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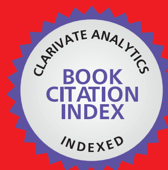

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Biotransformation of Aflatoxin B1 and Its Relationship with the Differential Toxicological Response to Aflatoxin in Commercial Poultry Species

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1. Introduction

Aflatoxin B1 (AFB1) is a highly toxic compound (LD_{50} = 1-50 mg/kg) for most animal species, although it is extremely toxic (LD50 < 1 mg/kg) for some highly susceptible species such as pigs, dogs, cats, rainbow trouts, and ducklings. The toxic effects of AFB1 are both dose and time dependent and two distinct forms of aflatoxicosis, namely acute and chronic, can be distinguished depending on the level and length of time of aflatoxin exposure. In many species acute poisoning is characterized by an acute hepatotoxic disease that manifests itself with depression, anorexia, icterus, and hemorrhages. Histologic hepatic lesions include periportal necrosis associated with bile duct proliferation and oval cell hyperplasia. Chronic aflatoxicosis resulting from regular low-level dietary intake of aflatoxins causes unspecific signs such as reduced weight gain, reduced feed intake, and reduced feed conversion in pigs and poultry, and reduced milk yield in cows. Another effect of chronic exposure is aflatoxin-induced hepatocellular carcinoma, bile duct hyperplasia and hepatic steatosis (fatty liver). However, these effects are species-specific and not all animals exposed to aflatoxin develop liver cancer. For example, the only poultry species that develops hepatocellular carcinoma after AFB1 exposure is the duck.

Differences in the susceptibility to acute and chronic AFB1 toxicosis have been observed among animals of different species. Animals having the highest sensitivity are the duckling, piglet, rabbit, dog and cat, while chickens, mice, hamsters, and chinchillas are relatively resistant. Further, mature animals are generally more resistant to AFB1 than young ones and females are more resistant than males. In general, in commercial poultry species, intake of feed contaminated with AFB1 results primarily in liver damage (the target organ of AFB1 is the liver), associated with immunosuppression, poor performance, and even mortality when the dietary levels are high enough. However, there is wide variability in specific species sensitivity to AFB1 and the susceptibility ranges from ducklings > turkey poults > goslings > pheasant chicks > quail chicks > chicks (Leeson et al., 1995). Even though there is still no clear explanation for this differential sensitivity, differences in susceptibility could be due to differences in AFB1 biotransformation pathways among species. The aim of the present chapter is to review the current knowledge on AFB1 biotransformation, with emphasis on

commercial poultry species, and to correlate this information with the *in vivo* susceptibility to AFB1 in these species.

2. Biotransformation of aflatoxin B1

In general, the metabolism or biotransformation of xenobiotics (chemicals foreign to the organism) is a process aimed at converting the original molecules into more hydrophilic compounds readily excretable in the urine (by the kidney) or in the bile (by the liver). It has traditionally been conceptualized that this process occurs in two phases known as Phase I and Phase II, although some authors argue that this classification is no longer tenable and should be eliminated (Josephy et al., 2005). Phase I metabolism consists mainly of enzyme-mediated hydrolysis, reduction and oxidation reactions, while Phase II metabolism involves conjugation reactions of the original compound or the compound modified by a previous Phase I reaction. The current state of knowledge on the metabolism of AFB1 in different avian and mammalian species is summarized in Figure 1. As Figure 1 shows, a wide array of metabolites can be

