area.](Image)

**Study design**

The present study is an epidemiological cross-sectional survey. The aims are: i) determination of chromium concentrations in the environmental compartments (water, soil, crops) of the study area (wide area of Asopos) and ii) the investigation of health
effects in the general population associated with long-term (over 30 years) consumption of drinking water containing Cr\(^{\text{VI}}\) in the range of 10 to 150 \(\mu g \cdot L^{-1}\). The study area will be classified according to the level of Cr\(^{\text{VI}}\) in drinking water and the selection of the study population will be based on these results to cover a wide range of Cr levels. The research protocol and the questionnaire have gained an approval by the Bioethics Committee of the University of Patras.

**Study population**
The study population is estimated to be 300 individuals. The inclusion criteria are: a) to live permanently in the study area for at least consecutive 7 years immediately prior the study (that equals to a minimum of 5 years of exposure), b) to be within the range of 30 – 65 years old (that equals to a maximum of 40 years of exposure). The study population will be selected so as to reflect the full range of Cr water concentrations in the area and would be matched per sex and age. After the first contact, through a brief letter explaining the aims and components of the project, subjects accepting to participate and fulfilling the inclusion criteria will be visited by the General Practitioner and the Health Visitor of the Health Centre of Aliartos. The individuals will sign a written consent before their participation.

**Environmental analyses**
Water samples from the groundwater aquifer, representative of the water that the population was consuming during the last 40 years, will be collected and analyzed for their physicochemical properties, major ions content and toxic and essential metals’ concentration including hexavalent chromium. 30% of samples will be screened for Persistent Organic Pollutants. Soil and crops from the study area will also be sampled and analyzed for total and hexavalent chromium and other toxic metals (cadmium, lead, nickel, mercury, arsenic). All the chemical analyses will be conducted in the Laboratory of Public Health, Medical School, University of Patras, Greece. The Laboratory is accredited by the Hellenic Accreditation System S.A (E.SY.D.) for parameters including the determination of chromium in water samples. A database will be constructed concerning Cr concentrations in the drinking water aquifer of the study area, as well as the history of drinking water supply by combining data either from the Lab of Public Health or published by other researchers. This database will serve the estimation of the Cr exposure via drinking water consumption in each study site.

**Personal data**
A). **Physical examination.** Study subjects will be examined by the Practitioner to measure current body weight and height, systolic and diastolic blood pressure. B). **Questionnaire.** A questionnaire will be administered by the Practitioner to collect the following information:
   a) Demographical characteristics: age, sex, duration of residence, marital status, educational level, economical status, working status.
   b) Medical history (morbidities, hereditary diseases).
   c) Lifestyle: physical activity, smoking and dietary habits, alcohol consumption.
   d) Source of drinking water supply (municipal/bottled/private well/other) currently and in the past.
   e) Occupational history.
The questionnaire will be tested through its administration to a pilot population of 30 individuals, which are going to be selected randomly in the city of Patras. C). **Neurological tests.** The practitioner will conduct an appropriate neurological test namely the Unified Parkinson’s Disease Rating Scale (UPDRS) _ Motor examination test, which is composed by 14 questions and assesses motor disability and impairment. D). **Biological samples-Biomarkers.**
Blood and hair samples will be taken from each individual. In blood samples, a full blood count analysis and a biochemical examination will be conducted. Moreover, total chromium and certain cytokines (inflammatory factors) will be determined. In the hair samples total chromium concentration will be determined, while in the first 10% of the samples the levels of other toxic metals (cadmium, lead, nickel, mercury, arsenic, zinc) will also be determined and will be compared with base line measurements previously established in Greek population (Leotsinidis and Kondakis 1990). In case of positive results, the determination of these parameters will be performed in all samples.

