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Biodetection of potential genotoxic pollutants entering the human food chain through ashes used in livestock diets

Laura Sanchez-Vicente [a], Elisa Herraez [a,c], Oscar Briz [a,c], Rogelio Nogales [b], Eduarda Molina-Alcaide [b], Jose J.G. Marin [a,c,*]

[a] Laboratory of Experimental Hepatology and Drug Targeting (HEVEFARM), IBSAL, University of Salamanca, Salamanca, Spain
[b] Estación Experimental del Zaidín (CSIC), Granada, Spain
[c] Centre for the Study of Liver and Gastrointestinal Diseases (CIBERehd), Carlos III National Institute of Health, Madrid, Spain

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ABSTRACT

Ash derived from energy generation is used as a source of minerals in livestock feeds. The microbial biosensor recApr–Luc2 was built to detect genotoxic hazard in recycled ash. *Escherichia coli* SOS gene (*recA*, *lexA*, *dinI* and *umuC*) expression in response to cisplatin-induced DNA damage led to the selection of the *recA* promoter. The biosensor required functional RecA expression to respond to genotoxic heavy metals (Cr > Cd ≈ Pb), and polluted ash induced a strong recApr–Luc2 response. In human liver and intestinal cells, heavy metals induced acute toxicity (Cr > Cd > Pb) at concentrations sufficient to activate recApr–Luc2. Cytostatic effects, including genotoxicity, were cell- and metal-dependent, apart from Cr. In agreement with the recApr–Luc2 bioassay, Cr had the strongest effect in all cells. In conclusion, recApr–Luc2 could be useful for evaluating the genotoxic risk of pollutants present in ash that might be concentrated in animal products and, thus, entering the human food chain.

1. Introduction

Although the recycling of by-products is a desirable goal of modern society, safety controls must be implemented to prevent the carry-over of noxious pollutants. These include genotoxic compounds that might be present in ash from renewable energy production that uses residual biomass. The source of this hazard is diverse; for instance, heavy metals are present in painted wood, fertilizers and pesticides, which together with soil pollution might contaminate agricultural by-products. These, together with wood from industrial waste or recycled materials, are commonly a source of ash used in the preparation of livestock feeds. This was the case for ash used in the present study, which was produced by combustion of wood and olive cake, a by-product from olive oil extraction. It is, therefore, important to detect genotoxic compounds that, otherwise, might be incorporated in the human food chain. Similar risks, due to the accumulation of heavy metals in raw cows' milk (Arianejad, Alizadeh, Bahrami, & Arefhoseini, 2015), hens' eggs (Bautista, Puschner, & Poppenga, 2014) and in the flesh of fish (El-Sadaawy, El-Said, & Sallam, 2013) have been reported previously.

The main threats to human health from heavy metals, such as chromium (Cr), cadmium (Cd) and lead (Pb), are associated with exposure through contact with products containing these metals or contaminated soil, air, water and food as a result of industrial processes or environmental pollution (Wasi, Tabrez, & Ahmad,

* Corresponding author at: Department of Physiology and Pharmacology, Campus Miguel de Unamuno ED-231, 37007 Salamanca, Spain.
E-mail address: jjgmarin@usal.es (J.J.G. Marin).

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2013; Chowdhury & Chandra, 1987; Glombitza & Reichel, 2014; Jarup, 2003; Nriagu & Pacyna, 1988; Tchounwou, Yedjou, Patlolla, & Sutton, 2012). The use of contaminated by-products in livestock feeds might lead to accumulation in dairy products and meat (Adetunji, Famakin, & Chen, 2014; Rychen, Jurjanz, Fournier, Toussaint, & Feidt, 2014). Exposure to heavy metals is an important problem because they have been classified as carcinogens by regulatory agencies including the WHO International Agency for Research on Cancer (IARC, 1990, 1993, 2006).

More specifically, the toxicity of heavy metals is due to interference with functional groups in biologically important molecules, such as proteins and nucleic acids, but the exact mechanisms of action for each metal differ and, in most cases, are not fully understood (Beyersmann & Hartwig, 2008). For example, DNA damage leading to carcinogenic changes might arise via several mechanisms, including chemical modification of nucleotides, strand breakage, crosslinking, micronucleus formation and aneuploidy induction. Many assays *in vitro* and *in vivo* have been used to evaluate genotoxic hazards by measuring their capacity to induce DNA damage (Kang, Kwon, Lee, & Seo, 2013). These include: (i) Ames test, which assesses mutagenic capacity based on whether the effects can be reversed (Ames, Durston, Yamasaki, & Lee, 1973), (ii) Comet assay, which detects DNA fragmentation, specifically single- and double strand breaks (Ostling & Johanson, 1984), (iii) tests based on the bacterial SOS response including the *umu*-test (Oda, Nakamura, Oki, Kato, & Shinagawa, 1985) and the SOS-Chromotest (Quillardet, Huisman, D'Ari, & Hofnung, 1982), which are two of the most commonly used; and (iv) assays based on the observation of structural DNA alterations, such as the chromosome aberration test, micronucleus test, and sister chromatid exchange assay (Perry & Evans, 1975).