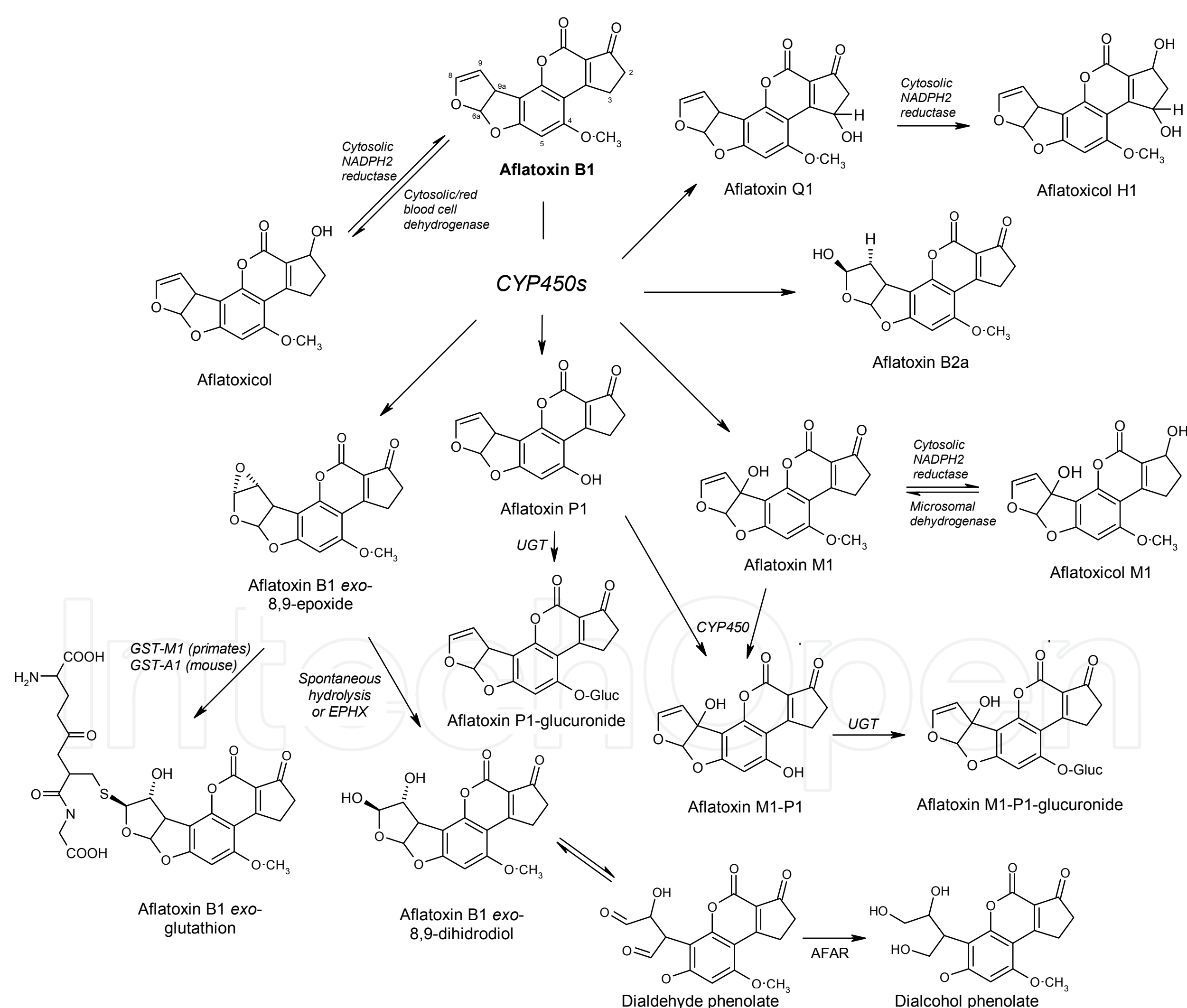

Fig. 1. Biotransformation reactions of aflatoxin B1 in poultry and mammals, including humans. The main CYP450s involved in these reactions are CYP1A1, CYP1A2, CYP2A6 and CYP3A4. Not all reactions occur in a single species.

produced directly from AFB1 (by oxidation and reduction reactions) or indirectly by further biotransformation of the metabolites formed. However, not all of these reactions occur in a single species and, in fact, only a few of them have been reported in poultry. Most AFB1 Phase I reactions are oxidations catalyzed by cytochrome P450 (CYP450) enzymes, but one reaction is catalyzed by a cytosolic reductase, corresponding to the reduction of AFB1 to aflatoxicol (AFL). Phase II reactions are limited to conjugation of the metabolite AFB1-*exo*-8,9-epoxide (AFBO) with glutathione (GSH, γ-glutamyl-cysteinyl-glycine), and conjugation of aflatoxins P1 and M1-P1 with glucuronic acid. Conjugation of AFBO with GSH is a nucleophilic trapping process catalyzed by specific glutathione transferase (GST) enzymes. The AFBO may also be hydrolyzed by an epoxide hydrolase (EPHX) to form AFB1-*exo*-8,9-dihydrodiol, although this reaction may also occur spontaneously. The dihydrodiol is in equilibrium with the dialdehyde phenolate form, which can be reduced by AFB1 aldehyde reductase (AFAR), an enzyme that catalyzes the NADPH-dependent reduction of the dialdehyde to dialcohol phenolate (Guengerich et al., 2001).

The translocation of xenobiotics across cell membranes by specific proteins known as transporters has been termed by some as “Phase III” metabolism. However, this process does not involve any modification of the xenobiotic structure and therefore it cannot be termed metabolism. This process, however, may have important implications on the toxic effect of a xenobiotic, particularly if the specific transporter involved in the translocation of the compound is not expressed normally, presents a genetic abnormality or becomes saturated. One transporter that has been identified as responsible for the translocation of a mycotoxin from the sinusoidal hepatic space into the hepatocyte is OATP (organic anion transporter polypeptide), which transports ochratoxin A (Diaz, 2000). However, no transporters for AFB1 have yet been described.

2.1 Phase I metabolism of aflatoxin B1

As mentioned before, the Phase I metabolism of AFB1 is carried out mainly by members of the CYP450 superfamily of enzymes. Their name comes from the absorption maximum at 450 nm when the reduced form complexes with carbon monoxide (Omura & Sato, 1964). CYP450s are membrane bound enzymes that can be isolated in the so-called microsomal fraction which is formed from endoplasmic reticulum when the cell is homogenized and fractionated by differential ultracentrifugation; microsomal vesicles are mainly fragments of the endoplasmic reticulum in which most of the enzyme activity is retained. The highest concentration of CYP450s involved in xenobiotic biotransformation is found in the endoplasmic reticulum of hepatocytes but CYP450s are present in virtually every tissue. CYP450s are classified into families identified by a number (e.g., 1, 2, 3, and 4), subfamilies identified by a letter (e.g., 2A, 2B, 2D, and 2E), and individual members identified by another number (e.g. CYP2A6, CYP2E1). Collectively, CYP450 enzymes participate in a variety of oxidative reactions with lipophilic xenobiotics and endogenous substrates including hydroxylation of an aliphatic or aromatic carbon, epoxidation of a double bond, heteroatom (*S*-, *N*- and *I*-) oxygenation and *N*-hydroxylation, heteroatom (*O*-, *S*-, and *N*-) dealkylation, oxidative group transfer, cleavage of esters, and dehydrogenation (Parkinson & Ogilvie, 2008). In regards to AFB1, CYP450s can hydroxylate, hydrate, *O*-demethylate, and epoxidate the molecule.

2.1.1 Hydroxylation and hydration of aflatoxin B1

CYP450s can produce at least three monohydroxylated metabolites from AFB1, namely aflatoxins M1 (AFM1), Q1 (AFQ1), and B_{2a} (AFB_{2a}) (Fig. 1). AFM1 was first isolated from the

milk of cows and rats fed AFB1-contaminated peanut meal and it was initially termed "milk toxin" (de Iongh et al., 1964). It was later discovered that AFM1 is not a metabolite exclusive of mammals and, in fact, it is produced by crude or isolated microsomal liver preparations from many non-mammalian species. For example, AFM1 was found in most tissues of chickens receiving a diet containing 2,057 ppb AFB1 for 35 days (Chen et al., 1984); the highest level was found in the liver and kidneys, which relates to the important role of these organs in the biotransformation and elimination of xenobiotics, respectively.

AFQ1 results from the 3α-hydroxylation of AFB1 and it was first discovered as a major metabolite of AFB1 from monkey liver microsomal incubations (Masri et al., 1974). The predominant enzyme responsible for AFQ1 formation in human liver microsomes is CYP3A4 (Raney et al., 1992b) and AFQ1 is considered to be a major metabolite of AFB1 in humans and monkeys *in vitro* (Hsieh et al., 1974). Although AFQ1 has been detected as a minor metabolite of chicken and duck microsomal preparations (Leeson et al., 1995) it is considered to be a significant detoxication pathway of AFB1 (Raney et al., 1992b). In fact, AFQ1 is about 18 times less toxic for chicken embryos than AFB1 and it is not mutagenic in the *Salmonella typhimurium* TA 1538 test (Hsieh et al., 1974).

