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Is oxidative stress involved in the sex-dependent response to ochratoxin A renal toxicity?

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin considered the most powerful renal carcinogen in rodents and classified as a possible human carcinogen. Though its mechanism of action is still unknown, indirect DNA reactivity mediated by oxidative stress has been hypothesized to play an important role. Moreover, large sex-differences have been observed in carcinogenicity studies, male rats being more sensitive than females. Male and female F344 rats were administered (p.o.) with bicarbonate or 0.5 mg OTA/kg b.w. for 7 days; or with bicarbonate, 0.21 or 0.5 mg OTA/kg b.w. for 21 days. Total glutathione (tGSH) and oxidized glutathione (GSSG) levels, glutathione S-transferase (GST) and superoxide dismutase (SOD) activities were analysed in kidneys. The standard alkaline comet assay was used in combination with Formamidopyrimidine-DNA glycosylase (Fpg) to detect oxidized DNA bases in kidney. No biologically relevant sex-differences were observed in all the oxidative-stress related parameters analysed. Indeed, no relevant oxidative-stress related response was observed between treated animals and controls. In accordance with the similar OTA levels and histopathological changes between both sexes observed previously in the same animals, and with other oxidative-stress related parameters measured by others, results support that there are no differences between sexes in the oxidative stress response to OTA.

1. Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite produced by several species of *Aspergillus* and *Penicillium*. Since this mycotoxin is present in a wide variety of human foodstuff and animal feed (EFSA, 2006; WHO, 2008), human exposure can occur both through consumption of contaminated food commodities or products from animals fed with contaminated feed. The most contaminated commodities are cereals and cereal products, pulses, coffee, beer, grape juice, dry vine fruits and wine as well as cacao products, nuts and spices (EFSA, 2006). In general, cereals, wine and juices have been considered as main contributors to OTA human exposure (EFSA, 2006) with worldwide occurrence data ranging from 0.01 to 1164 μg/kg for cereals (Lee and Ryu, 2017) and from 0.01 to 15.61 μg/L for wine and musts (Paterson et al., 2018). Thereby, human are continuously exposed to this mycotoxin (EFSA, 2006; Fink-Gremmels, 2005).

OTA has been proposed as a possible etiological agent of the Balkan Endemic Nephropathy (BEN) and it has also been associated with an increased incidence of urinary tract tumours in humans (Petkova-Bocharova et al., 1988; Pfohl-Leszkowicz et al., 2002; Plestina et al., 1990). However, there is still a lack of epidemiological evidence as other factors or co-factors might be involved in the aetiology of the diseases (Reddy and Bhoola, 2010).

OTA nephrotoxicity has been demonstrated in every laboratory species used, and it is also considered the most powerful renal carcinogen in rats (Lock and Hard, 2004). The available data obtained from different carcinogenicity studies in rodents show large sex-differences in susceptibility towards OTA-induced tumours: dosing Fischer (F344) rats with OTA for 2 years produced a ten-fold higher incidence of renal tumours in male rats when compared to female rats (Boorman et al., 1992; NTP, 1989), and this sex-biased response has also been observed in other studies using Dark-Agouti and Lewis rats (Castegnaro et al., 1998; Son et al., 2003). Other studies have deepened into the effect of sex on OTA toxicity *in vivo* from the toxicokinetic point of view (Vettorazzi et al., 2011, 2010; 2009; Zepnik et al., 2003).

Unfortunately, the exact mechanism of action by which OTA induces tumours is still unknown and several hypotheses have been proposed to contribute, totally or partially, to it (Kőszegi and Poór,

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2016; WHO, 2008). Among them, indirect DNA reactivity mediated by oxidative stress has been supported by several authors. Several studies have demonstrated that OTA inhibits the nuclear factor, erythroid 2-like 2 (Nrf2) oxidative stress response pathway, which would affect glutathione synthesis and recycling, oxidoreductases activity, and phase II metabolism inducibility, thus rendering the tissue more vulnerable to oxidative stress (Limonciel and Jennings, 2014). A downregulation of genes under transcriptional control of Nrf2 was also observed in the kidney of rats fed OTA up to 12 months (Marin-Kuan et al., 2006). The effects observed at mRNA level were later confirmed as biologically relevant as OTA also decreased the protein expression of several markers of the Nrf2-regulated gene battery in kidney *in vivo*, which resulted in oxidative damage to DNA, and *in vitro* in the NRK renal cell line and in primary hepatocyte cultures (Cavin et al., 2007). Arbillaga et al. (2007a), found that several genes implicated in the oxidative stress response were up-regulated in the human renal cell line (HK-2) following OTA exposure during 6 and 24 h, but identified down-regulation as the predominant effect in a repeated-dose study carried out in F344 rats (Arbillaga et al., 2008). A differential expression of genes involved in the response to oxidative stress was also seen *in vivo* and *in vitro* by Lühe et al. (2003). In the same line, using both the Eker rat model of increased susceptibility to renal tumour formation and the corresponding wild-type strain, Stemmer et al. (2007) found that OTA treatment down-regulated the expression of several phase I and phase II enzymes in both strains and deregulated the expression of several genes involved in the response to DNA damage (including oxidative stress) in Eker rats.

