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Short Communication

Frog as Sentinel of Human Cancer Incidence in Southern Italy's So-Called 'Terra dei Fuochi'

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Abstract

The southern Italy waste management crisis has reached epic proportions, posing grave health risks to the populations. Epidemiological data from Campania indicated that there might be a causal relationship between illegal dumping of toxic substances and incidence of cancer. Our data showed the relationship between the wrong management of municipal toxic waste and the effects on human health, demonstrated by monitoring the DNA damage in animal sentinel edible frog (*Pelophylax kl. esculentus*).

Keywords: frog; comet assay; toxic waste; human health

Introduction

In recent times "*Campania Felix*," the most fertile soil of Italy, has become one of the largest mass poisonings in Europe [1]. This depends on the tons of toxic wastes illegally dumped into the land of province of Naples and Caserta, areas sadly known as "Terra dei Fuochi*"* [2]. Naples and its province produce more rubbish than its coping capacity, and this problem is linked to the presence of organized criminal associations leading to illegal waste dumping. Moreover, hazardous waste from northern Italy and hidden in southern Italy brings out the widespread presence of toxic elements and compounds [3].

The prolonged persistence of this status generated negative effects on ecosystems, on water [4], soil [5-6], vegetables [3] and animals [7], and it has been linked to increasing rates of cancer [4,8] (Fig. 1). In fact, pollution can be considered one of the main causes of many of the DNA mutation-based diseases [9-12]. According to this, data from Campania Cancer Registry show that Naples and Caserta host the highest cases of cancer mortality, compared to the rest of the Campania region [13-15]. Epidemiological studies have been developed in this area, concerning the effect of waste treatment on human health [4, 16-17]. Even if it is a difficult task, to link illegal activities and human health assessing the cancer incidence and mortality, should be to develop studies for the correct management of land.

Humans inhabiting areas with wastewater discharges can suffer genetic damage as well as organisms (animal and plant) living in the same areas can be exposed to similar genotoxic effects [18]. This concept introduces an animal sentinel approach in which a sensitive species can be predictive of human exposure to a threat. A few studies have been developed on animals as bioindicators

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Fig. 1. Tyres, plastic and burnt toxic waste are left strewn on the sides of rivers and agriculture fields, near Nola, southern Italy, one of the villages inside the "Terra dei Fuochi" and a small edible frog with evident morphologic alterations.

in this area [19-20], but none concerns wild species in a predictive sentinel approach.

We show, in a previous work [7], that the edible frog *Pelophylax kl. esculentus* can be considered a good sentinel organism of the occurrence of genotoxic effects of waste dumping. This species has a large ecological spectrum because its life cycle takes place in an aquatic environment (as a larva) as well as in the terrestrial environment (as an adult), bioaccumulation capability, and is numerically abundant as well as easy to survey [21-23]. Here we try to link DNA damage, in animal sentinel edible frog, to the rate of cancer impact on human population from Caserta and Naples provinces.

Materials and Methods

In brief, we performed a comet assay on the nuclei of frog erythrocytes and matched the results with the impact of cancer in the human populations living in the same sites. The comet assay, or single cell gel electrophoresis (SCGE), is a standard method for determining in vivo/in vitro genotoxicity (Fig. 2). It offers a simple way of evaluating the damage caused by a clastogenic agent by measuring breaks in the DNA chain of animal and plant cells, for example in fish [24-25], mollusks [26-27], earthworms [28-30] or arthropods [31-33].

Additionally, the biomonitoring of contaminated sites recurring to the comet assay in amphibians has also been performed, namely, of chemically polluted lakes [21], coal mines [34], waste dumping sites [35], dredged sediments [7], polluted water bodies [36-37], and residues from municipal solid waste incineration [38].