The hydration of the vinyl ether double bond (C8-C9) of AFB1 produces the 8-hydroxy derivative or hemiacetal, also known as AFB_{2a}. This metabolite was discovered in 1966 and, interestingly, it can be produced enzymatically (by both higher organisms and microbial metabolism), by photochemical degradation of AFB1, and by the treatment of AFB1 with acid (Lillehoj & Ciegler, 1969). The formation of the hemiacetal is difficult to assess *in vitro* because of strong protein binding, which probably involves the formation of Schiff bases with free amino groups (Patterson & Roberts, 1972). The ability of certain species to metabolize AFB1 into its hemiacetal at higher rates than others constitutes an important aspect of the resistance to the toxin, since the toxicity of AFB_{2a} is much lower than that of the parent compound. For instance, AFB_{2a} has been shown to be not toxic to chicken embryos at levels 100 times the LD_{50} of AFB1 (Leeson et al., 1995), and the administration of 1.2 mg of AFB_{2a} to one-day-old ducklings does not produce the adverse effects caused by the same dose of AFB1 (Lillehoj & Ciegler, 1969).

It has been generally considered that the monohydroxylated metabolites of AFB1 are "detoxified" forms of the toxin, which is probably the case for aflatoxins B_{2a} and Q1; however, AFM1 cannot be considered a detoxication product of AFB1. AFM1 is cytotoxic and carcinogenic in several experimental models and in ducklings its acute toxicity is similar to that of AFB1 (12 and 16 μg/duckling for AFB1 and AFM1, respectively). Also in ducklings, both AFB1 and AFM1 induce similar liver lesions; however, AFB1 induces only mild degenerative changes in the renal convoluted tubules whereas AFM1 causes both degenerative changes and necrosis of the tubules (Purchase, 1967).

2.1.2 *O*-Demethylation of aflatoxin B1

Another CYP450-mediated reaction of rat, mouse, guinea pig and rabbit livers is the 4-*O*-demethylation of AFB1. The phenolic product formed was initially isolated from monkey urine (Dalezios et al., 1971) and named aflatoxin P1 (the P comes from the word primate). AFP1 can be hydroxylated at the 9a position to form 4,9a-dihydroxyaflatoxin B1 (AFM1-P1, see Fig. 1), although this compound can also originate from AFM1 (Eaton et al., 1988). AFP1 is generally considered a detoxication product, mainly because it is efficiently conjugated with glucuronic acid (Holeski et al., 1987). There is no evidence that AFP1 or its 9a-hydroxy derivative are produced by any avian species (Leeson et al., 1995).

2.1.3 Epoxidation of aflatoxin B1

Another metabolic pathway of the vinyl ether double bond present in the AFB1 furofuran ring is its epoxidation. The resultant product, AFB1-*exo*-8,9-epoxide (AFBO), is an unstable, highly reactive compound, with a half-life of about one second in neutral aqueous buffer (Johnson et al., 1996), that exerts its toxic effects by binding with cellular components, particularly protein, DNA and RNA nucleophilic sites. AFBO is considered to be the active form responsible for the carcinogenicity and mutagenicity of AFB1 (Guengerich et al., 1998). The *endo*-8,9-epoxide of AFB1 can also be formed by rat and human microsomes (Raney et al., 1992a), but this form of the epoxide is not reactive. Once AFBO is formed it may be hydrolyzed, either catalytically or spontaneously, to form AFB1-8,9-dihydrodiol (AFB1-dhd) or it may be trapped with GSH. If AFB1-dhd is formed it may suffer a base-catalyzed furofuran ring opening to a dialdehyde (AFB1 α-hydroxydialdehyde), which is able to bind to lysine residues in proteins. The enzyme AFAR (see section 2) can protect against the dialdehyde by catalyzing its reduction to a dialcohol which is excreted in the urine either as the dialcohol itself or as a monoalcohol (Guengerich et al., 2001). AFAR activity, however, does not correlate with *in vivo* sensitivity to AFB1 in selected mammalian models (hamster, mouse, rat and pig) as it was demonstrated by Tulayakul et al. (2005). AFAR has been evidenced by immunoblot in the liver of turkeys (Klein et al., 2002) but its activity has not been investigated in this or any other avian species.

2.1.4 Reduction of aflatoxin B1

The C1 carbonyl group present in the cyclopentanone function of AFB1 can be reduced to a hydroxy group to form the corresponding cyclopentol AFL (Fig. 1). This reaction is not catalyzed by microsomal enzymes but by a cytosolic NADPH-dependent enzyme that in the case of the chicken has an estimated molecular weight of 46.5 KDa and is inhibited by the 17-ketosteroids androsterone, dehydroisoandrosterone and estrone (Chen et al., 1981). Formation of AFL was first reported in chicken, duck, turkey and rabbit liver cytosol (Patterson & Roberts, 1971), and it also occurs in quail (Lozano & Diaz, 2006). However, little or no activity has been observed in guinea pig, mouse or rat liver cytosol (Patterson & Roberts, 1971). AFL can be oxidized back to AFB1 by liver cytosol (Patterson & Roberts, 1972) and by red blood cells from several species (Kumagai et al., 1983). For this reason, AFL is considered to be a "storage" form of AFB1. The ratio of AFB1 reductase activity to AFL dehydrogenase activity *in vitro* has been observed to be higher in species that are extremely sensitive to acute aflatoxicosis (Wong & Hsieh, 1978), but the significance of this finding in poultry species remains to be determined. AFL cannot be considered a detoxified product of AFB1 since it is carcinogenic and mutagenic, it is acutely toxic to rabbits and it is correlated with susceptibility to AFB1 in some species (Kumagai et al., 1983). Further, AFL has the ability of inducing DNA adduct formation because the double bond between C–8 and C–9 is still present in this metabolite (Loveland et al., 1987). Conjugation of AFL with either glucuronic acid or sulfate would potentially be a true detoxication reaction because this step would prevent AFL from being reconverted to AFB1.

