Protective effect of glutathione *S*-transferase enzyme activity against aflatoxin B_1 in poultry species: relationship between glutathione *S*-transferase enzyme kinetic parameters, and resistance to aflatoxin B_1

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ABSTRACT Comparative studies designed to investigate the role of glutathione *S*-transferase (**GST**) activity on the enzyme catalyzed trapping of aflatoxin B_1-8,9-epoxide (**AFBO**) with glutathione, and the relationship with aflatoxin B_1 (**AFB_1**) resistance have not been conducted in poultry. Hepatic cytosolic fractions of chickens, quail, turkeys and ducks were used to measure in vitro the enzymatic parameters maximal velocity (V_{max}), Michaelis-Menten constant (K_m) and intrinsic clearance (**CL_{int}**) for GST activity. AFB_1 used ranged from 2.0 to 157.5 μM and the AFB_1-GSH produced was identified and quantitated by HPLC. Significant differences were found in GST V_{max} values, being the highest in chickens, followed by quail, ducks and turkeys. The K_m values were also significantly different, with chickens < ducks < turkeys < quail. Chickens had the higher CL_{int} value in contrast to ducks. Differences by sex showed that duck females had a higher CL_{int} value than the turkey and quail, whereas duck males had a CL_{int} close to that of turkey. The ratio "AFBO production /AFB_1-GSH production" follows the order duck>turkey>quail>chicken, in agreement with the known poultry sensitivity. The extremely high "AFB_1 epoxidation activity/ GST activity" ratio observed in ducks might be the explanation for the development of hepatocellular carcinoma in this species.

Key words: aflatoxin B_1, aflatoxin B_1-8,9-epoxide, glutathione *S*-transferase enzyme activity, aflatoxin B_1-glutathione, aflatoxin B_1 resistance

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INTRODUCTION

Aflatoxin B_1 (**AFB_1**) is a secondary metabolite produced by some species of *Aspergillus* fungi, including *A. flavus, A. parasiticus, A. nomius* and *A. pseudonomius* (Varga et al., 2009, 2011; Diaz, 2020). Hepatic biotransformation of AFB_1 through mammalian and avian cytochrome P450 enzymes produces aflatoxin B_1-8,9-epoxide (**AFBO**), with 2 possible stereoisomers: aflatoxin B_1-8,9-*exo*-epoxide and aflatoxin B_1-8,9-*endo*-epoxide; only the *exo*-epoxide is capable of adducting the guanine-7 position of DNA, leading to carcinogenesis (Eaton and Gallagher, 1994; Guengerich et al., 1998). A major detoxication pathway that prevents DNA adduct formation is the nucleophilic trapping of AFBO with glutathione (**GSH**) to form aflatoxin 8,9-dihydro-8-(*S*-glutathionyl)-9-hydroxyaflatoxin B_1 (**AFB_1-GSH;** Deng et al., 2018). This reaction is catalyzed by cytosolic glutathione *S*-transferase (**GST**, EC 2.5.1.18; Figure 1). GSTs are a superfamily of enzymes that speed up the nucleophilic trapping of the thiolate group of GSH with electrophilic groups present in a wide variety of substrates (Oakley, 2011). Although GST enzymes can be found in different subcellular compartments like cytosol, mitochondria, endoplasmic reticulum, nucleus and plasma membrane (Raza, 2011), only the cytosolic GSTs are responsible for electrophile detoxication (Hayes et al., 2005; Wu and Dong, 2012). These GSTs are comprised of either two subunits of the same type (homodimers) or 2 subunits of different type (heterodimers), each subunit with a molecular mass close to 25 kDa (Mannervik and Jensson, 1982; Mannervik and Danielson, 1988).

Studies have shown that the differences in sensitivity to AFB_1-induced carcinogenesis are related to specific GST activity against the AFBO (Neal et al., 1987; Quinn et al., 1990; Hayes et al., 1991). In the mouse, the very high GST-AFBO conjugating activity is associated to a GST Alpha-class enzyme (Quinn et al., 1990; Ramsdell and Eaton, 1990), more specifically to an

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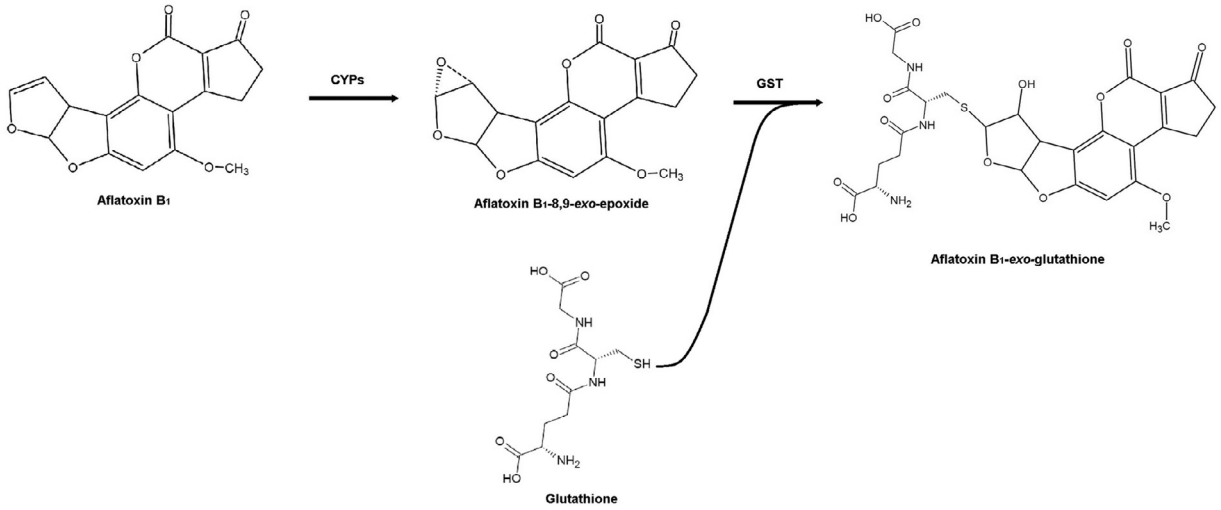