None of the frogs used for this study were killed. Blood sampling was conducted with cardiac puncture, without any effect on animal vitality. The project was approved by the Institutional Animal Care and Use Committee of the University of Naples Federico II (prot. numb. 2013/0032826). Twenty adult frogs were sampled from each of the 4 sites: Nola, Castel Volturno, Giugliano in Campania and Aversa (N = 80) during August/September 2008 (Fig. 3).

We performed a comet assay on edible frog erythrocytes to evaluate DNA damage following methods in Maselli and co-workers [7].

Images of a minimum of 50 randomly selected nuclei were analyzed from each slide. Thus, a minimum

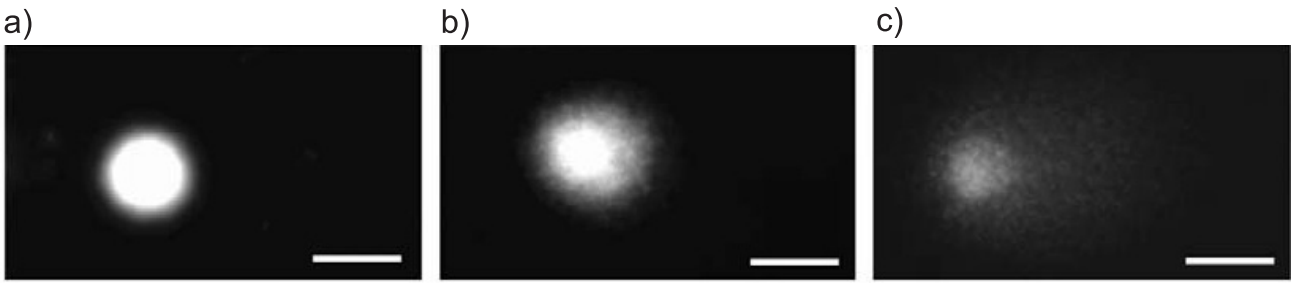

Fig. 2. Examples of nuclei (comet) with Tail Moment (TM, the product of tail length and tail intensity) increasing from A to C. Cells were stained with DAPI and capture at fluorescent microscope. Scale bar = 20 μm.

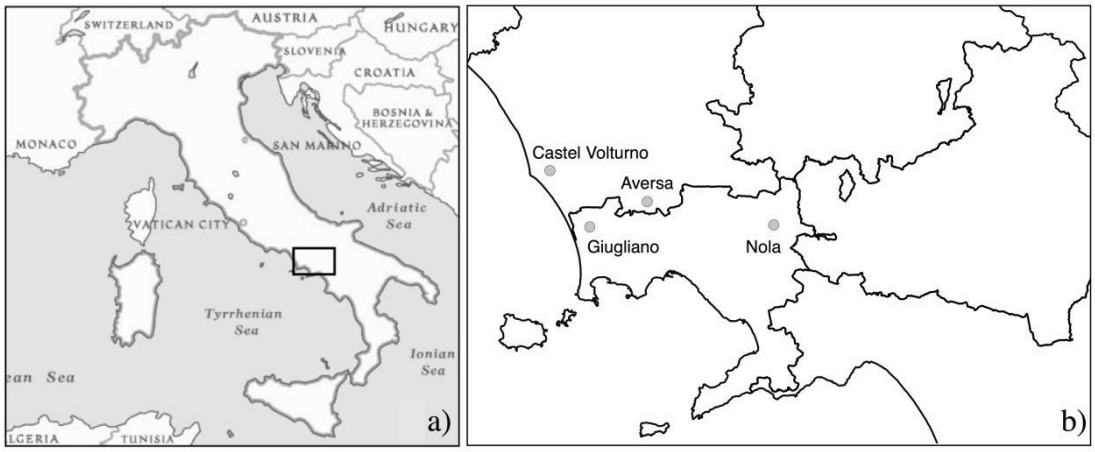

Fig. 3. a) Location of the study area in North Campania (in the box); b) municipalities of Nola, Castel Volturno, Giugliano and Aversa in Campania

of 2,000 blood cells from each group of frogs were examined. The slides were displayed on a fluorescence microscope (Leica DMLB microscope with digital camera Leica DFC340FX, Nussloch, Germany). Comet images were randomly captured from the center of the slide; overlapping figures were avoided. Quantitative assessment of DNA damage in erythrocyte nuclei was performed using CometScore 1.5 Image Analysis (TriTek Corporation, Sumerduck, Virginia, USA) software, which computes the integrated intensity profile for each cell. To compare different populations, *Tail Moment* value was selected because their correlation was widely used in genotoxicity studies on wild animal populations [7].