2.1.5 Reduction of aflatoxin B1 metabolites

The hydroxylated metabolites AFM1 and AFQ1 can also undergo the cytosolic reduction of the C1 carbonyl group in a reaction analogous to the reduction of AFB1 to AFL. The reduced metabolites of AFM1 and AFQ1 have been named aflatoxicol M1 (Salhab et al., 1977; Loveland et al., 1983) and aflatoxicol H1 (Salhab & Hsieh, 1975), respectively. Aflatoxicol H1

is a major metabolite of AFB1 produced by human and rhesus monkey livers *in vitro* (Salhab & Hsieh, 1975). Aflatoxicol M1 can also be produced from AFL and it can be oxidized back to AFM1 by a carbon monoxide-insensitive dehydrogenase activity associated with human liver microsomes (Salhab et al., 1977).

2.2 Phase II metabolism of aflatoxin B1

The most studied Phase II biotransformation reaction of any AFB1 metabolite is the nucleophilic trapping process in which GSH reacts with the electrophilic metabolite AFBO. Conjugation of AFBO with GSH is catalyzed by glutathione transferases (GST, 2.5.1.18), a superfamily of enzymes responsible for a wide range of reactions in which the GSH thiolate anion participates as a nucleophile. These intracellular proteins are found in most aerobic eukaryotes and prokaryotes, and protect cells against chemically-induced toxicity and stress by catalyzing the conjugation of the thiol group of GSH and an electrophilic moiety in the substrate. GSTs are considered the single most important family of enzymes involved in the metabolism of alkylating compounds and are present in most tissues, with high concentrations in the liver, intestine, kidney, testis, adrenal, and lung (Josephy & Mannervik, 2006). The soluble GSTs are subdivided into classes based on sequence similarities, a classification system analogous to that of the CYP450s. The classes are designated by the names of the Greek letters: Alpha, Mu, Pi, and so on, abbreviated in Roman capitals: A, M, P, etc. Within the class, proteins are numbered using Arabic numerals (e.g. GST A1, GST A2, etc.) and specific members are identified by the two monomeric units comprising the enzyme (e.g. GST A1-1, GST A2-2, GST M1-1, etc.). The microsomal GSTs (MGSTs) and its related membrane-bound proteins are structurally different from the soluble GSTs, forming a separate superfamily known as MAPEG (membrane-associated proteins in eicosanoid and GSH metabolism). MGSTs are not involved in the metabolism of AFB1 metabolites.

Another conjugation reaction reported for AFB1 metabolites is the conjugation of AFP1 and its 9a-hydroxy metabolite (aflatoxin M1-P1) with glucuronic acid. This conjugation has only been reported in rats and mice (Holeski et al., 1987; Eaton et al., 1988) and leads to the synthesis of detoxified products. Conjugation with glucuronic acid is catalyzed by enzymes known as UPD-glucuronosyltransferases (UGTs, Josephy & Mannevick, 2006), but the specific UGT involved in the conjugation of AFP1 and AFM1-P1 has not been described yet.

3. Biotransformation of aflatoxin B1 in poultry and its relationship with *in vivo* sensitivity

The role of poultry in mycotoxin research in general and aflatoxin research in particular is historically highly relevant since aflatoxins were discovered after a toxic Brazilian peanut meal caused the death of more than 100,000 turkeys of different ages (4-16 weeks) in England during the summer of 1960 (Blount, 1961). This mycotoxicosis outbreak was the first one ever reported for any animal species and for any mycotoxin. Initially only turkeys were affected but later ducklings and pheasants were also killed by the same misterious "X disease". Interestingly, no chickens were reported to have died from this new disease. Research conducted with poultry after the discovery of aflatoxins (reviewed by Leeson et al., 1995) has clearly shown that the *Gallus sp.* (which includes the modern commercial meat-type chickens and laying hens) is extremely resistant to aflatoxins while other commercial poultry species are highly sensitive. For instance, whereas ducklings and turkey poults exhibit 100% mortality at dietary levels of 1 ppm (Muller et al., 1970), chicks can tolerate 3 ppm in the diet without showing any observable adverse effects (Diaz & Sugahara, 1995). Interestingly, chickens are

not only highly resistant to the adverse effects of AFB1 but some studies have reported a modest enhancement in the body weight of chickens exposed to dietary aflatoxins, a finding that has been characterized as an hormetic-type dose-response relationship (Diaz et al., 2008). At the molecular level, at least four mechanisms of action could potentially play a role in the resistance to AFB1: low formation of the putative reactive metabolite (AFBO) and/or AFL, high detoxication of the AFBO and/or AFL formed, intestinal biotransformation of AFB1 before it can reach the liver ("first-pass action"), and increased AFB1 (or toxic metabolites) efflux from the cells. It is important to note that translocation of xenobiotics and their metabolites from the hepatocytes (efflux) mediated by specific basolateral and canalicular transporters (Diaz, 2000) -a process sometimes referred to as Phase III metabolism-, has not been investigated for AFB1 in any species. However, both Phase I and Phase II metabolism appear to have a profound effect on the differential *in vivo* response to AFB1 in commercial poultry species. The formation of AFBO (by CYP450s) and AFL (by a cytosolic reductase) as well as the scarce information available about detoxication of AFBO through nucleophilic trapping with GSH in poultry will be discussed below.

3.1 Phase I metabolism of aflatoxin B1 in commercial poultry species

Research conducted by our group (Lozano & Diaz, 2006) showed that the microsomal and cytosolic biotransformation of AFB1 in chickens, quail, ducks and turkeys results in the formation of two major metabolites: AFBO (microsomes) and AFL (cytosol). The relative *in vivo* sensitivity to AFB1 in these species corresponds to ducks > turkeys > quail > chicken, and the aim of this work was to try to correlate the toxicological biochemical findings with the reported *in vivo* sensitivity. Using liver microsomal incubations it was demonstrated that turkeys produce the highest amount of AFBO (detected either as AFB1-dhd or AFB1-GSH) while chickens produce the least; duck and quails produce intermediate amounts (Fig. 2). AFB1 consumption (rate of AFB1 disappearance from the microsomal incubations) was also highest in turkeys, lowest in chickens and intermediate in quail and ducks. Interestingly, these two variables (AFBO production and AFB1 consumption) were highly correlated in the four species evaluated (Fig. 2).