Figure 1. Scheme of the bioactivation of aflatoxin B_1 (AFB_1) into aflatoxin B_1 8,9-epoxide (AFBO) through cytochrome P450 enzymes and the enzyme catalyzed nucleophilic trapping of AFBO by glutathione (GSH) through glutathione *S*-transferase (GST) enzyme.

Alpha-class 3-3 GST (Hayes et al., 1992). In experimental rodents such as mice, hamsters, and rats, little differences in their in vitro AFBO-DNA adduct production are found. However, the ability to conjugate AFBO with GSH is higher in the mouse, intermediate in the hamster and lower in the rat (Esaki and Kumagai, 2002). This ability reflects the expected sensitivy to AFB_1-induced carcinogenesis since the rat is highly prone to develop hepatic tumors in vivo, the hamster is much less prone, and the mouse is resistant to AFB_1-induced hepatic carcinogenesis (Newberne and Butler, 1969). Indeed, after subchronic exposure to AFB_1 (6 wk), 50 out of 50 rats given the toxin by oral gavage 5 times/wk developed hepatocellular carcinomas by 46 wk of age, while only 2 of 49 AFB_1-treated hamsters developed hepatocellular carcinomas (Moore et al., 1982). In similar studies, mice have been found to be completely refractory to the carcinogenic properties of AFB_1 (Croy and Wogan, 1981). These differences between rat and mouse AFB_1 sensitivity are apparently related to conjugation of AFBO stereoisomers. Rats conjugate the aflatoxin B_1-8,9-*endo*-epoxide more efficiently than mouse, and mouse seems to exclusively conjugate the active isomer aflatoxin B_1-8,9-*exo*-epoxide (Raney et al., 1992).

The information about GST-mediated AFBO-GSH trapping in poultry species is scarce, and no comparative studies among poultry have been conducted. Studies carried out with turkeys, which have an intermediate sensitivity to AFB_1 between chickens and ducks (Monson et al., 2015; Diaz, 2020), have shown that their GST activity is slightly higher than that of the rat, but much lower than the mouse GST activity (Klein et al., 2000). Further, domestic turkeys seem to have a lower AFBO-GST conjugation activity than wild turkeys (Kim et al., 2013). GST enzymes identified in quail (Dai et al., 1996), a species almost as resistant to AFB_1 as the chicken, have a lower conjugating activity compared to those of mice or rats (O'Brien et al., 1983), whereas the most resistant poultry species (the chicken) seems to have a GST activity only slightly higher than that of the rat (Maurice et al., 1991).

Unpublished studies conducted in our laboratory failed to detect any UDP-glucuronosyltransferase or sulfotransferase enzyme conjugation activities for biotransformation products such as aflatoxin B_1-8,9-dihydrodiol (**AFB_1-dhd**), the metabolite responsible for the acute toxicity of AFB_1 (Diaz and Murcia, 2019) or aflatoxicol, a metabolite related with resistance in poultry species like chicken (Murcia and Diaz, 2020). Thus, we focused our study in GST activity due to the lack of information about AFBO-GST enzyme kinetics in poultry species and its relationship with AFB_1 resistance. The present study was carried out in order to evaluate and compare the enzyme kinetic parameters of AFB_1-GSH production and to investigate a possible relationship between AFBO enzymatic trapping by GSH and the known in vivo sensitivity to AFB_1 in the major poultry species: ducks > turkeys > quail > chickens.

MATERIALS AND METHODS

Reagents

Glucose 6-phosphate sodium salt, glucose 6-phosphate dehydrogenase, nicotinamide dinucleotide phosphate (**$NADP^+$**), ethylenediaminetetraacetic acid (**EDTA**), bicinchoninic acid solution (sodium carbonate, sodium tartrate, sodium bicarbonate and sodium hydroxide 0.1 N pH 11.25), copper sulphate pentahydrate, formic acid, sucrose, glycerol, bovine serum albumin, L- glutathione reduced, dimethyl sulfoxide (**DMSO**) and ethanol (spectrophotometric grade) were from Sigma-Aldrich (St. Louis, MO). Aflatoxin B_1 was from Fermentek Ltd. (Jerusalem, Israel). Sodium chloride and magnesium chloride pentahydrate were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Sodium phosphate monobasic monohydrate and sodium phosphate dibasic anhydrous were from Merck (Darmstadt,

Germany). Methanol, acetonitrile and water were all HPLC grade.

