Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gche20

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Available online: 04 Nov 2011

To cite this article: M. P. Santacroce, M. Narracci, M. I. Acquaviva, V. Zacchino, R. Lo Noce, G. Centoducati & R. A. Cavallo (2011): Effects induced *in vitro* by aflatoxin B_1 on *Vibrio fischeri* and primary cultures of *Sparus aurata* hepatocytes, Chemistry and Ecology, 27:sup2, 67-76

To link to this article: http://dx.doi.org/10.1080/02757540.2011.625938

Chemistry and Ecology
Vol. 27, No. S2, December 2011, 67–76

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Effects induced *in vitro* by aflatoxin B_1 on *Vibrio fischeri* and primary cultures of *Sparus aurata* hepatocytes

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(*Received 28 January 2011; final version received 16 September 2011*)

Aflatoxins are one of the most widespread and worrisome sources of feed contamination worldwide, and have a considerable impact on fish farm production, leading to high mortality and a gradual decline in fish stock quality in aquaculture. In this study, we investigated, for the first time, the effects induced *in vitro* by aflatoxin B_1 (AFB_1) on *Sparus aurata* hepatocyte culture and we compared our results with the Microtox® system using *Vibrio fischeri*. At AFB_1 doses ranging from 1 to 10 $\mu g \cdot mL^{-1}$, the results showed signs of primary necrotic cell death in hepatocytes and a very toxic evaluation with Microtox®; between 0.005 and 1 $\mu g \cdot mL^{-1}$, the cytotoxic effects and apoptotic delayed death in eukaryotic cells corresponded with an evaluation of no toxicity or biostimulatory effect using *V. fischeri*. Overall, our results highlighted equivalent toxic responses and overlapped with values of EC_{50}/IC_{50}. Hence, these two *in vitro* systems could be considered as a useful starting point in the design of new batteries to evaluate the toxicity of potentially dangerous feed-borne substances.

Keywords: aflatoxins; Microtox®; *Sparus aurata* hepatocytes

1. Introduction

Aflatoxins are the most potent natural toxic metabolites produced by some toxigenic strains of mycelial fungi, all belonging to the genus *Aspergillus*, contaminating foods, feed components and products of animal origin [1].

At present, increased use of plant-derived ingredients (cottonseed, peanuts, rice) in aquafeed formulations has intensified the potential for aflatoxicosis in fish farming systems due to the carryover of high loads of aflatoxin from vegetable sources [2–5]. As a result, the problem of mycotoxin contamination in aquaculture has amplified [4–6]. In addition, diet has an important effect on water quality and culture systems deterioration, and must be formulated to keep any antinutritional components below concentrations that would impede the performance and health of the fish [7,8].

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ISSN 0275-7540 print/ISSN 1029-0370 online

http://dx.doi.org/10.1080/02757540.2011.625938
http://www.tandfonline.com

Among all known naturally occurring aflatoxins, AFB_1 is the most prevalent and biologically active [1,9,10]. Different studies have revealed that AFB_1 residues can be retained in aquatic animal tissues, giving rise to potential public health risks when people consume these tissues [11–13]. AFB_1 has been shown to be a strong hepatocarcinogen for humans, and is classified as a group I carcinogen by the International Agency for Research on Cancer [14].

The European Union (EU) established 2 ppb as the maximum allowable concentration of AFB_1 in human food [15]. Because of its carcinogenic, teratogenic, immunosuppressive properties and severe adverse effects, AFB_1 has become a growing threat to the health of humans and animals, including fish [9,16–21].

Nevertheless, the presence of aflatoxins in aquaculture and their impact on fish health are still underestimated. Although serious cases of aflatoxicosis (acute or chronic) have been reported in both cultured aquatic vertebrates and crustaceans, very few studies are available on farmed marine vertebrates [3,19,22–24].

The biological effects in fish are thought to be directly related to the amount of AFB_1 in the feed, animal age and species [25,26]. The sensitivity of different fish to AFB_1 has been investigated in freshwater species and seems to be correlated with interspecies variation in biotransformation efficiency [1,26]. However, susceptibility to AFB_1, as well as its adverse effects on health, welfare and flesh quality in Mediterranean reared species, have been poorly investigated [12].

In vitro systems are used primarily to screen and create toxicological profiles, providing important tools to improve our understanding of the effects of hazardous chemicals and predict their effects on humans [27].

Nowadays, toxicity bioassays based on the inhibition of bacterial luminescence are used in ecotoxicology for environmental assessment [28–31]. In addition, the application of this type of bioassay has great potential in the determination of contaminants in food and feed.