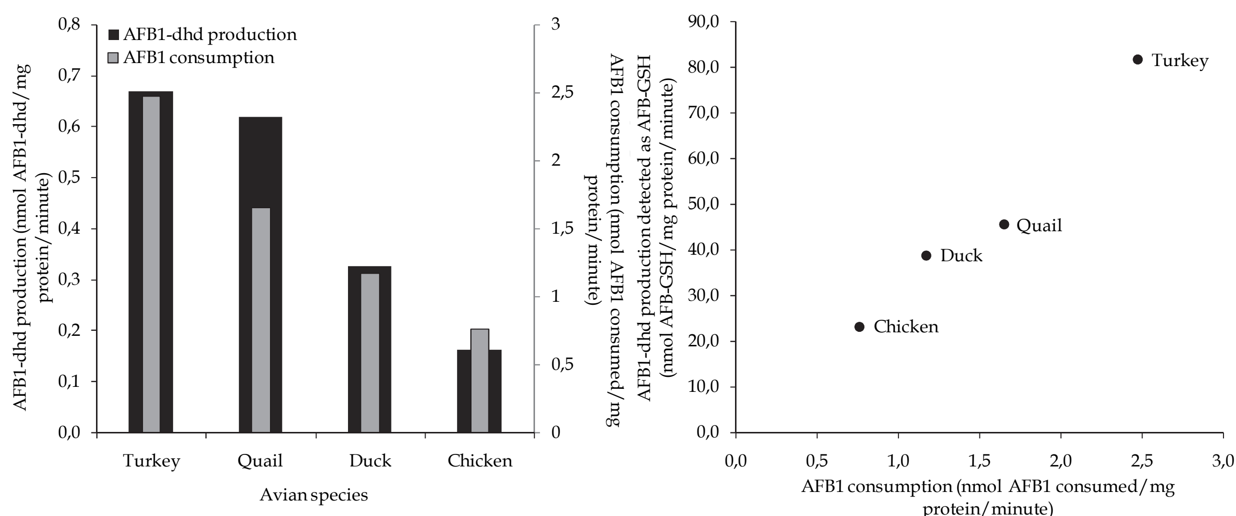

Fig. 2. AFBO production (measured as AFB1-dhd) and AFB1 consumption in turkey, quail, duck and chicken microsomal incubations (left) and relationship between AFBO formation (measured as AFB1-GSH) and AFB1 consumption (right).

Both biotransformation variables (AFBO formation and AFB1 disappearance) correlate well with the *in vivo* sensitivity observed for turkeys, quail and chickens (turkeys being highly sensitive, chickens being the most resistant and quail having intermediate sensitivity). However, other factor(s) besides AFBO formation and AFB1 consumption must play a role in the extraordinary high sensitivity of ducks to AFB1 because these biochemical variables did not correlate with the *in vivo* sensitivity for this particular species [ducks exhibit the highest *in vivo* sensitivity to AFB1 among these poultry species, not turkeys, as Rawal et al. (2010a) affirm].

The cytosolic metabolism of AFB1 in the same four poultry species shows a different trend compared with the microsomal metabolism (Fig. 3). Turkeys are again the largest producers of the cytosolic metabolite AFL but are followed by ducks, chickens and quail (instead of quail, ducks and chickens as it is observed for microsomal metabolism). As discussed before (see section 2.1.4), AFL is a toxic metabolite of AFB1 and it cannot be considered a detoxication product; therefore, it would be expected that sensitive species produce more AFL than resistant ones. However, no correlation between AFL production and *in vivo* sensitivity was observed. For instance, quail produced the lowest amount of AFL and it exhibits intermediate sensitivity to AFB1, while ducks, which are the most sensitive species, produced much less AFL than turkeys. AFB1 consumption by cytosol (rate of AFB1 disappearance from cytosolic incubations) was highest for the chicken, followed by turkeys, ducks and quail and there was no correlation between AFL formation and AFB1 consumption (Fig. 3). Further, as it was observed for AFL formation, there was no correlation between AFB1 disappearance from cytosol and *in vivo* sensitivity to AFB1. Investigation of the potential conjugation reactions of AFL might clarify the role of AFL formation on the *in vivo* sensitivity to AFB1 in poultry. It is possible that the high resistance of chickens to AFB1 might be due to an efficient reduction of AFB1 to AFL followed by conjugation and elimination of the AFL conjugate. Interestingly, it has been demonstrated that chick liver possesses much higher AFB1 reductase activity than duckling or rat liver (Chen et al., 1981).

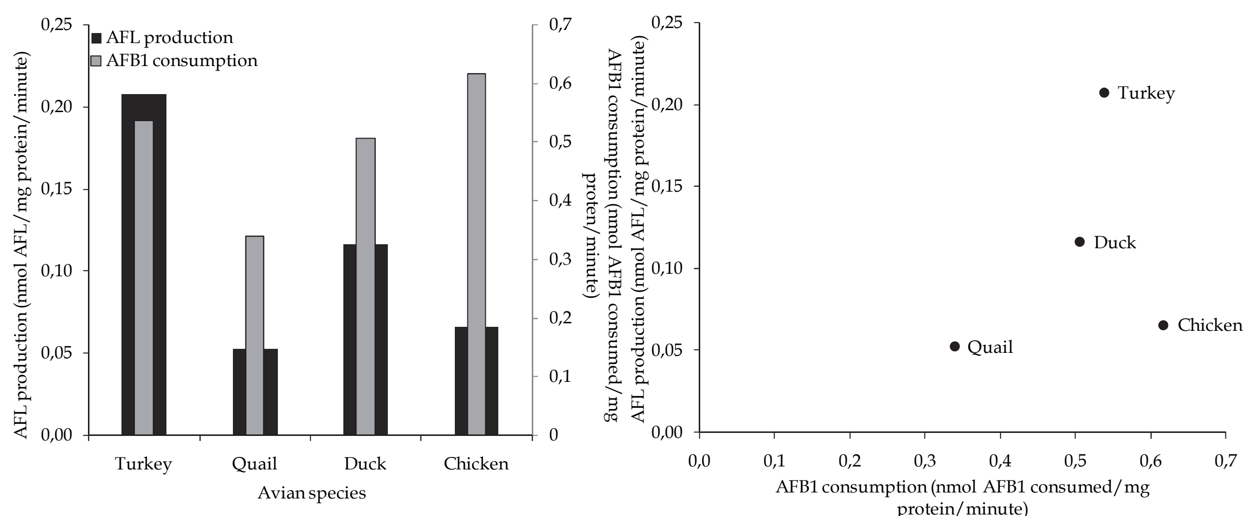

Fig. 3. AFL production and AFB1 consumption in turkey, quail, duck and chicken cytosolic incubations (left) and relationship between AFL formation and AFB1 consumption (right).

