Effects of Aflatoxin B1 on liver phase I and phase II enzymes induced in vitro on Sparus aurata hepatocytes primary culture

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Abstract

Among all known mycotoxins, aflatoxin B1 (AFB1) has been one of the most extensively studied due to its hepatotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressant effects (IARC, 1993; Smela et al., 2001; Puschner, 2002; Bennet and Klich, 2003). Aflatoxins have a significant impact on fish farming production since they can cause high mortality and a decline in fish stock quality, and are, therefore, a considerable problem in aquaculture systems (Naylor et al., 2009; Hassan et al., 2010). Many feed ingredients used in aquaculture (cottonseed, peanuts, corn, soybean, maize, rice, dried fish, shrimp and fishmeals) are considered the main source of AFB1, contamination (Ellis et al., 2000; Cagauan et al., 2004), and because of the growing use of plant origin ingredients in aquafeed formulations, the problem of mycotoxin contamination in aquaculture has grown (Tacon et al., 1995; EFSA, 2004; Spring, 2005). In the last decade, several authors investigated the modulation of AFB1 metabolism in salmonid fish such as rainbow trout, coho salmon, as well as in zebrafish and channel catfish (Santacroce et al., 2008). Nevertheless, metabolic and toxicological studies on aflatoxins in marine farmed species are fragmented, restricted to in vivo trials and only examine a few fish species (Santacroce et al., 2011a). In addition, there is very limited information regarding the impact of AFB1 on Sparus aurata in literature, except for in vivo experiments (El-Sayed and Khalili, 2009). AFB1 is metabolised in liver cells and the pathway is mainly characterised by two catalyst systems: the phase I or activation phase, mediated by cytochrome P450-dependent monooxygenases (CYPs enzymes), and phase II or detoxification phase, comprising the uridine diphosphate-(UDP), glucuronyl-transferase (UDPGTs enzyme) and glutathione-(GSH) S-transferase (GSTs enzymes) (Livingstone, 1998). The family of cytochrome P450s comprises a group of isoenzymes involved in the bioactivation and detoxification process of xenobiotic metabolism. They oxidise AFB1; determining both the toxic metabolite aflatoxin B2,8,9-epoxide (AFBO) by epoxidation, the less toxic metabolites AFM1 and AFQ1 by hydroxylation, and AFB1 by demethylation enzymatic reaction (Ramsdell et al., 1991). Several CYP isoforms have been shown to take part in AFB1 metabolism in trout as in humans (Williams and Buhrer, 1983; Gallagher et al., 1996). Buhrer et al. (1994) discovered that the majority of AFB1, 8,9-epoxide in trout was catalysed by CYP2K1. Subsequently, Yang et al. (2000) reported that CYP2K1, similarly to the human counterpart CYP1A2, was primarily responsible for carcinogenic exo-epoxide metabolite production. The cytosolic GSTs are a multigene family of enzymes that catalyse the conjugation of reduced glutathione to substrates as AFBO (Berhane et al., 1994). GSTs have been widely studied in rodents who account for at least 20 coding genes (Leaver et al., 1997). Less is known about GSTs from other organisms; therefore, records concerning fish GSTs are relatively poor (Gallagher et al., 1999). Overall, most of those fish studies concerned the molecular identification of GST sequences and their purification and characterisation (Kim et al., 2010). However, to date, at least four classes of GST isoforms (α, μ, θ and π) have been identified in several fish species (George and Taylor, 2002; Blanchette et al., 2007), such as salmonids (θ-class GST) (Dominey et al., 1991), channel catfish (θ- and π-class GST) (Gallagher et al., 1996), sea bass (Dicentrarchus labrax) (α- and μ-class GST) (Angelucci et al., 2000), plaice (Pleuronectes
(0-like class GST-A) (Leaver et al., 1993a), English sole (Parophrys vetulus) (0-like class GST-B) (Leaver et al. 1993a, 1993b, 1997; George, 1994), and starry flounder (Platichthys stellatus) (0-like class GST-A) (Gallagher et al., 1999). Gallagher et al. (1999) also found that English sole and starry flounder, two closely related species of benthic fish, both showed hepatic GST-mediated catalytic activity toward AFB1. Overall, the selective sensitivity towards AFB1 in fish appears to be due to different enzymes involved in AFB1 metabolism (Leaver et al., 1992). According to the recent European directives on the desirable replacement of the use of animals with new screening and testing systems based on fish in vitro models (Castàno et al., 2003; EC, 2006), this study uses a new metabolically active in vitro liver model, such as S. aurata hepatocytes in primary culture (Santacroce et al., 2011b). Primary liver cells represent an innovative tool and offer a significant functional advantage over cell lines in both dose-response and metabolic studies because they are considered to be more sensitive, with a high metabolic capacity compared to cell lines (Mothersill and Austin, 2003). Primary cells keep most of their physiological properties and could be used as a bridge between cell lines and in vivo systems (Chen and Bunce, 2003).