In the specific case of mycotoxins, application of a luminescence-based bioassay can be a useful alternative, low-cost and reliable screening tool for indirect mycotoxin detection [32]. However, there are very few reports on the use of these bioassays to evaluate AFB_1 toxicity [32,33]. Microtox® is a well-known, rapid, standardised and frequently used system in ecotoxicology and is used to evaluate acute toxicity and cytotoxicity. However, *in vitro* cytotoxicity tests are useful and necessary, not only to define basal cytoxicity, but also for preliminary searches to establish the dangerous concentration range on which more detailed assays can be based. This enables us to obtain important information regarding parameters such as genotoxicity, the induction of mutations or programmed cell death. In particular, the use of cell cultures for *in vitro* toxicological evaluation can be considered a good approach to highlight new and sensitive endpoints which can then be used as biomarkers to predict the impact of toxins *in vivo* [34].

Among the experimental non-mammalian animal models based on *in vitro* systems, hepatocytes in primary culture from aquatic species are considered the gold standard for studying cellular, biochemical and metabolic responses, as well as for investigations on functional genomics, molecular and environmental toxicology [35–37]. Most fish cell lines currently available for research are obtained from freshwater species; the number of cell lines from marine fish is very low, in particular, there are no liver cell lines [38–42]. *Sparus aurata* is one of the most economically important fish species, and is intensively farmed in the Mediterranean and north-east Atlantic. Although widely studied because of its economic value, its sensitivity to AFB_1 has never been tested *in vivo* or *in vitro.*

The aim of this study was to investigate the effects induced *in vitro* by AFB_1 on *Vibrio fischeri*, using the Microtox® system, and on primary cultures of *S. aurata* hepatocytes, in order find the toxicity range, and ascertain the boundary between acute and subacute effects. The two different test systems used in this study succeeded in highlighting equivalent and overlapping toxic responses. The use of these two *in vitro* systems will provide new information on the exposure risk of *S. aurata* to AFB_1-contaminated feedstuffs.

2. Materials and methods

2.1. *Microtox® bioassay*

The Microtox® Basic Test (BT) was performed according to standard operating procedures [43]. Bacteria (*V. fischeri*) were obtained from Azur Environmental as freeze-lyophilised cells.

Bacteria were exposed to different AFB_1 concentrations ranging from 0.005 to 10 $\mu g \cdot mL^{-1}$ (0.005, 0.313, 0.469, 0.625, 0.938, 1.250, 1.875, 2.500, 3.750, 5.000, 7.500 and 10 $\mu g \cdot mL^{-1}$) using AFB_1 purchased from Sigma Aldrich and diluted with diluent reagent for Microtox® and Osmotic Adjusting Solution (OAS) in order to adjust the osmotic pressure of the sample to ~2% NaCl.

The light emissions from the bacteria were measured after 5, 15, 30 min and 3.5 h incubation at 15 °C and were compared with a control.

All measurements were performed using a M500 luminometer equipped with the appropriate cells. The instrument was interfaced with PC operating Microtox® Omni 1.16 software for Windows 98 for acquisition and data handling.

2.2. *Aflatoxin B_1 exposure of hepatocytes in primary culture*

S. aurata juveniles (30 ± 4 g mean body weight, $n = 45$) were used to obtain the primary hepatocyte culture, according to a recently developed method [44,45]. On arrival, fish were anaesthetised by immersion in seawater plus tricaine methane sulfonate (MS222, 0.02%; Sigma) and rapidly killed by decapitation. Liver tissue, weighing ~0.3–0.4 g, was removed aseptically and quickly processed for hepatocyte isolation in a laminar vertical flow safety hood.