**Statistical analysis**

Oral Cr\(^{6+}\) exposure will be calculated according to Water or Food Ingestion Exposure Dose equation (ATSDR 2005)

\[
D = \frac{(C \times IR \times EF)}{BW}
\]

Where 
- \(D\) = exposure dose (mg·kg\(^{-1}\)·day\(^{-1}\))
- \(C\) = contaminant concentration (mg·L\(^{-1}\) for water or mg·g\(^{-1}\) for food)
- \(IR\) = intake rate of contaminated water (L·day\(^{-1}\)) or food (g·day\(^{-1}\))
- \(EF\) = exposure factor (unitless)
- \(BW\) = body weight (kg)

The drinking water intake rate for each subject will be asked through the questionnaire. Exposure factor will be equal to 1, since average daily consumption is considered (ATSDR 2005). Moreover, the food intake of Cr\(^{6+}\) through local vegetables' consumption will be taken into consideration, according to ATSDR 2005. Descriptive statistics will be used to describe the various parameters (Cr and Cr\(^{6+}\) water concentrations, water chemical parameters, Cr blood and hair concentrations, metals hair concentrations, blood count parameters, blood biochemical parameters, inflammatory factors). Correlations will be examined and regression models will be constructed to describe the association between the Cr\(^{6+}\) exposure and the outcomes. Logistic regression analysis will be considered for dichotomous outcomes.

**Chronodiagramm**

Step 1. Sampling of water, soil and crops. Determination of total and hexavalent chromium concentrations.

Step 2. According to the water analyses results, the selection of the study participants will be performed to represent a wide range of Cr levels.

Step 3. Contact and recruitment of the participants. In total 300 individuals will be selected from villages/towns with various water Cr concentrations.

Step 4. Visit of the medical doctor and the Health Visitor to each individual. The medical doctor will conduct the physical examination, as well as the neurological tests and administer the questionnaire. The Health Visitor will collect the blood and the hair sample.

Step 5. Determination of biomarkers in biological samples.

Step 6. Construction of the database. Statistical analysis will be performed. Descriptive statistics will be used to describe the various parameters. Correlations and multivariate models will be applied to investigate associations, if any.


3. **DISCUSSION**

This epidemiological study is of great importance at national and international level. There is limited evidence of the health effects that can be induced through long-term low level consumption of water contaminated with Cr\(^{6+}\). The extended area of Asopos is an ideal study area for this purpose and provides a valuable tool that can be utilized in order to get more information and fill the gap that exists in that scientific area. The main strength of the present research is that it will be the first epidemiological study on ingested Cr\(^{6+}\) with an individual-based design.
The researchers think that the most likely and stronger finding is the revelation of a positive correlation between chromium concentration in human hair and chromium concentration in the water aquifer. That is because hair is a tissue which provides a print of one’s biochemistry and previous exposures to pollutants.

Ingested Cr has a short elimination half-time in blood, approximately 25–35 days (Dever et al. 1989). However, because of the Cr^{+6} sequestration within erythrocytes, chromium excretion in blood remains detectable for the lifespan of the red blood cell (approximately 130 days). Nevertheless, in case of long-term repetitive exposure and given that there are subjects among the study population that continue consuming drinking water with Cr^{+6}, it is expected that blood Cr concentration will reveal, at least, slight associations.

Changes in inflammatory cytokines are expected to be detected among subjects being exposed to high levels of Cr^{+6}, in accordance with previous studies (Cohen et al. 1998), but it would be difficult to be attributed to Cr^{+6} exposure, since there are a lot of substances that provoke inflammation response.

Although there is biological plausibility to find associations between Cr exposure and neurological/neurobehavioral outcomes, there is no previous evidence from epidemiological studies and our results will provide the first evidence concerning this concept. It is expected that the publications that will come out from this research work will extend the current knowledge about the effects on human health of oral intake of hexavalent chromium via drinking water and will constitute a “tool” on establishing guidelines for drinking water.

Limitations. Besides the limitations of the study as a cross-sectional one, its main limitation is the lack of records for the Cr^{+6} levels in all sites and for the whole 30 years time period. Consequently, the current Cr^{+6} concentrations will be used to assess the Cr^{+6} exposure. Nevertheless, this limitation can be partly overcome by utilizing the previous records that exist for some sites for the last 5-10 years. The epidemiological study would be stronger if the time fluctuations in Cr^{+6} concentrations were known, even if they are not expected to be really large. The exact time point that contamination of the water aquifer occurred in each site is also unknown.

Another limitation is that there have already passed approximately 4 years since the change in water supply reservoir in some sites, but not all, under study. So, there is some gap in Cr^{+6} exposure for some of the subjects. This will also decrease the strength of blood examinations, since Cr has a short elimination half-time in blood. Nevertheless, there would be other subjects that are still under Cr^{+6} exposure.

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