The economic importance of S. aurata as one of the most farmed species in the Mediterranean, and the lack of in vitro studies in literature, justifies the relevance of this research concerning the effects of AFB1 on this species. Furthermore, S. aurata has been shown to be a very sensitive species in terms of liver biotransformation responses (Banni et al., 2009). Therefore, this study aimed to examine the effects of acute and chronic AFB1 exposure on CYP1A and GST enzymes induced in vitro on S. aurata hepatocytes primary culture by immunoblot analysis, and apoptosis induction by immunofluorescence analysis.

Materials and methods

Animals

Gilthead seabream juveniles (Sparus aurata) (n=45) with mean body weight 25±4 g were obtained from a local fish farm (Panitica Pugliese, Brindisi, Italy), caged in oversaturated natural seawater (O2 at 10 mg/L, 37.3% salinity) holding tanks at a temperature of 18±1°C with a density of 10 kg/m³, for no more than 90 min, and then transferred to the laboratory. On their arrival, fish were anesthetized by immersion in a tank with seawater plus tricaine methane sulphonate (MS 222, 0.02%; Sigma), were taken out singularly, wiped with 70% ethanol, and rapidly sacrificed by decapitation.

Isolation and culture of liver cells from S. aurata

Liver cells were isolated and cultured as described by Centoducati et al. (2009). In brief, cells were isolated by immersion of the dissected, washed and mashed livers with a digestion medium (7 mM CaCl₂, 200 U/mL penicillin, 200 μg/mL streptomycin, 200 μg/mL amphotericin B, 100 μg/mL gentamicin, 10 mM HEPES, 25 mM NaHCO₃ in Leibovitz’s L-15) supplemented with a cocktail of four enzymes (0.1% collagenase type IV, 0.05% hyaluronidase type IV-S, 0.4% dispase type II, 0.03% DNase type I). The digestion mixture was blocked and then filtered throughout 230 μm and 104 μm stainless steel filters, centrifuged twice in cold 1 x phosphate-buffered saline (PBS) at 80 g at 4°C for 5 min. Collected pellets were washed in cold PBS with 0.45 μg/mL Dnase I to dissociate cell clumps, and centrifuged. Following digestion phases, purified hepatocytes were suspended in basal nutrient culture medium (L-15 with 2 mM glutamine, 10% FBS, 100 U/mL penicillin/100 μg/mL streptomycin/100 μg/mL amphotericin/50 μg/mL gentamicin, 1 mM Na pyruvate, 5 mM d-glucose, 10 mM HEPES, 12 mM NaHCO₃) supplemented with 0.05% ITS plus, 0.01 mM MEM non-essential amino acid (MEM-NEA; BioWhittaker), 0.01 mM MEM-vitamin mix (BioWhittaker), 0.1 mM ascorbic acid, 0.01 μg/mL epidermal growth factor (EGF) and 0.005 μg/mL hepatocyte growth factor (HGF), and seeded at a density of 3x10⁵ cells/cm² in 96-well plates pre-coated with collagen I and cultivated in a refrigerated incubator (3% CO₂) at 18°C. Cells were allowed to attach for 12 h, then fresh medium was added and then replaced every 48 h, till the complete monolayer formation on Day 4 after seeding. Unless specified, all chemicals were from Sigma Aldrich Ltd., Milan, Italy.

AFB1 exposure of S. aurata hepatocytes in primary culture

Based on previous results (Santacroce et al., 2011a), two main AFB1 doses were checked in order to evaluate the effects at cell lethality concentration (5 μg/mL) and at NOEC (no observable adverse effect concentration) value (5 ng/mL), the latter being identified as the first cut off for apoptosis induction. Confluent primary monolayer cultures of hepatocytes were exposed to AFB1 at doses of 5 μg/mL and 5 ng/mL for 24, 48 and 72 h in order to evaluate the acute and chronic, cytotoxic and metabolic responses. The experiments were performed in triplicate for each dosage group plus one control group. After each exposure, hepatocytes were examined for morphological alterations using a phase-contrast microscope (Motic AE30X1 inverted Epi-Fluorescence microscope) with a MOTICAM 3000C-cooled CCD camera (Seneco, Milan). In addition, apoptosis was evaluated by assessing the phosphatidylserine inversion at the cell surface of dying apoptotic cells, using the Annexin V-Cy3.18/ 6-carboxyfluorescein diacetate (CFDA) kit (Sigma Aldrich Ltd., Milan, Italy) by fluorescence microscopy. This assay allowed us to identify the sub-acute cytotoxicity by differentiating early apoptotic cells from viable and necrotic ones.