In the present study, an *in vitro* method was developed to evaluate genotoxicity based on SOS response in bacteria exposed to DNA-damaging agents. This method was employed to measure genotoxicity risks resulting from heavy metal pollutants in ash used as a supplement in livestock feeds. The bacterial DNA repair system is activated by expression of SOS genes that form part of a regulon (Michel, 2005). Two proteins, inducer RecA and repressor LexA, have key roles in regulation of the SOS response (Little, 1982). During normal growth, LexA forms a dimer that binds to a specific sequence in the SOS box of the SOS genes promoters, suppressing the expression of these genes (Thliveris, Little, & Mount, 1991). In response to DNA damage, RecA binds to single-stranded DNA (ssDNA) to form a nucleoprotein filament (Zhang, Pigli, & Rice, 2010). Once bound to DNA, the RecA protein is activated and acts as a co-protease, triggering the self-cleaving activity of LexA (Horii et al., 1981). The cleaved repressor then dissociates from DNA, allowing the transcription of SOS genes (Michel, 2005).

Although it would be useful to develop a functional test that could detect any toxic effect related to heavy metals in a single assay (as opposed to individual assays for the different metals), this is not possible. Instead, in the present study, we developed a method able to detect pollutants with genotoxic activity. To validate this approach, we selected four model sources of ash from renewable energy production using residual wood and olive cake biomass.

2. Materials and methods

2.1. Chemicals and ashes

Ampicillin, $CdCl_2$, cisplatin or cis-diamminedichloroplatinum(II), dimethyl sulfoxide (DMSO), MEM, sodium bicarbonate, sodium pyruvate and thiazolyl blue tetrazolium bromide were obtained from Sigma-Aldrich Quimica (Madrid, Spain). The antibiotic–antimycotic solution and fetal calf serum were obtained from Gibco (Thermo Fisher Scientific, Madrid, Spain). Agar, tryptone and yeast extract were obtained from Pronadisa (Laboratorios Conda, Madrid, Spain). $K_2Cr_2O_7$ and $Pb(NO_3)_2$ were obtained from Panreac Quimica (Barcelona, Spain). Ashes were obtained from combustion of wet (OK1: Baena, Cordoba, Spain) and dry (OK2: La Loma, Jaen, Spain) olive cake, and from recycled/residual wood (RW1: Tradema, Linares, Jaen, Spain; RW2: Ence, Huelva, Spain), which were provided by the Estación Experimental del Zaidín (Granada, Spain). Ashes (0.5 g/ml) and heavy metal salts (up to 3 mM depending on the metal and the experimental setting) were prepared freshly in ultrapure H_2O and filtered (0.22 μm-pore size filter) immediately prior to use. There was no need to adjust the pH after the addition of heavy metal salts or aqueous extracts of ashes to bacterial or mammalian cell cultures.

2.2. Chemical composition of ashes

The components of ashes, except for Cr, were analyzed by inductively coupled plasma mass spectrometry after samples were digested in concentrated aqua regia, as previously reported (McGrath & Cunliffe, 1985). In brief, 0.5 g of ash samples were treated with $HCl:HNO_3$ (4:1 v/v) at room temperature for 24 h and heated at 150 °C until they were dry. Cr content was determined by flameless atomic absorption spectrophotometry (Z-8100 Polarized Zeeman; Hitachi, Tokyo, Japan), after the samples were treated with 37% HCl at room temperature for 24 h and then heated at 150 °C until they were dry. Then, to precipitate Fe, and prevent interference with the Cr measurements, the samples were dissolved in 20 mM HCl at 60 °C for 4 h and mixed with NH_4Cl (20 g/l). All samples were filtered (Whatman no. 42 filters) and diluted to 100 ml with deionized, distilled water prior to analysis. Blank solutions were also prepared without addition of ash.

2.3. Bacteria strains, growth conditions and toxicity tests

DH5α and BL21(DE3) strains of *Escherichia coli* were donated by the Department of Microbiology and Genetics, University of Salamanca (Spain). Recombinant *E. coli* cells were selected on Luria Agar (LA) plates containing 100 μg/ml of ampicillin and used to inoculate Luria Broth (LB) media supplemented with the same antibiotic. Non-genetically modified *E. coli* cells were grown without this antibiotic. Bacterial cultures in LB medium were incubated with shaking (220 rpm) at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.3 (Spectrophotometer Hitachi U-2000 with 1 cm path-length cuvettes). Bacterial cultures were then considered to be in the early exponential phase of growth and were placed in vented test tubes for the different assays.

To perform the toxicity assays, 5 ml of *E. coli* BL21(DE3) culture at OD_{600} = 0.3 were placed in test tubes containing 10–50 μl of different heavy-metal salt solutions in ultrapure H_2O at the desired final concentration (0–2 mM). Bacterial growth was analyzed using OD_{600}.

2.4. Cloning of the expression vector pcDNA6.2–recApr–Luc2

A 110-bp sequence located in the 5′-flanking region of the *recA* gene, which was previously identified as the proximal *recA* promoter (recApr) (Horii, Ogawa, & Ogawa, 1980), was selected for cloning using genomic DNA from *E. coli* BL21(DE3). The recApr sequence was amplified by PCR using high-fidelity AccuPrime *Pfx* DNA polymerase (Invitrogen; Thermo Fisher Scientific, Madrid, Spain) and specific oligonucleotide primers (Supplementary Table 1), to which *att*B sites were added to obtain cDNA adapted for Multisite Gateway® cloning (Invitrogen). The pGL4.10[Luc2] plasmid (Promega, Madrid, Spain) was used as a template with