Microsomal and Cytosolic Fraction Processing

Liver fractions were obtained from 12 healthy birds (6 males and 6 females) from each of the following species and age: 7-wk-old Ross and Rhode Island Red chickens (*Gallus gallus ssp. domesticus*), 8-wk old Nicholas turkeys (*Meleagris gallopavo*), 8-wk-old Japanese quails (*Coturnix coturnix japonica*), and 9-wk-old meat-type Pekin ducks (*Anas platyrhynchos ssp. domesticus*). Birds were raised with no additives or medication added to the diets provided. The diets were formulated with the same ingredients (corn, extruded full-fat soybeans, soybean meal, vegetable oil, calcium phosphate, calcium carbonate, sodium chloride, lysine, methionine, tryptophan, choline, vitamin and mineral premix) formulated to reach or exceed the nutrient requirements of each poultry species studied (Supplementary Table 1). The feed was tested for residual AFB_1 and no detectable residues were found. For in vitro production of AFB_1-GSH standard, a mouse liver was used (*Mus musculus*, ICR outbred stock). The birds were sacrificed by cervical dislocation and the mouse by CO_2 overdose, and their livers extracted immediately, washed with cold PBS buffer (50 mM phosphates, pH 7.4, NaCl 150 mM), cut into small pieces and stored at −70°C until processing. The experiment was conducted following the welfare guidelines of the Poultry Research Facility and was approved by the Bioethics Committee, Faculty of Veterinary Medicine and Zootechnics, National University of Colombia, Bogotá D.C., Colombia (approval document CB-FMVZ-UN-033-18). Frozen liver samples were allowed to thaw, and 2.5 g were minced and homogenized for 1 min with a tissue homogenizer (Cat X120, Cat Scientific Inc., Paso Robles, CA) with 10 mL of extraction buffer (phosphates 50 mM pH 7.4, EDTA 1 mM, sucrose 250 mM). The homogenates were then centrifuged at 12,000 × *g* for 30 min at 4°C (IEC CL31R Multispeed Centrifuge, Thermo Scientific, Waltham, MA). After this first centrifugation, the supernatants (approximately 10 mL) were transferred into ultracentrifuge tubes kept at 4°C and centrifuged for 90 min at 100,000 × *g* (Sorval WX Ultra 100 Centrifuge, Thermo Scientific, Waltham, MA). The pellets from the Rhode Island Red chickens (corresponding to the microsomal fraction) were resuspended in 3 mL of storage buffer (phosphates 50 mM pH 7.4, EDTA 1 mM, sucrose 250 mM, 20% glycerol) and fractioned in microcentrifuge tubes. The Rhode Island Red chicken microsomal fraction was used because its AFBO production is higher than that if any other rodent or poultry fractions tested. The supernatants from all species (corresponding to the cytosolic fraction) were also fractioned in microcentrifuge tubes. All fractions were stored at −70°C and previously an aliquot of each sample was taken to determine its protein content by using the bicinchoninic acid protein quantification method according to Redinbaugh and Turley (Redinbaugh and Turley, 1986). No further enzyme purification was done, and the incubations were carried out with the cytosolic and microsomal fractions obtained as previously described.

Aflatoxin B_1-GSH Enzyme Kinetics

For AFB_1 GST enzyme activity (nucleophilic trapping of AFBO by GSH: AFBO + GSH → AFB_1-GSH), a discontinuous direct in vitro assay was done with incubations made per each animal at 7 different substrate concentrations, with each concentration run in duplicate according to the method proposed by Mannervik and Jemth (Mannervik and Jemth, 2002) with some modifications. Incubations were carried out in 1.5 mL microcentrifuge tubes kept at 39°C (the normal body temperature for the age of the birds used) containing 5 mM glucose 6-phosphate, 0.5 mM $NADP^+$, 0.5 I.U. glucose 6-phosphate dehydrogenase, 100 μg of Rhode Island Red microsomal protein (AFBO generating system), 1 μL of AFB_1 in DMSO at concentrations (in order to saturate GST enzyme) ranging from 8.6 to 157.5 μM for chicken breeds, from 5.3 to 96.9 μM for quail and turkey, from 2.0 to 36.7 μM for duck, and 800 μg of cytosolic protein for chicken breeds, 1,600 μg for quail, 4,800 μg for turkey and duck and 5 μg for mouse. In the case of mouse incubations, AFB_1 ranged from 13.9 to 256 μM. All volumes were completed to 250 μL with incubation buffer (phosphates 50 mM pH 7.4, MgCl 5 mM, EDTA 0.5 mM), and the reaction stopped after 10 min with 250 μL of ice-cold acetonitrile. The stopped incubations were centrifuged at 15000 x *g* for 10 min and 2 μL were analyzed by high-performance liquid chromatography as described below.

The production of AFB_1-GSH in each incubation was quantitated in a Shimadzu Prominence system (Shimadzu Scientific Instruments, Columbia, MD) equipped with a DGU-20A3R degassing unit, two LC-20AD pumps, a SIL-20ACHT autosampler, a CTO-20A column oven, an RF-20AXS fluorescence detector, and a CBM-20A bus module, all controlled by "LC Solutions" software. The chromatography was carried out on an Alltech Alltima HP C18 chromatographic column, 150 mm × 3.0 mm (Alltech Associates Inc., Deerfield, IL) kept at 40°C. The mobile phase was a linear gradient of solvent A (water - 0.1% formic acid) and B (acetonitrile: methanol, 1:1 - 0.1% formic acid), as follows: 0 min: 17% B, 1 min: 17% B, 10 min: 20% B, 10.01 min: 100% B, 12 min: 100%, 12.01 min: 17% and 18 min: 17% B. The flow rate was 0.6 mL/min and the fluorescence detector was set at excitation and emission wavelengths of 360 and 440 nm, respectively. The in-vial concentration of AFB_1-GSH was quantitated using an enzyme synthetized AFB_1-GSH standard. The linearity of the response for AFB_1-GSH was confirmed with a calibration curve for AFB_1-GSH with in column amounts ranging from 6 to 300 fmol, for which an R^2 value of 0,9993 was obtained. Analytical method precision was estimated by the Relative Standard Deviation of the results obtained

for determinations of AFB_1-GSH at the intermediate level of the calibration curves in triplicate. Relative Standard Deviation values for AFB_1-GSH was 7%. Recovery was estimated at 100% since the concentration of the analyte AFB_1-GSH found in blank incubations corresponded to the amount expected from the calculation based on the external standard calibration curves. This result was expected since the matrix corresponded to incubation buffer that was not subjected to any type of extraction or clarification procedures.