Preparation of cell lysates from AFB1 exposed hepatocytes primary cultures

After analysis of morphological alterations, cells were lysed by adding lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% Triton X-100) directly to the monolayer cultured in the well. The obtained suspension was then collected in an ice-cooled tube and centrifuged at 13,000 rpm for 20 min at 4°C to remove cell debris. Supernatants were then quickly stored at -80°C until further processing. Protein concentration was determined according to Bradford (1976) using Bio-Rad assay reagent (Bio-Rad Laboratories, Italy).

Western blot analysis of liver enzymes

Western blot analysis was performed to evaluate the expression of CYP1A and GST enzymes involved in the liver biotransformation process after acute and chronic exposure of S. aurata hepatocyte primary cultures to AFB1. Cell lysates were thawed, concentrated by ice-cold acetone precipitation for 1 h at -20°C and centrifuged at 13,000 rpm at 4°C for 10 min. Pellets were solubilised in 2 x loading buffer containing 125 mM Tris pH 6.8, 10% glycerol, 4% sodium dodecylsulfate, 1% b-mercaptoethanol, and 0.003% bromophenol blue. Subsequently, proteins (40 μg/lane) were separated by 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970) in a Bio-Rad Mini-Protein 3 system at 120 V for 1 h. Transfer of the protein to polyvinylidene difluoride (PVDF) membrane was performed in a Bio-Rad Mini-Blot apparatus at 100 V for 1 h in modified Towbin’s Transfer
Buffer (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3). Ten µL of pre-stained broadrange Precision Plus Protein WesternC molecular standards (range 10-250 KDa, Bio-Rad Laboratories) were used as protein weight marker. The blots where then probed overnight with a 1:200 diluted anti-fish CYP1A (rabbit pAb; Biosense) and 1:500 diluted anti-GST (rabbit pAb; Santa Cruz) primary antibodies, and incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG antibody (1:30,000 diluted) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The immunoreactions were visualised using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, and bands were revealed by fluorography on Kodak BioMax light films (Sigma Aldrich, Milano, Italy) exposed for 30 min in a Kodak BioMax cassette.

**Results and discussion**

Morphological and immunocytochemical analysis of AFB$_1$ exposed hepatocytes primary culture.

In the present study, we examined both short- and long-term (24 and 72 h, respectively) survival of $S$. aurata hepatocytes in primary culture upon AFB$_1$ incubation at low and high toxic concentrations. Non-exposed hepatocytes, representing the control group, retained their plasticity and monolayer integrity (Figure 1 A,B). Results showed that AFB$_1$ cytotoxicity increased in a time and dose dependent manner. At the highest concentration, both in short- and long-term incubation, AFB$_1$ exposure resulted in a rapid loss of hepatocyte survival, revealing that the potency increased over time. In cells exposed to 5 µg/mL of toxin, contrast-phase analysis promptly showed a significant lethal effect within 24 h (Figure 1C). Hepatocyte primary culture showed loss of cell membrane integrity and cell death for primary necrosis (Figure 1C) and this was confirmed by immunofluorescence cell death assay with Annexin V-Cy3.18 positive staining (Figure 2C). After 72 h, cell death became more severe as a consequence of prolonged AFB$_1$ exposure (Figure 1D), exhibiting almost 100% of Annexin V-Cy3.18 positive staining compared to the 70% of hepatocytes treated for 24 h (Figure 2D). At low concentration (NOEC value 5 ng/mL), although the monolayer apparently appeared unaffected after 24 h of exposure, cell shrinkage and pyknosis were observed (Figure 1E). Such cell sufferance appeared greater after 72 h as revealed by areas of extensive cell detachment (Figure 1F). We then assessed whether cell death was due to primary or secondary necrosis, the second hypothesis involving an apoptotic event. Hepatocytes that apparently did not respond quickly to AFB$_1$ exposure, remaining viable within the first 24 h, showed early signs of apoptosis (Figure 2E). After 24 h at 5 ng/mL, at least 50% of cells were orange-coloured and marked positively for both Annexin V-Cy3.18 and CFDA, thus demonstrating a timely induction of apoptosis (Figure 2E). These cells died during the following 72 h, appearing completely red coloured, signifying a delayed mortality for late apoptosis (Figure 2F). This suggests that the toxic damage is not reversible but permanent, as the cellular repair systems are unable to recover the induced toxic insult and, finally,