Even though the studies of Klein et al. (2000) in turkeys, and Lozano & Diaz (2006) in turkeys, chickens, ducks and quail had clearly demonstrated that hepatic microsomes from poultry were capable of bioactivating AFB1 into AFBO, there was only scarce information on the specific CYP450 enzymes responsible for this biotransformation reaction and it was limited to turkeys (Klein et al., 2000; Yip & Coulombe, 2006). In contrast, in humans, at least three CYP450s had been identified as responsible for AFB1 bioactivation to AFBO (CYP1A2, CYP2A6 and CYP3A4) (Omiecinski et al., 1999; Hasler et al., 1999), and there was evidence that the CYP3A4 human enzyme was the most efficient (Guengerich & Shimada, 1998). In view of this lack of information a series of studies were conducted by our group (Diaz et al., 2010a, 2010b, 2010c) in order to investigate which specific avian CYP450 orthologs were responsible for the bioactivation of AFB1 into AFBO. These studies were conducted by using specific human CYP450 inhibitors (α-naphthoflavone for CYP1A1/2, furafylline for CYP1A2, 8-methoxypsoralen for CYP2A6 and troleandomycin for CYP3A4), by correlating AFBO formation with human prototype substrate activity (ethoxyresorufin *O*-deethylation for CYP1A1/2, methoxyresorufin *O*-deethylation for CYP1A2, coumarin 7-hydroxylation for CYP2A6 and nifedipine oxidation for CYP3A4) and by investigating the presence of ortholog proteins in avian liver by immunoblot using antibodies specific against human CYP1A1, CYP1A2, CYP2A6 and CYP3A4. These series of studies revealed that the avian CYP2A6 ortholog is the main CYP450 enzyme responsible for the bioactivation of AFB1 into its epoxide form in all poultry species investigated. Evidences for this conclusion include the fact that AFBO production was inhibited by the CYP2A6 inhibitor 8-methoxypsoralen and that a significant correlation existed between coumarin 7-hydroxylation and AFB1 epoxidation activity in all species studied (Table 1). The finding of a protein by immunoblot using rabbit anti-human CYP450 polyclonal antibodies directed against the human CYP2A6 enzyme confirmed the existence of an immunoreactive protein in all birds studied (the putative CYP2A6 avian ortholog). These studies demonstrated for the first time the existence of the CYP2A6 human ortholog in avian species and they were the first reporting the role of this enzyme in AFB1 bioactivation in avian liver.

Poultry Species	7-Ethoxyresorufin-*O*-demethylation (CYP1A1/2)	7-Methoxyresorufin-*O*-demethylation (CYP1A2)	Coumarin 7-hydroxilation (CYP2A6)	Nifedipine oxidation (CYP3A4)
Turkey	0.32	-0.76	**0.90**	0.73
Quail	-0.09	0.21	**0.78**	0.07
Duck	**0.81**	**0.82**	**0.68**	**0.88**
Chicken	0.25	0.46	**0.83**	-0.24

Table 1. Pearson correlation coefficients for aflatoxin B1 epoxidation vs. prototype substrate activities of selected human CYP450 enzymes. Correlations in bold numbers are statistically significant ($P \leq 0.01$).

In turkeys, quail and chickens the CYP1A1 ortholog seems to have a minor role in AFB1 bioactivation, while in ducks there are evidences that AFB1 bioactivation is carried out not only by the CYP2A6 and CYP1A1 orthologs but also by the CYP3A4 and CYP1A2. The fact that four CYP450 enzymes are involved in AFB1 bioactivation in ducks could partially explain the high sensitivity of this species to AFB1. In turkey liver, AFB1 was

reported to be activated to AFBO by a CYP 1A ortholog (Klein et al., 2000) that later was identified as the turkey CYP1A5 on the basis of its 94.7% sequence identity to the CYP1A5 from chicken liver (Yip & Coulombe, 2006). This enzyme was suggested to correspond to the human ortholog CYP1A2 (Yip & Coulombe, 2006). However, using human prototype substrates and inhibitors, Diaz et al. (2010a) found evidence for AFB1 bioactivation by CYP1A1 but not by CYP1A2 in turkey liver microsomes. Interestingly, the turkey CYP1A5 has a high amino acid sequence homology not only with the human CYP1A2 (62%) but also with the human CYP1A1 (61%) as reported by the UniProtKB database (http://www.uniprot.org) and the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). It is possible that the turkey CYP1A5 enzyme cloned by Yip & Coulombe (2006) may in fact correspond to the human CYP1A1 ortholog or, even more interesting, to both the CYP1A1 and 1A2 human orthologs. Murcia et al. (2011) found a very high correlation between EROD (CYP1A1/2) and MROD (CYP1A2) activities in turkey liver microsomes ($r=0.88$, $P<0.01$) a finding that suggests that CYP1A1 and CYP1A2 activities in turkey liver are catalyzed by the same enzyme (i.e., the avian CYP1A5). The role of CYP1A5 turkey activity on the bioactivation of AFB1 in turkeys is further supported by the work of Guarisco et al. (2008) who found that dietary supplementation of the antioxidant butylated hydroxytoluene (BHT) partially protected against the adverse effects of AFB1, an effect that was accompanied by a reduction in EROD and MROD activities in the liver.