Aflatoxin B_1-GSH Standard Synthesis

Production of AFB_1-GSH standard was done in vitro in four 2 mL vials kept at 39°C, each containing 5 mM glucose 6-phosphate, 0.5 mM $NADP^+$, 2 I.U. glucose 6-phosphate dehydrogenase, 1.2 mg of Rhode Island Red microsomal protein, 4.8 mg of mouse cytosolic protein and 4 μL of AFB_1 256 μM in DMSO. Volume was completed to 1 mL with incubation buffer (phosphates 50 mM pH 7.4, MgCl 5 mM, EDTA 0.5 mM), and the reaction stopped after 40 min with 1 mL of ice-cold acetonitrile. Stopped incubations were pooled, vacuum-dried to 1 mL, centrifuged at 15,000 x *g* for 10 min and then run on an Agilent Technologies InfinityLab LC system (Agilent Headquaters, Santa Clara, CA) equipped with a G1314B 1260 VWD VL variable wavelength UV/Vis detector, a G1316A 1260 TCC thermostatted column compartment, a G1329B 1260 ALS standard autosampler, and a G1311C 1260 Quaternary Pump VL, all modules controlled by "LC Openlab CDS ChemStation Edition" software. The chromatography was carried out on a Waters preparative chromatographic column μBondapack C18 125 Å 10 μm 7.8 × 300 mm (Waters Corporation, Milford, MA) kept at 50°C. The mobile phase was a linear gradient of A (water 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) as follows: 0 min: 22% B, 7 min: 22% B, 7.01 min: 100% B, 10 min: 100% B, 10.01 min: 22% B and 12 min: 22% B, 17min: 18% B. The flow rate was 2.5 mL/min and the UV detector was set at 360 nm. Aliquots of 100 μL from the incubation vials were injected until the whole synthesis volume was run in the HPLC system. The fractions corresponding to the AFB_1-GSH adduct were collected, taken to dryness using a rotary evaporator (Hei-Vap Advantage, Heidolph Instruments GmbH & CO, Schwabach, Germany) and resuspended in ethanol for UV quantitation. Concentration of AFB_1-GSH was estimated from AFB_1 extinction coefficient (ε = 21800 M^{-1} cm^{-1}; (Budavari, 1996). To confirm the AFB_1-GSH identity, the monoisotopic protonated mass of the adduct was determined by HPLC-MS by means of a 3200 QTrap mass spectrometer (Applied Biosystems, Toronto, Canada) using a thermospray ionization probe in positive mode and the following settings: probe voltage: 4,800 V, declustering potential: 140 V, entrance potential: 10 V, curtain gas value: 30, collision energy: 81 V and collision cell exit potential: 5 V.

Statistical Analysis

The enzymatic parameters K_m and V_{max} were determined by non-linear regression using the Marquardt method adjusting the data to the Michaelis-Menten enzyme kinetics using the equation: $v = V_{max}[S]/K_m + [S]$, where v is the enzyme reaction velocity, [S] represents substrate concentration, $\mathbf{V_{max}}$ represents maximal velocity and $\mathbf{K_m}$ represents the Michaelis-Menten constant. Intrinsic clearance ($\mathbf{CL_{int}}$; mL/mg protein/minute) was calculated as the ratio V_{max}/K_m. The calculated CL_{int} only applies for the selected enzymatic activity and not for the hepatic clearance, since GST enzyme was not purified from liver extracts. In all cases the kinetic parameters are "apparent" because hepatic extracts and not purified enzymes were used. Interspecies differences in enzymatic kinetic parameters were determined by using the Kruskal-Wallis test, while nonparametric multiple comparisons were made by using the Dwass-Steel-Critchlow-Fligner method, with a significance level of 5% ($P < 0.05$). All analyses were performed using the Statistical Analysis System (SAS version 9.4) software (SAS Institute Inc., 2013).