We consulted the Cancer Registry of Campania Region 2003-2011 (Istituto Superiore di Sanità; 2015, Rapporti ISTISAN 15/27) to assess human population health, considering mortality and malformations at the municipality level. We considered separately males and females affected by cancer. The Standardized Mortality Ratio (SMR) expresses, as either a ratio or percentage, the increase or decrease in mortality of a study cohort with respect to the general population [39].

Results and Discussion

Today, the northern region of Campania, dramatically called Terra dei Fuochi for the numerous illegal fires that are set to the illegal rubbish dump, represents a problem of a very large and incisive dimensions magnitude, on human populations living inside and both agricultural and pastoral products there are spread all over Europe [40-42].

Finding a survey strategy able to work as a sentinel of danger to human health is a central objective and should mobilize many toxicology studies using animal and plant bioindicators.

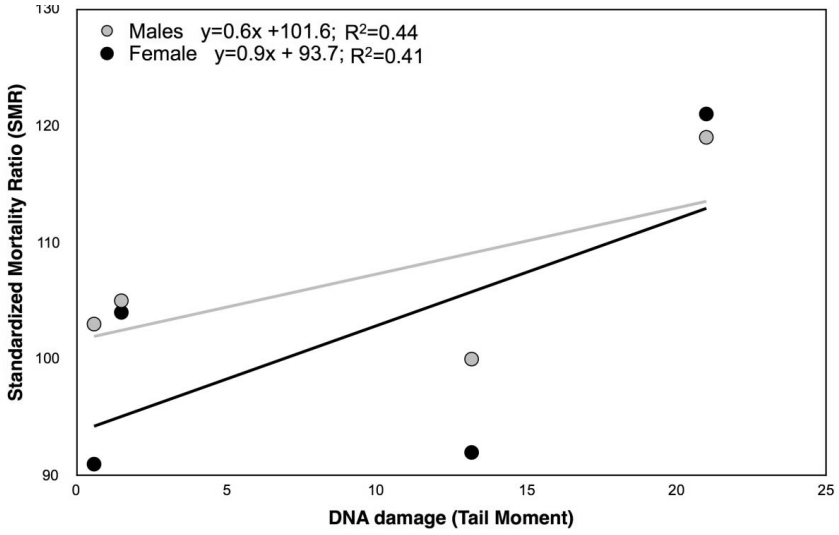

Fig. 4. Regression analysis on the value of DNA damage (Tail Moment on frogs) and Standardized Mortality Ratio (SMR). From left to right: Aversa, Giugliano in Campania, Castel Volturno and Nola; in gray data from males, in black data from females.

Our analysis is based on four municipalities, providing a clear indication about the extension of the epidemiological problem and response of animal sentinel. The study area considered involves 21.415 hectares, more than 20% of a core area in the so-called Terra dei Fuochi. This land is characterized by intense pressure from human activities of illegal waste storage, according to investigations carried out by ARPAC [43-45]. All values of frog DNA damage are higher than the control value (population of frogs stored in the laboratory with a Tail Moment values equals 0.001).

In this area, there are many water points and the proposed animal sentinel (edible frog) can be found very easily. Moreover, its wide and diversified dispersion ensures accurate monitoring throughout the territory [46].