Dissected livers, placed on a sterile Petri dish, were washed three times by immersion and shaking in sterile pre-cooled Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, supplemented with 10 mM HEPES, 0.5 mM EDTA, 25 mM $NaHCO_3$, 200 $IU \cdot mL^{-1}$ penicillin, 200 $\mu g \cdot mL^{-1}$ streptomycin, 200 $\mu g \cdot mL^{-1}$ amphotericin B and 100 $\mu g \cdot mL^{-1}$ gentamicin (washing solution; WS). Unless specified, all chemicals were from Sigma Aldrich Ltd, Milan, Italy. Livers were flushed by a syringe with WS until complete blood clarification. Liver masses were crushed into a stainless-steel sieve with 380-μm mesh for mechanical disruption. Cells were filtered and collected with digestion medium (DM) (7 mM $CaCl_2$, 200 $IU \cdot mL^{-1}$ penicillin, 200 $\mu g \cdot mL^{-1}$ streptomycin, 200 $\mu g \cdot mL^{-1}$ amphotericin B/100 $\mu g \cdot mL^{-1}$ gentamicin, 10 mM HEPES, 25 mM $NaHCO_3$ in Leibovitz's L-15). The digestion step started by adding to the cell homogenate a cocktail of four enzymes including 0.1% collagenase type IV/0.05% hyaluronidase type IV-S/0.4% dispase type II/0.03% DNase type I. The enzyme digestion was blocked after 20 min of incubation at 20 °C by adding L-15 medium supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Lonza Walkersville Inc., Italy). The digestion mixture was filtered through 230-μm and 104-μm stainless-steel filters and centrifuged twice in cold 1× phosphate-buffered saline (PBS) at 80 ***g*** for 5 min at 4 °C. In order to separate parenchymal, non-parenchymal and nucleated red blood cells, a subcellular fractionation in double density gradient of pre-cooled Percoll at 90–50% was performed by isopycnic centrifugation at 150 ***g*** for 10 min at 4 °C. The white ring of hepatocytes was recovered, washed in cold PBS with 0.45 $\mu g \cdot mL^{-1}$ Dnase I to dissociate cell clumps, and centrifuged at 70 ***g*** for 5 min at 4 °C. The collected pellets were resuspended in 10% FBS/L-15 medium and cell number was counted. The viability of the hepatocytes was estimated by Trypan Blue exclusion. Liver cell suspension yielded 2.6×10^7 viable $cells \cdot g^{-1}$ liver weight with a viability of 95.4 ± 0.05%. The suspension of purified hepatocytes was adjusted to a density of 0.5×10^6 $cell \cdot mL^{-1}$ in basal nutrient culture medium (BM) consisting of L-15 with 2 mM L-glutamine, 10% FBS, 100 $IU \cdot mL^{-1}$ penicilin/100 $\mu g \cdot mL^{-1}$ streptomycin/100 $\mu g \cdot mL^{-1}$

amphotericine/50 $\mu g \cdot mL^{-1}$ gentamycin, 1 mM Na pyruvate, 5 mM D-glucose, 10 mM HEPES, 12 mM $NaHCO_3$, supplemented with 0.05% ITS *plus* (insulin/transferrin/sodium selenite plus oleic acid/linoleic acid/BSA), 0.01 mM MEM non-essential amino acid (MEM-NEAA; BioWhittaker), 0.01 mM MEM–vitamin mix (BioWhittaker), 0.1 mM ascorbic acid, 0.01 $\mu g \cdot mL^{-1}$ epidermal growth factor (EGF) and 0.005 $\mu g \cdot mL^{-1}$ hepatocyte growth factor (HGF). The BM osmolality was adjusted to sea bream serum osmolarity by adding 20 mM NaCl [45].

Thereafter, purified hepatocytes were seeded at a density of 3×10^4 cell$\cdot cm^{-2}$ in 96-well plates pre-coated with collagen I and cultivated in BM and cultivated in a refrigerated incubator at 18 °C in an atmosphere of 3% CO_2/97% air.

On the third day after seeding, primary monolayer cultures of hepatocytes were exposed to decreasing doses of AFB_1 in the range 0.005–10 $\mu g \cdot mL^{-1}$ for three exposure times (24, 48 and 72 h). After each exposure, hepatocytes were examined for morphological alterations, cytotoxic responses and apoptosis induction. Cytotoxicity was assessed by measuring the retention of Neutral Red (NR), to check lysosomal function upon AFB_1 exposure. Viability response values normalised to control were plotted against the logarithm of AFB_1 concentration to produce conventional dose–response curves. The cytotoxic effect was determined as the half-maximal inhibiting concentration (IC_{50}) resulting in 50% reduction in cell viability, at each time point. IC_{50} values were determined by fitting data to a four-parameter logistic model using a Hill function non-linear regression analysis. The GraphPad Prism software package (GraphPad Software Inc. v.5.00, San Diego, CA, USA) was used to plot the dose–response curves and to calculate IC_{50} values.

To recognise the boundary between lethal and sublethal effects, release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was quantified.

Apoptosis was evaluated by assessing the phosphatidylserine inversion at the cell surface of dying apoptotic cells, using annexin V–Cy3.18 binding, by fluorescence microscopy. This assay allows us to identify the subacute cytotoxicity by differentiating early apoptotic cells from viable and necrotic ones.

The experiments were repeated at least three times for each *in vitro* system. In the cytotoxicity assay on hepatocytes, each dosage group plus one control group were performed in triplicate. Statistical significance among time and dose was calculated using two-way analysis of variance (ANOVA) and a post-hoc test [least squares difference (LSD)] with Bonferroni correction.