![Figure 1. Morphological damage of AFB$_1$ on hepatocytes primary culture exposed for 24 and 72 h at doses of 5 µg/mL and 5 ng/mL. Contrast-phase analysis: original magnification (A,B) 200x; (C-F) 400x. CN, control.](image1)

![Figure 2. AFB$_1$ apoptotic damage on hepatocytes primary culture after 24 and 72 h by immunofluorescence analysis: original magnification (A, B, D-F) 200x; (C) 400x. A-B) Normal control (CN) without AFB$_1$ exposure (annexin V-/6-CFDA+; living cells green). Hepatocytes cultured in 96-well plates were exposed to the toxin at doses of 5 µg/mL for 24 and 72 h (C-D) (annexin V+/6-CFDA+; necrotic cells red coloured); 5 ng/mL for 24 h (E) (annexin V+, 6-CFDA+; early apoptotic cells orange coloured) and 72 h (F) (annexin V+, 6-CFDA+; necrotic cells red coloured). Methanol-fixed cells were stained with Annexin V- Cy3.18.](image2)
cells died from secondary necrosis. Chronic exposure to sub-lethal doses of AFB1 can irreparably affect seemingly viable hepatocytes at a gene level resulting in cell death due to the subsequent apoptotic process, since it is generally assumed that the apoptosis event represents an early DNA lesion. Our results demonstrate that adverse effects induced by AFB1 exposure significantly worsen over time even at a lower dose. Other authors have previously shown that AFB1 triggers a cytotoxic effect in a time and dose dependent manner in mammal, rat and cattle hepatocytes (Metcalfe and Neal, 1983; Kuilman et al., 2000). In fish, AFB1 damage has been intensively investigated in vivo with short-term trials (Jantrarotai and Lovell, 1990; Chávez-Sánchez et al., 1994; Takahashi et al., 1996; Sahoo et al., 2003) while only recently long-term experiments have been performed in tilapia and sea bass (El-Sayed and Khalil, 2009; Deng et al., 2010).

Western blot analysis of liver enzymes

The analysis of the effects of AFB1 on hepatic metabolism in S. aurata has been performed evaluating the expression of two important enzymes involved in the liver biotransformation process, CYP1A and GST. Several types of CYP1A and GST isoforms were detected with protein expression profiles varying in a dose and time dependent manner. In particular, expression profiles of CYP1A showed that, after exposure to 5 µg/mL of AFB1, the band corresponding to CYP1A precursor (250 KDa) was constantly detected for all the exposure times compared to control groups where immunodetection decreased over time (Figure 3A and B). Other CYP1A isoforms were detected at 24 h with the band of 57 KDa more intensely expressed than control over all other bands (75, 50 and 46 KDa). No changes in immunodetection were observed for bands of 75 and 57 KDa over the following 48 h while a peak of expression was detected at 46 and 50 KDa bands compared to those at 24 h. At the end of exposure time (72 h), the expression pattern for all the isoforms had decreased (Figure 3B). At 5 ng/mL of AFB1, after 24 h, bands of 250 and 57 KDa showed greater expression than control, while the other isoforms showed a similar pattern. Furthermore, the expression of another two bands at 40 and 130 KDa was observed. After 48 h, the expression of high molecular weight bands (250 and 130 KDa) decreased, while expression of all the others was increased compared to the 24 h group. Finally, after 72 h, there was no change in the expression pattern for all the isoforms compared to the 48 h group (Figure 3C).