In regards to CYP3A4, Klein et al. (2000) found that this enzyme plays a minor role in the bioactivation of AFB1 in turkeys. This finding, however, could not be substantiated by Diaz et al. (2010a) who found no correlation between nifedipine oxidation (an indicator of CYP3A4 activity) and AFBO formation, and no effect on AFBO formation when the prototype inhibitor of human CYP3A4 activity troleandomycin was used. Induction of CYP3A4 activity by BHT in turkeys (as evidenced by increased nifedipine oxidation) was correlated with decreased *in vivo* adverse effects of AFB1 (Guarisco et al., 2008), which further supports the notion that CYP3A4 is not involved in AFB1 bioactivation in turkeys. This finding is of interest since CYP3A4 has been shown to be an activator of aflatoxins B1 and G1 in humans and other species (Parkinson & Ogilvie, 2008); however, in humans, CYP3A enzymes can form the AFBO only at relatively high substrate concentrations (Ramsdell et al., 1991). In contrast with turkeys, however, CYP3A4 does appear to play a role on AFB1 bioactivation in ducks (Diaz et al., 2010b). Duck microsomes show a high correlation between nifedipine oxidation and AFB1 epoxidation (Table 1) but the use of the specific human CYP3A4 inhibitor troleandomycin did not reduce AFBO production (Diaz et al., 2010b). A recent study reports the cloning of a turkey CYP3A37 expressed in *E. coli* able to biotransform AFB1 into AFQ1 (and to a lesser extent to AFBO) with an amino acid sequence homology of 76% compared with the human CYP3A4 (Rawal et al., 2010b). In this study the use of the inhibitors erythromycin (specific for human CYP3A1/4) and 17α-ethynylestradiol (specific for human CYP3A4) completely inhibited the production of AFBO. The results of the studies conducted with the CYP3A4 turkey ortholog indicate that the turkey enzyme is not sensitive to the CYP3A4 human inhibitor troleandomycin but that it is sensitive to erythromycin and 17α-ethynylestradiol. If this lack of sensitivity to troleandomycin also applies for the duck CYP3A4 ortholog, this could explain the results of Diaz et al. (2010b) previously described. In regards to the findings of Rawal et al. (2010b), it is important to note that the fact that a cloned gene expressed in a heterologous system (e.g.

E. coli) biotransforms AFB1 does not necessarily mean that this is a reflection of the situation in a biological system. Heterologously expressed enzymes typically exhibit a much different behavior than native ones. For instance, the enzyme affinity for nifedipine oxidation activity in turkey liver microsomes is much higher than that of the heterologously expressed turkey CYP3A37 (K_M values of 21 and 98 µM, respectively) (Murcia et al., 2011; Rawal et al., 2010b). Both *in vivo* studies and *in vitro* hepatic microsomal metabolism suggest that the turkey ortholog of the human CYP3A4 is most likely not involved in AFB1 bioactivation.

Large interspecies differences in enzyme kinetics and enzymatic constants for AFB1 epoxidation also exist among poultry species (Diaz et al., 2010a, 2010b, 2010c). Non-linear regression of these variables showed that turkey enzymes have the highest affinity and highest biotransformation rate of AFB1, as evidenced by the lowest K_M and highest V*max* values compared with quail, duck and chicken enzymes (Fig. 4). This finding correlates well with the high *in vivo* sensitivity of turkeys to AFB1. In contrast, chicken enzymes showed the lowest affinity (highest K_M) and lowest biotransformation rate (lowest V*max*), findings that also correlate well with the high resistance of chickens to AFB1. Quail, a species with intermediate sensitivity to AFB1, also exhibited intermediate values for enzyme affinity and catalytic rate. In ducks, however, the enzymatic parameters of AFB1 biotransformation could not explain their high sensitivity to AFB1 since they had the second lowest catalytic rate (V*max*) and the third lowest enzyme affinity (K_M) for AFB1 (it was expected that ducks had the highest V*max* and lowest K_M).

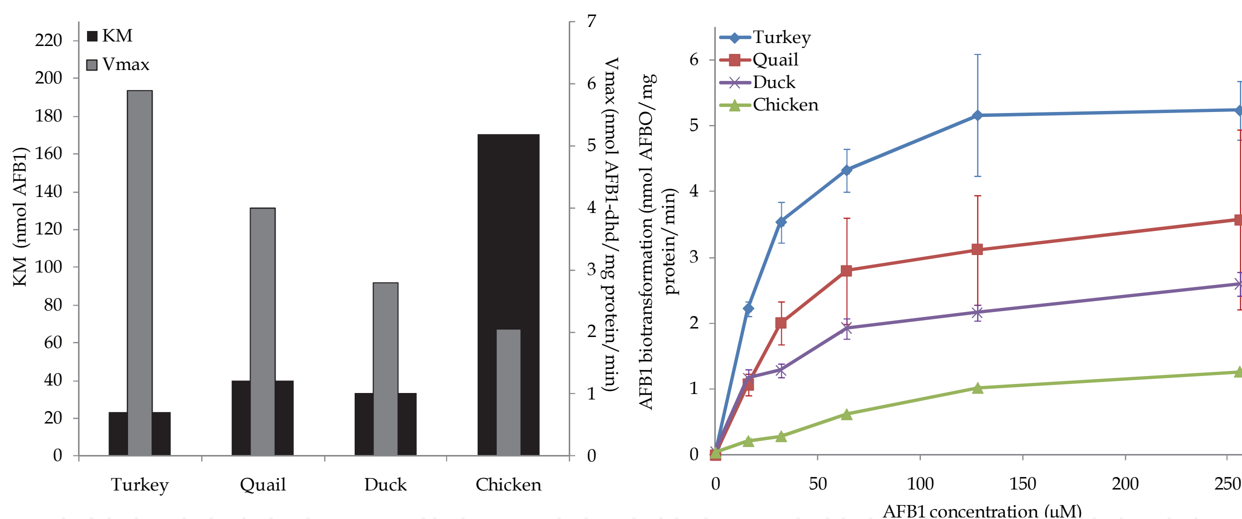

Fig. 4. Enzymatic constants K_M and V*max* (left) and enzyme kinetics (right) of aflatoxin B1 epoxidation activity in liver microsomes of four poultry species.