RESULTS

The expected monoisotopic molecular mass of the AFB_1-GSH adduct obtained from in vitro synthesis, was confirmed by mass spectrometry since the putative peak eluting at t_R = 7.96 min corresponded to a molecular protonated mass value of 636.2 Da (Figure 2). Figure 3 shows the enzyme kinetic parameters of cytosolic AFB_1-GSH production, including the saturation curve, maximal velocity (V_{max}), Michaelis-Menten constant (K_m) and intrinsic clearance (CL_{int}). Saturation of GST activity (Figure 3A) occurs at lower AFB_1 concentrations (<60 μM) in duck and turkey (sensitive species) compared with the more resistant chicken breeds. Apparently, quail seems to reach a plateau at a concentration of AFB_1 close to 100 μM and Ross and Rhode Island Red chickens reach V_{max} beyond 160 μM of AFB_1. The V_{max} value per species was the highest for the chicken breeds (1.28 ± 0.26 and 1.40 ± 0.12 pmol AFB_1-GSH/mg protein/minute for Ross and Red Island Red chickens, respectively), followed by quail (0.85 ± 0.27 pmol AFB_1-GSH/mg protein/min), duck (0.34 ± 0.17 pmol AFB_1-GSH/mg protein/min) and turkey (0.18 ± 0.08 pmol AFB_1-GSH/mg protein/minute; Figure 3B). Differences by sex were found only for ducks, with values of 0.44 ± 0.15 and 0.23 ± 0.11 pmol AFB_1-GSH/mg protein/min for females and males, respectively. In regard to the K_m value, it was found that the lowest values corresponded to the chicken breeds (47.41 ± 7.11 and 65.66 ± 14.4 μM of AFB_1-GSH for Rhode Island Red and Ross, respectively), followed by the duck (69.09 ± 51.48 μM of AFB_1-GSH), the turkey (87.54 ± 24.43 μM of AFB_1-GSH) and the quail (92.66 ± 25.20 μM of AFB_1-GSH; Figure 3C). Differences by sex were found only in ducks (26.83 ± 10.11 and 111.36 ± 37.94 μM AFB_1-GSH for females and males respectively) and Rhode

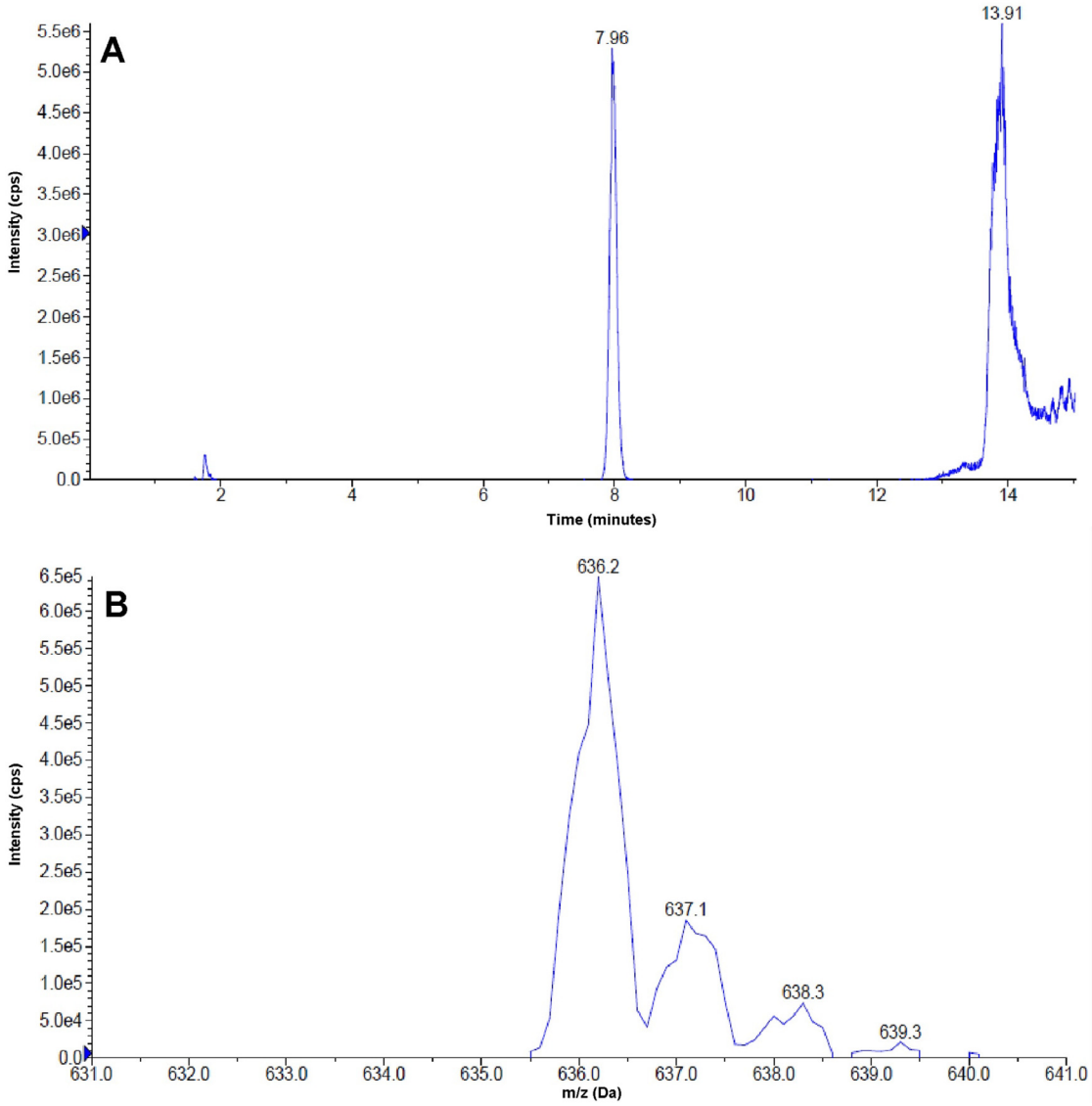

Figure 2. Identification of aflatoxin B_1-glutathione (AFB_1-GSH) conjugate by HPLC-MS. (A) Chromatogram of the purified AFB_1-GSH obtained from enzymatic synthesis. The peak at $t_R = 7.96$ min shows the putative AFB_1-GSH product. (B) Protonated monoisotopic mass found in the 7.96 min peak, corresponding to a value of 636.2 Da.

