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Genotoxicity evaluation of carvacrol in rats using a combined micronucleus and comet assay

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ABSTRACT

Genotoxic data of substances which could be incorporated into food packaging are required by the European Food Safety Authority. Due to its antioxidant and antibacterial properties carvacrol is one of these compounds. This work aims to study for the first time the *in vivo* genotoxic effects produced in rats orally exposed to 81, 256 or 810 mg cavacrol/kg body weight (bw) at 0, 24 and 45 h. A combination of the micronucleus assay (OECD 474) in bone marrow and the standard (OECD 489) and enzyme-modified comet assay was used to determine the genotoxicity on cells isolated from stomach and liver of exposed animals. In addition, a histopathological study was performed on the assayed tissues, and also in the lungs due to the volatility of carvacrol. Direct analytical pyrolysis was used to search for carvacrol in viscera and to ensure that the compound reaches stomach and liver cells. Results from MN-comet assay revealed that carvacrol (81–810 mg/kg bw) did not induce *in vivo* genotoxicity or oxidative DNA damage in any of the tissues investigated. Moreover, no histopathological changes were observed. Altogether, these results suggest lack of genotoxicity of carvacrol and therefore its good profile for its potential application as food preservative.

1. Introduction

Food products are often sold in areas of the world far away from their production sites, hence, several reactions, such as microbial spoilage or oxidation processes, may alter the safety and organoleptic properties of perishable products (Llana-Ruiz-Cabello et al., 2015a). The use of plant extracts and essential oils (EOs) to extend the shelf life of foods are increasing in the food industry, such as in the case of active packaging (Echegoyen and Nerín, 2015; Llana-Ruiz-Cabello et al., 2015b).

Carvacrol, the main compound of Oregano EO is registered as a flavoring in Europe; however, its use for other applications, such as in active food packaging, may require higher concentrations that will increase the concern regarding exposure to these compounds (Llana-Ruiz-Cabello et al., 2014a). In this sense, the European Food Safety Authority (EFSA) panel on Food Contact Materials, Enzymes, Flavorings and Processing Aids (CEF) has recently published a Scientific Opinion on the developments of risk assessment in which the amount of toxicity data needed should be related to the expected human exposure level (EFSA, 2016). This draft remarks that genotoxicity must be evaluated, even if low exposure is expected for substances migrating from food contact materials (FCMs) and recommends performing two tests in the first step-approach: a bacterial reverse mutation (Ames test) and an *in vitro* micronucleus (MN) test (EFSA, 2011, 2016).

The potential mutagenic and carcinogenic activities of carvacrol *in vitro* have been studied previously, and contradictory results have been published (Llana-Ruiz-Cabello et al., 2015a). For the Ames test, carvacrol at different concentrations exhibited a mutagenic response in *Salmonella thyphimurium* strains in absence and presence of S9 (Ipek et al., 2005; Llana-Ruiz-Cabello et al., 2014b), whereas negative results were found by Stammati et al. (1999). On the other hand, for MN test, contradictory results have been also reported: a significant increase of MN was observed in L5178Y/*TK*$^{\pm}$ cells in the absence of S9 (Maisanaba et al., 2015), while negative

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responses have been reported in different cell models, such as HepG2 cells or human lymphocytes (Melusová et al., 2014; Türkez and Aydin, 2013). Moreover, no genotoxic response was found in the mouse lymphoma assay (MLA) after 4 h and 24 h of exposure to carvacrol (Maisanaba et al., 2015). The comet assay has also been recommended by EFSA to evaluate the genotoxicity of substances intended to be used in food packaging (EFSA, 2011, 2016). In this regard, negative results have been reported when different cell lines were exposed to carvacrol (Horváthová et al., 2006; Ündeger et al., 2009; Llana-Ruiz-Cabello et al., 2014b). However, results from the enzyme-modified comet assay, using endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG), developed to detect oxidative pirimidine or purine damage, respectively, showed that carvacrol exerted oxidation in purine DNA bases of Caco-2 cells (Llana-Ruiz-Cabello et al., 2014b). All these contradictory results increase the concerns about carvacrol genotoxicity and make it necessary to elucidate its genotoxic potential.

Kirkland et al. (2011) concluded that there is no convincing evidence that any genotoxic rodent carcinogen or *in vivo* genotoxin would remain undetected in an *in vitro* test battery consisting of Ames and MN assays; however, in the case of inconclusive or contradictory results, as well as positive results from these *in vitro* tests, it may be appropriate to conduct further *in vivo* tests (EFSA, 2011). The CEF panel recommends three different assays to evaluate the genotoxicity of substances: the *in vivo* MN test, the *in vivo* comet assay or the transgenic rodent gene mutation assay (EFSA, 2016). In order to comply with the 3Rs principles (Replace, Reduce and Refine), Bowen et al. (2011) proposed a combined multi-end point *in vivo* assay and evaluated the combination of bone marrow MN test with the comet assay. Previous studies have employed MN as well as comet assay simultaneously to detect the genotoxic effects of various chemicals (Kitamoto et al., 2015), nanoparticles (Downs et al., 2012), drugs (Mughal et al., 2010) or toxins (Corcuera et al., 2015). This combination can improve the sensitivity as well as the reliability of toxicity studies (Mughal et al., 2010).