By comparing same times of exposure at the two treatment doses, expression of bands appeared inversely related to dose, since immunostaining detection significantly decreased in parallel to an increase in dose. At 5 µg/mL, the expression of CYP1A decreased with an increase in exposure time. By contrast, at 5 ng/mL, CYP1A expression was directly related to exposure time. Similarly, Deng et al. (2010) observed a decrease in CYP1A activity in tilapia dietary exposed to high doses of AFB1 over a long period, supporting the hypothesis of an indirect correlation between the induction of CYP1A and the dose and time of exposure. The decrease in expression of CYP1A over time, along with high toxic concentration, could probably support the hypothesis of a detoxification function of this enzyme in AFB1 metabolism, thus allowing the biotransformation of aflatoxin B1-8,9-epoxide in a less toxic metabolite such as AFM1 as pointed out by Ramsdell et al. (1991). In addition, Bailey et al.
(1996) identified another CYP isoform in rainbow trout, called CYP2K1, involved in the biotransformation of AFB1, able to generate toxic reactive intermediates reliable for the adverse effects that invariably result in toxic effects. Again, Takahashi et al., (1995) found that adding indole-3-carbinol to the diet of rainbow trout induced hepatic CYP1A, thus reducing AFB1 hepatocarcinogenesis. Therefore, in our results, the lethal damage caused by a high dose of AFB1 on cells over time can be related to the decrease in CYP1A induction, with its important function in detoxification.

According to the expression profiles of GST, after 24 h of exposure to 5 µg/mL of AFB1, the isoforms of 250, 65 and 49 KDa were equally detected compared to the control group (Figure 4A,B). After 48 h, the expression of the band of 250 KDa decreased; there was no change in isoform of 65 KDa, whereas the band of 49 KDa reached the peak of expression. Thereafter, at 72 h, the GST 250 and 65 KDa bands were weakly detected while expression of the isoform of 49 KDa (the mature protein form) was greatly enhanced compared to the 24 h group and the control group (Figure 4A and B). At 5 ng/mL, after 24 h, equal expression of the bands of 250 and 65 KDa was seen whereas there was much less expression of the 49 KDa band compared to the control group. After 48 h, there was a decrease in expression of the 250 KDa GST precursor while the remaining isoforms reached their peak of expression. Finally, after 72 h, there was less expression of the 250 and 65 KDa bands while in contrast, 49 KDa isoform maintained its previous preeminent induction (Figure 4A and C). Other authors have already reported the existence of different GST isoforms in fish that modulate their response and expression upon xenobiotic exposure in different ways (George, 1994; Leaver et al., 1997; Gallagher et al., 1999; George and Taylor 2002; Doi et al., 2004). For example, in in vivo studies, Kim et al. (2010) provided the expression profiles of seven GST genes from river pufferfish (Takifugu obscurus) exposed to cadmium, the induction of which was strictly time dependent. According to our GST expression profiles, the different isoforms identified in AFB1 treated S. aurata hepatocytes could be used as a marker of acute and chronic exposure. In fact, at 5 µg/mL, there was a decrease in expression of 65 KDa isoform along with the increase in exposure time, showing a very low signal after 72 h. The same isoform reached its peak at 5 ng/mL within 48 h. Therefore, it could be hypothesized that this GST isoform is a marker of acute exposure because its induction reached its maximum early, in just 24-48 h. By contrast, the 49 KDa band was strongly expressed after 72 h in both AFB1 concentrations tested; therefore suggesting the use of this GST isoform as a marker of chronic exposure. Hence, the GST expression pattern found in this study can be considered time and dose dependent, in agreement with reports both in in vitro and in vivo AFB1 studies in mammals (Liebert et al., 2006; Costa et al., 2008). Several GST isoforms have recently emerged as promising biomarkers of chronic damage involved in early lesions to DNA, since they have been associated both with the inhibition of cell death for apoptosis induction and high tumor prevalence (Townsend and Tew, 2003).

Conclusions

Aflatoxin B1 is the most known biologically active toxin and has been found to produce hepatotoxic, carcinogenic mutagenic, teratogenic and immunosuppressant effects in aquatic animals. Since susceptibility to the hepatotoxic and carcinogenic effects of AFB1 differs between fish, further research is needed in order to better understand AFB1 metabolism and bioaccumulation in a species that has undergone very little investigation, such as S. aurata. Our data showed an indirect correlation between induction of CYP1A and dose and time of exposure. The decrease in expression of CYP1A over time, along with high toxic concentration, could probably support the hypothesis of a detoxification function of this isoform in sea bream and explain the lethal damage caused on hepatocytes, as highlighted by contrast phase and immunofluorescence analysis. In addition, the different GST isoforms detected could be a promising target for acute and chronic response to AFB1 exposure.

These encouraging results, have shown this new cell model from S. aurata to be a useful and valid tool to be used in further research to investigate the modulated response of liver phase I and II enzymes to AFB1.

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