Island Red chickens (42.42 ± 5.53 and 52.40 ± 4.57 μM of AFB_1-GSH for females and males respectively). Enzyme efficiency, estimated by the CL_{int} value, was highest for the chicken breeds (0.03 ± 0.004 and 0.02 ± 0.003 mL/mg protein/min for Rhode Island Red and Ross, respectively), followed by duck (0.01 ± 0.008 mL/mg protein/min), quail (0.009 ± 0.001 mL/mg protein/min) and turkey (0.002 ± 0.001 mL/mg protein/min; Figure 3D). Differences by sex were found only for the ducks (0.017 ± 0.004 and 0.002 ± 0.001 mL/mg protein/min for females and males, respectively) and for Rhode Island Red chickens (0.032 ± 0.004 and 0.028 ± 0.002 mL/mg protein/min for females and males, respectively).

Because the net toxic effect of AFB_1 depends on both activation and deactivation reactions, the AFB_1 activation/deactivation ratio was estimated using the CL_{int} values for these reactions. The ratio CL_{int} AFB_1 activation (AFBO production)/AFB_1 deactivation CL_{int} (AFB_1-GSH production) was calculated using the AFBO production values previously obtained from the same set of samples and published elsewhere (Diaz and Murcia, 2019). From lowest to highest, the calculated ratios were as follows: Ross chickens, 2.36 ± 0.95; Rhode Island Red chickens, 3.50 ± 0.63; quail, 15.12 ± 7.12, turkey, 65.79 ± 40.20; and duck, 470.82 ± 54.97.

DISCUSSION

The results of the present study suggest that, just like in rodents, the kinetics of AFB_1-GSH production in poultry is related to the in vivo resistance to AFB_1, which follows the order chickens > quail > turkeys > ducks (Monson et al., 2015; Diaz, 2020). The V_{max} kinetic parameter values for AFB_1-GSH production were found to follow the order chickens > quail > turkey = duck, which indicates that the higher V_{max}

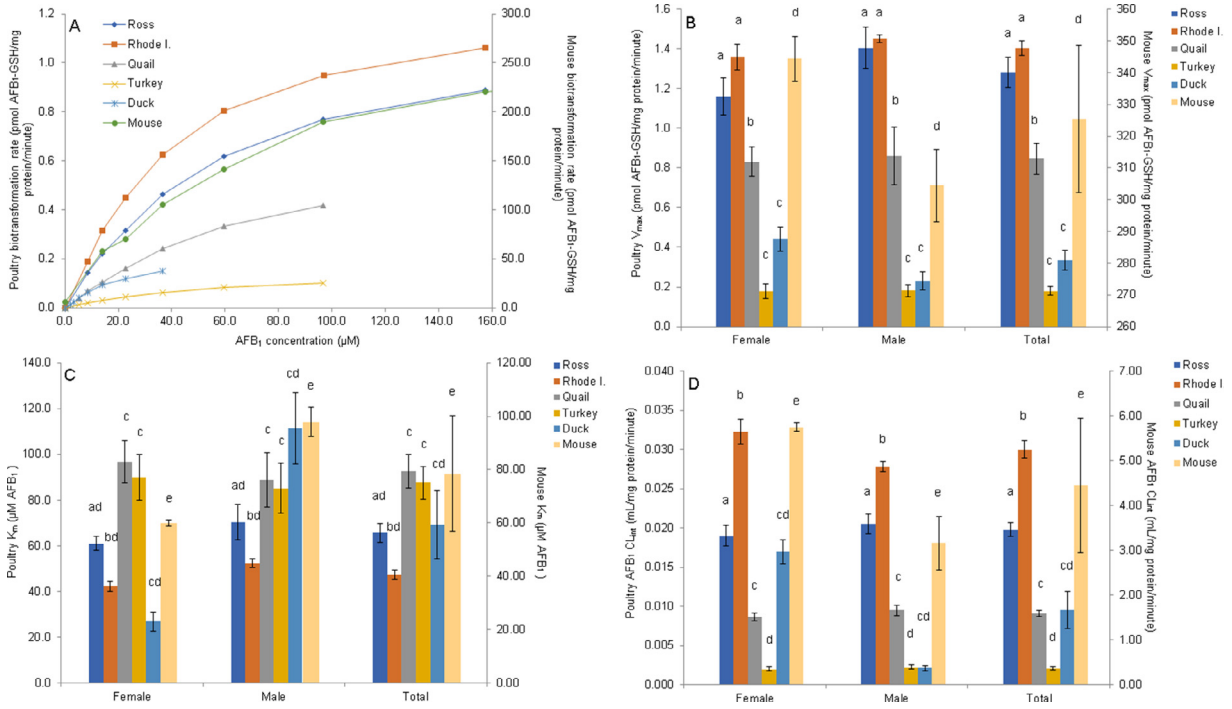

Figure 3. Enzyme kinetic parameters of hepatic cytosolic in vitro aflatoxin B_1-glutathione (AFB_1-GSH) conjugate production. (A) Saturation curve at AFB_1 concentrations ranging from 2.0 to 157.5 μM. (B) Maximal velocity (V_{max}). (C) Michaelis-Menten constant (K_m). (D) Intrinsic clearance (CL_{int}; V_{max}/K_m). Species mean values with the same letter do not differ significantly. Statistical differences ($P < 0.05$) were calculated using the Kruskal-Wallis test and nonparametric multiple comparisons were done by the Dwass-Steel-Critchlow-Fligner method. Values are means ± SEM of 6 birds per sex (n = 12 birds total).