Considering all this background, the present work aims to perform for the first time an *in vivo* genotoxic evaluation for carvacrol using the combined MN-comet assay in rats. The target tissues selected were bone marrow for MN (OECD 474), and stomach and liver cells from orally exposed rats for the comet assay (OECD 489). Moreover, the comet assay has been combined with bacterial repair enzymes Endo III and FPG, which allow detection of DNA oxidative damage. A histopathological examination was performed on stomach, liver and lungs, in order to complete the genotoxic evaluation and provide useful information to perform the accurate risk assessment required by EFSA for additives in FCMs. Finally, to ensure that carvacrol effectively reached the target tissues, a detailed analytical pyrolysis study was conducted.

2. Materials and methods

2.1. Supplies and chemicals

Carvacrol (98%) was purchased from Sigma–Aldrich (Madrid, Spain). All chemicals, including Endo III and FPG, were purchased from Sigma-Aldrich (Madrid, Spain), VWR Eurolab (Madrid, Spain), C-viral S.L. (Seville, Spain) and BioWhittaker (Madrid, Spain). Endo III (EC3.1.21.5) was purchased from C-Viral S.L. (Sevilla, Spain), and FPG (EC3.2.2.23) from Sigma–Aldrich (Madrid, Spain).

2.2. Animal housing and feeding conditions

The Ethics Committee on Animal Experimentation of the University of Sevilla approved the *in vivo* experiments. Moreover, in compliance with the Directive 2010/63/EU for the protection of animals used for scientific purposes all animals received humane care.

Young adult male and female Wistar rats, strain RjHan:WI (type outbred rats), between 8 and 10 week-old were purchased from the University of Sevilla Center for Animal Production and Experimentation (Espartinas, Spain). Animals were weighed after arrival and housed in polycarbonate cages with stainless steel covers. Then, the animals were acclimatized to the environmental conditions for one week before the experiments, 12 h dark/light cycle, temperature 23 ± 1 °C, relative humidity 55 ± 10%, standard diet (Harlan, 2014; Harlan Laboratories, Barcelona, Spain) and water *ad libitum*.

2.3. Experimental design and treatment

The MN and comet assay OECD protocols (OECD 474 and 489, respectively) recommend a preliminary range-finding study to identify the maximum tolerated dose (MTD), which was defined by Derelanko (2000) as the highest dose to produce toxic effects without causing death and to decrease body weight (bw) by no more than 10% relative to controls. For this purpose, the Acute oral toxicity OECD 425 guideline for testing of chemicals (OECD, 2008) was followed, taking into account the historical report of the LD_{50} in rats described by Jenner et al. (1964). Moreover, considering the OECD 474 (2014a) and OECD 489 (2014b) guidelines, the doses used in the combined MN-comet assay were the MTD and two lower doses (Bowen et al., 2011) appropriately spaced by less than $\sqrt{10}$ (OECD 489, 2014b).

In this study, 23 male and 23 female rats were randomly divided into five groups, two control and three treatment groups: the negative control group (C−) (5 males and 5 females rats) was treated with corn oil (vehicle); the positive control group (C+) (3 males and 3 females rats) was exposed to 200 mg/kg bw ethylmethanesulfonate (EMS) and three exposed groups (5 males and 5 females rats per group) received 81, 256 or 810 mg carvacrol/kg bw, according to the MTD results obtained for carvacrol. The number of animals included in each group was selected according to the OECD 474 (2014a) and OECD 489 (2014b) guidelines: a minimum of 5 analyzable animals of each sex for group (OECD 474) and a minimum of 3 animals of each sex treated with a positive control (OECD 489). All doses were prepared in corn oil at a final volume of 1 mL. According to Bowen et al. (2011), animals for the combined MN and comet endpoints were dosed at 0, 24 and 45 h by gavage using an enteral feeding tube (Vygon, Ecouen, France). Then, animals were sacrificed 3 h after the final dose administration.

During the treatment period, clinical signs, body weight, and food and water consumption were recorded daily.

2.4. Sample collection

Samples of bone marrow, stomach, liver and lungs were collected according to Mellado-García et al. (2016).

2.5. MN assay

The principles of the OECD guideline 474 (OECD, 2014a) and the recommendations of Corcuera et al. (2015) were followed to perform the mammalian erythrocyte MN test. Briefly, the bone marrow cells were smeared on a glass slide, fixed in absolute methanol air dried and stained with Giemsa.

The polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes (NCE) + PCE) ratio and the PCE among NCE ratio were calculated by counting 500 erythrocytes per animal. The incidence of micronucleated immature erythrocytes