On the other hand, other effects related to oxidative stress production have been observed after OTA exposure. Omar et al. (1990) came to the conclusion that OTA stimulates lipid peroxidation by complexing Fe^{3+}, which may facilitate its reduction, although the specific specie responsible for initiating lipid peroxidation was not identified. It has been established that OTA leads to lipid peroxidation both *in vitro* (Klarić et al., 2007) and *in vivo* (Abdel-Wahhab et al., 2005; Ferrante et al., 2006; Ozçelik et al., 2004), to a decrease of glutathione (GSH) levels *in vitro* (Klarić et al., 2007; Schaaf et al., 2002) and *in vivo* (Meki and Hussein, 2001), and to an increase of the kidney's protein carbonyl levels after 21 days of OTA-treatment (Domijan et al., 2005). It has also been observed that OTA causes a dose-dependent increase of reactive oxygen species (ROS) (Baldi et al., 2004) as well as oxidative damage to DNA *in vitro* (Arbillaga et al., 2007b; Schaaf et al., 2002), and it is considered to significantly increase oxidative damage to DNA *in vivo* (Kamp et al., 2005; Mally et al., 2005). OTA exposure to HepG2 cells decreased the intracellular zinc concentration (considered a potential antioxidant), induced ROS production, 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation and decreased superoxide dismutase (SOD) activity (Zheng et al., 2013). In fact, treatment of OTA-pretreated Wistar rats with SOD and catalase prevented enzymuria, proteinuria, creatinemia and increased urinary excretion of OTA (Baudrimont et al., 1994).

Thus, it appears clear that oxidative stress might be implicated in OTA toxic response. However, the influence of sex in OTA-mediated kidney oxidative stress response has not been specifically studied. Indeed, this might be an important aspect to evaluate as sex differences regarding oxidative stress response have been observed in other scenarios such as after giving a high cholesterol diet to Wistar albino rats (Al-Rejaie et al., 2012), acetaminophen to CD-1 mouse (Hoivik et al., 1995) or cisplatin to Swiss albino mice (Naseem et al., 2015). Besides, Lash et al. (1998) found that the rates of *S*-(1,2,2-trichlorovinyl)glutathione (TCVG) formation in isolated kidney cells from male and female F344 rats were similar, but kidney cytosol and microsomes from males exhibited higher amounts of TCVG formation than the corresponding fractions from females, for both F344 rats and B6C3F1 mice. Regarding *in vitro* studies, vascular smooth muscle cells (VSMC) isolated from male rat aorta were found to be much more susceptible to radiation-induced stress (measured by ROS production) than the female ones (Malorni et al., 2008).

Several studies have measured kidney oxidative stress status using different rat strains and with different dosages after OTA administration. However, all of them have used male rats. To the authors knowledge, only one study (Hibi et al., 2011) analysed oxidative damage to DNA (8-hydroxy-2′-deoxyguanosine levels) in both sexes after 4 and 13 weeks of OTA administration in diet (approximately 0.4 mg/kg b.w.). No differences between sexes were found in oxidative damage to DNA or at histopathological level. This is in agreement with our recently published study carried out in male and female F344 rats (Pastor et al., 2018), where slightly higher signs of toxicity were found in kidney histopathology in males after 7 days of 0.5 mg OTA/kg b.w. daily administration but no differences were found after 21 days of treatment.

Due to the different tumour incidence found between the sexes (NTP, 1989) after OTA administration, the role of oxidative stress in cancer induction (Klaunig and Kamendulis, 2004) and the above-mentioned evidences suggesting a role of oxidative stress in OTA mechanism of action, the objective of the present study was to try to understand the molecular mechanisms related with oxidative stress that may underlay and precede the different response to OTA toxic insult that has been described in male and female rats. To this aim, different oxidative stress-related parameters such as glutathione-S-transferase (GST) activity, total (tGSH) and oxidised (GSSG) glutathione levels and superoxide dismutase activity (SOD) in kidney of both male and female F344 rats were evaluated. Moreover, in order to relate these endpoints with oxidative damage to DNA, the comet assay in combination with Formamidopyrimidine-DNA glycosylase (Fpg) was carried out in kidney tissue.

2. Materials and methods

2.1. Chemicals and reagents

Commercial available kits were used for the determination of total (cytosolic and microsomal) GST activity (Glutathione S-Transferase Assay Kit®, Item No. 703302, Cayman Chemical), tGSH and GSSG glutathione (Glutathione Assay Kit®, Item No. 703002, Cayman Chemical), and SOD activity (SOD determination kit®, Item No. 19160, Sigma-Aldrich). For protein quantification, Protein Assay Dye Reagent Concentrate (Item No. 500-0006, Bio-Rad) and Standard Bovine Serum Albumin (BSA, Item No. A3803, Sigma) were used. The SOD standard from bovine erythrocytes (Item No. S2515), 2-vinylpyridine (Item No. 132292) and EDTA were purchased from Sigma-Aldrich. Dulbecco's Phosphate Buffered Saline (PBS) without Ca^{+2} and Mg^{+2} 10x from Lonza (Item No.BE17-515Q) was used to prepare PBS 1x washing solutions for tissues or comet assay slides. Saline for washing tissues for SOD activity determination was purchased from Grifols (Item No. 825083). The salts KH_2PO_4 and $K_2HPO_4{\cdot}3\ H_2O$ used to prepare buffer A and B for tissue preparations were obtained from Panreac AppliChem and Merck KGaA, respectively. For sucrose buffer (pH 7.4), 0.25 M sucrose, 10 mM Tris and 1 mM EDTA were purchased from Sigma.

For the comet assay, low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, BSA, methyl methanesulfonate (MMS) and 4′,6-diamidino-2-phenylindole (DAPI) (Item No. D9542, Sigma) were purchased from Sigma-Aldrich. NaCl, NaOH, Na_2HPO_4 and KCl were purchased from PanReac AppliChem and Dulbecco's Phosphate Buffered Saline (DPBS) 1x (Ref. 14190-094) for mixing cell suspensions with agarose was purchased from Gibco. Fpg was a gift from Prof. Andrew Collins (University of Oslo). Ro 19-8022 (Ro), which specifically produces oxidised purines (mainly 8-oxoguanine) in the presence of visible light, was kindly given by Hoffmann-La Roche.