values found in the chicken breeds is related with a greater capacity to biotransform AFBO into AFB_1-GSH. On the other hand, the lower K_m values found in chickens indicate that V_{max} is reached at lower AFB_1 concentrations compared to ducks, turkeys or quail. Since chicken GST activity towards AFBO reach the V_{max} at lower concentrations of AFB_1, GST activity operates at a wider range of AFB_1 concentrations, in contrast to duck or turkey, were inactivation of AFBO is limited to a narrow low AFB_1 concentration range. In the case of Rhode Island Red chicken and duck, although K_m values are slightly different, it is evident that saturation of duck GST activity occur at very low AFB_1 concentrations (less than 60 μM of AFB_1) limiting AFB_1 GST-AFBO conjugation capability and exposing duck hepatocyte to the AFBO harmful effects. Because the duck is the highest AFBO producer among poultry species (Diaz and Murcia, 2019), at high AFB_1 concentrations in the hepatocyte cytosol, the high K_m and low V_{max} GST parameters in the duck would lead to the production of high amounts of free AFBO capable to adduct to hepatocyte DNA, leading to DNA damage. Another way to visualize the impact of GST activity in AFBO management between poultry species is the CL_{int} parameter. The intrinsic clearance, as a parameter of GST conjugating efficiency, shows how chickens produce more efficiently the AFB_1-GSH conjugate than the duck, a sensitive species. The CL_{int} value for chicken is more than 2 times higher than the quail, 10 times higher than the turkey and 20 times higher than the duck. Despite the large differences between the resistant chickens and the sensitive ducks and turkeys, it is still not clear how different poultry species would develop different GST enzyme efficiencies against AFB_1. It has been postulated that the evolutionary biology of each particular species has determined its ability to cope (or not) with aflatoxins; for example, chickens (*Gallus gallus* spp. *domesticus*) are originally from southeast Asia, a geographic area with a large prevalence of aflatoxins, whereas turkeys (*Meleagris gallopavo*) are originally from the northern regions of the United States and southern Canada, where aflatoxins are very rare. According to this theory, continuous exposure to aflatoxins through thousands of years of evolution led to the hepatic enzyme adaptation that has conferred the chickens their extraordinary resistance to aflatoxins (Diaz, 2020). Further, as different polymorphisms have been associated with changes in GST enzyme kinetic parameters (Ping et al., 2006), it seems that natural selection has picked out those polymorphisms in the chicken GST enzymes that have enhanced the appropriate kinetic parameters to efficiently inactivate AFBO and deal with AFB_1. . However, in order to fully charaterize the avian GST enzymes it is necessary to isolate all possible isoforms through glutathione-Sepharose affinity beads (Harper and Speicher, 2011), as this would likely strip out all the class Alpha, Mu and Pi GSTs present in poultry cytosolic extracts and possibly unravel the level of expression of each GST-class enzymes. In fact, activity levels for GSTM have been reported for the turkey (Bunderson et al., 2013).

By calculating the ratio between AFB_1 bioactivation (AFBO formation) and AFBO inactivation (conjugation with GSH), an estimation of the net detoxification of AFB_1 can be obtained. In a study conducted with laboratory rodents, this calculation resulted in values of 0.54 for the mouse and 1.62 for the rat, respectively (Degen and Neumann, 1981). In another trial, the values obtained were 0.13 and 6.2 for the mouse and the rat, respectively (Eaton et al., 1994). In both reports, the more resistant mouse had a ratio lower than 1, meaning that the deactivation reaction is favored over the activation reaction. In the present study, the ratios AFB_1 CL_{int} (AFBO production) / AFB_1 CL_{int} (AFB_1-GSH production) followed the order duck > turkey > quail > chicken. Interestingly, the order of these ratios is in perfect agreement with the known in vivo sensitivity of these poultry species. Further, the calculated ratio for the duck is more than 134 times higher than that of the chickens, 31 times higher than the quail ratio and 7 times higher than the turkey ratio. These results clearly show that the duck liver lacks the ability to trap the AFB_1 active metabolite AFBO as efficiently as other poultry and might explain why this is the only poultry species that develops hepatic carcinoma upon AFB_1 exposure (Diaz, 2020).

Beyond the differences in the enzymatic parameters between poultry species, differences between sexes were also found in the ducks and the Rhode Island Red chicken breed. In both species, females were found to be more efficient in converting AFBO into AFB_1-GSH than males. In ducks, the difference for CL_{int} was 8.5 times higher for females. In Rhode Island Red the difference was slightly higher, being 14% greater in females. In rats, production of AFB_1-GSH has been found to be higher in the female than in the male rat (Esaki and Kumagai, 2002). Further, the AFB_1 LD_{50} in female Porton rat is 3 times higher than in males (18.0 and 6.25 mg/kg of body weight, respectively; Cullen, 1994). In 3 wk-old Fisher 344 rats, it has been found that GSH AFBO conjugating activity of female rat over male rat is 25.7% higher, increasing to a value of 98.7% at 7 wk (Hayes et al., 1994). Also, in turkey embryos it has been reported a sex-specific response to AFB_1 (Monson et al., 2016). In adult rat liver, the sex-dependent expression of GST enzymes has been linked to the effect of growth hormone, which enhances expression levels in females over males (Srivastava and Waxman, 1993; Ahluwalia et al., 2004). This fact opens a window to explore the effect of growth hormone on GST sex-dependent expression in poultry.