Information for some CYP450 enzymes in turkey, chicken and quail can be found in the databases mentioned before (i.e. UniProtKB and GeneBank). Sequences for turkey CYP1A5 and CYP3A37, chicken CYP1A1, CYP1A4, CYP1A5 and CYP3A80, and Japanese quail CYP1A1, CYP1A4 and CYP1A5 have been reported. Surprisingly, however, there are no sequences reported for CYP2A6 despite the biochemical evidence for its existence in birds. As expected, a comparison of the human and avian CYP450 enzymes reveals a higher similarity among avian orthologs compared to human orthologs. Differences in protein structure between avian and human CYP450 enzymes could explain the differential response of the avian CYP450 orthologs to the human prototype substrate and inhibitors, which, nevertheless, are still useful tools in the investigation of CYP450 enzymes in birds.

3.2 Trapping and conjugation of aflatoxin B1 metabolites in commercial poultry species

Even though the ability to bioactivate AFB1 into AFBO is critical in the toxicological response to AFB1, in some species it is the ability to trap AFBO with GSH which ultimately determines the degree of AFB1-induced liver damage. For instance, both rats and mice exhibit high bioactivation rates of AFB1; however, mice are resistant to the hepatic carcinogenic effects of AFB1 while rats develop hepatocellular carcinoma. The reason for this differential response lies in the constitutive expression of high levels of an Alpha-class GST that catalyzes the trapping of AFBO in the mouse that is only expressed at low levels in the rat (Esaki & Kumagai, 2002). In fact, the induction of this enzyme in the rat leads to resistance to the development of hepatic carcinoma. Interestingly, in non-human primates it is Mu-class GSTs the ones responsible for AFBO trapping with GSH (Wang et al., 2000).

Turkeys are the only poultry species in which the role of GST-mediated trapping of AFBO with GSH has been investigated (Klein et al., 2000; 2002). At least six Alpha-class GSTs have been isolated, amplified and fully characterized from turkey, which exhibit similarities in sequence with human Alpha-class GSTs ranging from 53% to 90% (Kim et al., 2010). However, no soluble GST activity towards microsomally activated AFB1 has been found in liver cytosol from one-month old male turkeys (Klein et al., 2000), a finding that was later confirmed in male turkeys 9, 41, and 65 days of age (Klein et al., 2002). GSTs from the liver of one-day-old chicks (Chang et al., 1990) and nucleotide sequences of Alpha-class (Liu et al., 1993), Mu-class (Liu & Tam, 1991), Theta-class (Hsiao et al., 1995) and Sigma-class (Thomson et al., 1998) GSTs from chicken liver have been characterized, but there are no reports for their role in AFBO trapping with GSH.

The role of other conjugation reactions on AFB1 metabolism in poultry is still uncertain. Liver UGT activity and sulphotransferase (SULT) activity have been reported in bobwhite quail (Maurice et al., 1991), and in chickens, ducks and geese (Bartlet & Kirinya, 1976). However, no research on glucuronic acid conjugation or sulfate conjugation of AFB1 metabolites has been conducted in any commercial poultry species.

4. Concluding remarks

Research conducted recently has shown that there are clear differences in oxidative/reductive AFB1 metabolism that could explain the differential responses to AFB1 observed *in vivo* among turkeys, quail and chickens, but not ducks. The existence of a clear toxicological biochemical pattern that explains AFB1 sensitivity in three out of four species may be related to their different phylogenetic origins: Turkeys, quail and chickens are phylogenetically close to each other (all belong to the order Galliformes, family Phasianidae), but distant from ducks (order Anseriformes, family Anatidae). It is also interesting to note that while CYP2A6 (and to a lesser extent CYP1A1) is the major enzyme responsible for AFB1 bioactivation in the Galliformes studied, four enzymes (CYP1A1, 1A2, 2A6 and 3A4 ortholog activities) appear to be responsible for AFB1 bioactivation in ducks.

In regards to conjugation reactions, it has been demonstrated that turkeys do not express the GSTs responsible for AFBO trapping. However, the role of AFBO trapping by GSH has not been investigated in other poultry species and no information on the possible conjugation reactions of AFL has been reported for any avian species, either. Another

pathway of AFBO metabolism that has not been investigated in poultry is the formation of AFB1-dhd and dialcohol (Fig. 1). Formation of AFB1-dhd may occur either spontaneously or through the action of a microsomal epoxide hydrolase (EPHX) and the possible role of EPHX in AFB1 biotransformation in birds is still unknown. The alternative pathway for AFB1-dhd, that is, the formation of an aflatoxin dialcohol through the action of the cytosolic enzyme AFAR, has not been investigated either. This topic is important to investigate since the dialcohol does not bind with proteins and therefore constitutes a true detoxication product.

Extra-hepatic localization of enzymes responsible for the biotransformation of AFB1 may also play a role in the differential response to AFB1 in birds. For instance, in humans CYP3A4 is the major enzyme involved in AFB1 bioactivation (Ueng et al., 1995) and this enzyme is highly expressed not only in the liver but also in the gastrointestinal tract. This "first-pass" effect may affect the absorption of unaltered AFB1 and therefore its ability to reach its target organ in humans. Finally, the so-called Phase III metabolism (basolateral and canalicular transport of xenobiotics) has been shown to determine sensitivity or resistance to xenobiotics in several experimental models. For instance, collie dogs are extremely sensitive to ivermectin due to the low expression of the transporter protein MDR1 (Diaz, 2000). The role of the translocation of AFB1 and its metabolites on AFB1 sensitivity/resistance needs to be investigated.

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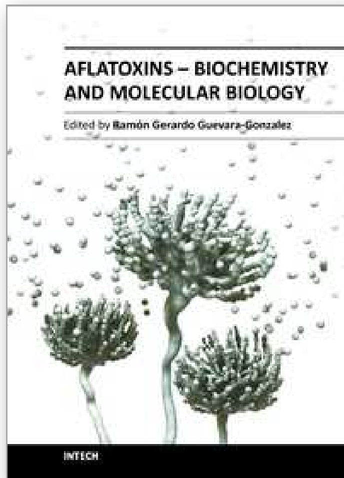

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Aflatoxins â€“ Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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