All the erythrocyte populations of the sampled frogs showed comets of nuclear material, and the highest values of Tail Moment were observed at the municipality with the highest death rate from cancer causes (Nola, SMR Males = 119; SMR Females = 121). Instead, frog population characterized by the lower Tail Moment values are Aversa and Giugliano in Campania correspond to the lower value of SMR (Aversa, SMR Males = 103; SMR Females = 91; Giugliano in Campania, SMR Males = 105; SMR Females = 104). An intermediate result was observed in Castel Volturno for both Tail Moment and SMR values (SMR Males = 100; SMR Females = 92).

The regression line linking the effects on animal sentinel as an independent variable and Standardized Mortality Ratio as the dependent variable, shows a rising trend for both males and females data (Spearman's Rank Correlation r_s=0.8 for Males, r_s = 0.4 for Females, $p<0.05$; Fig. 4).

Interestingly, except for Nola, in all examined cases, the incidence of cancer diseases affects more males than females [47].

The results support the hypothesis that assayed parameters, on the sentinel organism, reflect the incidence of health damage on human populations. Our study did not attempt to define which specific contaminants in the polluted river water were responsible for genetic damage. Our more modest objective was to detect overall genotoxic effects from multiple contaminants, because our sites are subjected to multiple sources of municipal pollution. Probably the effects we find are dependent from heavy metals (as shown by some of our recent surveys [48]) and also because heavy metals have posed a serious threat to the aquatic ecosystem [49-51].

Assessing environmental risk requires systems that quantitatively and qualitatively reflect the effects of exposure. Organisms that are in direct contact with contaminated environmental compartments (sediment, water, air) are well suited for inclusion in such systems [52]. Frogs are especially useful for assessing the biological risk of hazardous and toxic wastes in water and sediment since they are in direct contact with the contaminants in their environment and are sensitive to anthropogenic compounds [53-54].

Some studies developed on animal models, useful to reveal negative effects of waste, have been based on domestic species affected by such pathologies as cancer [19]. This approach is not only predictive, because it analyses the outcome of environmental hazards when its effects have already occurred, although on a nearby species and not on humans. Other studies were conducted on hair and blood from healthy pet dogs, assaying the concentration of heavy metals [55]. Likewise, we suggest concentrating investigations on DNA damage in wild animals closer to legal and illegal waste dumping, and they represent a previous step to the disease on human populations [56]. This result is a further confirmation of how our animal (*Pelophylax kl. esculentus*) species can have more immediate responses to pollutants than the human population. For this reason we highlight the advantage to use a predictive sentinel approach.

Our results clearly showed the effectiveness of animal modeling and screening technique as predictive and sensitive. In fact, confirming our hypothesis, the high increase of SMR is significantly related to Tail Moment, even if we consider an interval of Standardized Mortality Ratio relatively higher than the variation observed in healthy and polluted regions. Although our contribution can be considered a pilot study, it should be an encouragement to increase analyses on the territory, of both epidemiological and wildlife in a toxicological view. This will be a necessary effort to address the environmental emergency and plan a suitable waste management strategy in the Campania region.

Conclusions

Land in the provinces of Naples and Caserta is deemed heavily contaminated. According to scientific literature current knowledge available for the Campania region in terms of environmental monitoring is better than that available for other areas that are facing similar problems due to anthropic activities, including illegal waste disposal. Despite this, epidemiological studies on the effects of illegal waste toxicity can be biased by lack of reliable assessment of exposure, uncertain evaluation of the eco-systems, and missing information on relevant confounding factors.

For this reason, additional studies are necessary to better define not only the precise distribution of hot spots of pollution, but also the real impact of such exposure on the health of animal and human populations, in term of cancer incidence. An extensive survey on the DNA damage recorded in the edible frog could represent an initial step to identify priority areas in which to concentrate management actions.

A comprehensive biomonitoring programme should integrate several methods distributed along the biomonitoring chain, allowing us to detect exposure,

threats and impacts, using not only animals, plants and lichen [3], but providing useful biological outputs for decision-makers to establish correct sustainable waste management.

Conflict of Interest

The authors declare no conflict of interest.

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