Besides the sex-dependent expression of GST enzymes, significant differences in GST efficiency were also found between chicken breeds. As it has been reported, domestication of turkeys has apparently reduced GST activity (Reed et al., 2019). Wild turkeys (*Meleagris gallopavo silvestris*) and domesticated turkeys (*Meleagris gallopavo*) show a distinct hepatic GSTA3 expression level when fed control versus AFB_1 contaminated diets, having GSTA3 activity a higher expression level in wild turkeys compared to domesticated turkeys (Reed et al., 2018). Furthermore, spleen gene response to AFB_1 intake is also divergent between domesticated and wild turkeys (Reed et al., 2019). Further, duck interbreed differences have also been reported, since significant histopathological differences have been found in different duck breeds after AFB_1 exposure and these differences are probably associated to AFB_1 breed metabolism variability (Hetzel et al., 1984). Accordingly, we speculate that the lower GST enzyme activity efficiency found in Ross breed compared to Rhode Island Red breed could be attributed to lineage development for commercial purposes in the case of Ross breed.

Although resistance to AFB_1 is highly correlated to GST activity, resistance should not be attributed solely to this enzyme activity, since AFB_1 metabolism includes different metabolic steps. For example, aflatoxin B_1 aldehyde reductase enzyme activity is capable of reducing AFB_1 dialdehyde to AFB_1 monoalcohol and AFB_1 dialcohol, hence reducing the amount of adducts with lysine in proteins and consequently AFB_1 dialdehyde cytotoxicity (Murcia and Diaz, 2020). In addition to aflatoxin B_1 aldehyde reductase enzyme activity, the reduction of AFB_1 into AFL reduces the AFB_1 available for epoxidation through cytochrome P450 enzymes and becomes a biochemical strategy to avoid AFBO production in tolerant poultry species like the chicken (Murcia and Diaz, 2020). Even more, GST activity deals with the genotoxic effect of AFBO, but the amount of AFBO enzymatically produced through cytochrome P450 enzymes that is not trapped by GSH or is not adducted to DNA, spontaneously hydrolyzes and produces AFB_1-dhd. This way AFB_1-dhd contributes to the acute signs observed in poultry, especially in highly sensitive species like duck (Diaz and Murcia, 2019). Therefore, the net toxicity of AFB_1 should be considered as a multifactorial mechanism in which different metabolic pathways in AFB_1 biotransformation are interconnected, and different biotransformation products are involved.

The last issue to consider is the induction of xenobiotic metabolism enzymes by chemicals. Even though in vitro AFB_1-GSH enzymatic production was normalized by cytosolic protein amount, the level of expression of GST could act as a possible factor involved in species resistance to AFB_1 toxic effects. It is of general knowledge that coumarin, ethoxyquin and other compounds like phenolic antioxidants and isothiocyanates act as inductors of xenobiotic metabolism enzymes like GST or AFAR enzymes (McLellan et al., 1994; Hayes et al., 1998; Kelly et al., 2000). Some poultry diets contain different antioxidants as vitamin E, selenium, taurine, and polyphenolics to protect poultry against oxidative stress (Surai, 2020). Due to the inductive effect of some of these compounds on xenobiotic metabolism enzymes, it becomes necessary to use experimental diets with no additives in order to obtain no biased results. In addition to enzyme induction, time-dependent expression has been reported in poultry species. In broiler chickens at 7,

21, and 42 d old, it was observed the age-dependent increase in GST expression levels, more specifically the GSTA3 and GSTA4 Alpha-class enzyme isoforms, at a higher magnitude in the AFB_1 contaminated diets than in control diets (no AFB_1; Wang et al., 2018). This age effect highlights the importance of considering the animal's age in order to compare enzyme kinetic parameters between poultry species.

Several studies have related AFB_1 GST enzyme activity to resistance to the carcinogenic effects of AFB_1 in rodents; however, no comparative studies evaluating the ability to detoxify AFBO by GST activity had been conducted in poultry. In the present study we found that more resistant species like the chicken have a more efficient AFBO GSH trapping catalyzed by GST than sensitive species like the duck. Our finding strongly suggest that the duck is the only poultry species that develops hepatic carcinoma upon AFB_1 exposure because of its poor deactivation of enzyme produced AFBO. While chicken GST enzyme activity can deal with a wide range of AFB_1 concentrations, the duck GST activity seems to be unable to cope with elevated AFB_1 concentrations. Despite the possible explanation of hepatocarcinoma development in ducks, knowledge on the metabolism of AFB_1 in poultry still has many gaps to fill. Recognizing the nucleophilic trapping of AFBO by GST enzyme as the main pathway to deactivate AFBO, other biotransformation steps should be integrated in a model that includes the enzyme kinetic parameters obtained in each metabolic step and this model should be run as an in silico simulation. This model could include the efficiency of the DNA repairing system among poultry species, in regards of the evidence around the role of AFB_1 biotransformation and DNA repair as determinants of AFB_1 induced carcinogenesis (Bedard and Massey, 2006). Further, polymorphisms in genes associated to DNA repair are determinants of the repair capacity of AFB_1-induced DNA damage (Xia et al., 2013). A synergistic effect between high AFBO production and low capacity to repair DNA damage caused by AFBO in ducks, could explain their unique feature : their ability to develop hepatocarcinoma. Finally, polymorphisms found in poultry and contrasted to mouse GST catalytic H-site reveals potential residues that could explain the great differences between poultry and mouse, and between the tolerant chicken and the very sensitive duck.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101235.

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