3. Results

3.1. *Microtox® bioassay*

The toxic responses of the 12 dilutions of AFB_1 employed in the Microtox® basic test were determined and are presented in Figure 1.

A time-dependent decrease in light levels, resulting from increased toxicity, was observed in all samples analysed. AFB_1 induced no toxicity assessment or hormesis from 0.005 to 0.9375 $\mu g \cdot mL^{-1}$, whereas 1.25 $\mu g \cdot mL^{-1}$ resulted in low toxicity. Moreover, along with the increase in AFB_1 concentration, we observed a decrease in bioluminescence: with the increasing concentration, toxicity changed from medium to high and then very high.

In particular, very high toxicity effects were observed after only 5 min of AFB_1 exposure at 5–10 $\mu g \cdot mL^{-1}$, whereas a dose of 3.75 $\mu g \cdot mL^{-1}$ elicited the same results after 3.5 h.

At low AFB_1 concentrations, the biostimulatory response showed an increase in light output from 0.17 to 9% compared with controls. The EC_{50} values calculated ranged from 1.931 $\mu g \cdot mL^{-1}$ at 3.5 h to 2.915 $\mu g \cdot mL^{-1}$ at 15 min of exposure, with a medium value of 2.53 $\mu g \cdot mL^{-1}$.

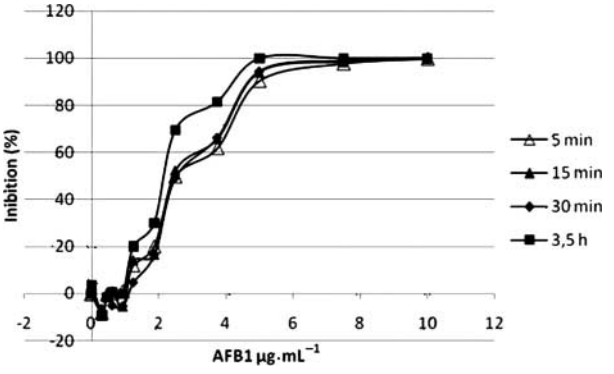

Figure 1. Concentration–response curves for AFB_1 exposures using Microtox®.

3.2. *Cytotoxicity on* S. aurata *hepatocytes*

A significant increase in cell lethality was assessed at doses ranging from 0.005 to 10 $\mu g \cdot mL^{-1}$ at all exposure times (Figure 2).

After 72 h, cell death remained constant in almost 75% of sea bream hepatocytes without any further damage.

Cytotoxic effects, and delayed secondary cell death, were registered at AFB_1 concentrations ranging from 0.02 to 0.005 $\mu g \cdot mL^{-1}$, with signs of apoptosis. The threshold dose at which lethality first appears (LOEC) was estimated to be 10 $ng \cdot mL^{-1}$. However, at concentrations approaching the no observable adverse effect concentration (NOEC; 5 $ng \cdot mL^{-1}$) and below (data not shown), delayed mortality for apoptosis induction was observed. At this apparent safe range, we registered the most sensitive sublethal response to AFB_1.

The lethality threshold was confirmed by the immunocytochemical analysis; direct immunofluorescence with the annexin V–Cy3.18 staining revealed a loss of cell membrane integrity for primary necrosis or delayed secondary cell death to apoptosis. Figure 3 shows hepatocytes cultured in a four-well slide exposed to the toxin (500 $\mu L \cdot well^{-1}$) for 24 h at doses of 0 $\mu g \cdot mL^{-1}$ (Figure 3A) as normal control (annexin V−/6-CFDA+), living cells are visualised in green and marked with arrow; at 1.9 $\mu g \cdot mL^{-1}$ (Figure 3B) (annexin V+/6-CFDA−) necrotic cells are red,

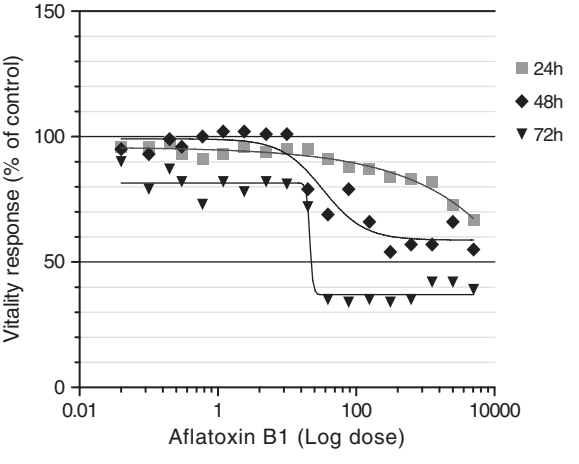

Figure 2. Concentration–response curves for AFB_1 exposures on *Sparus aurata* hepatocytes.

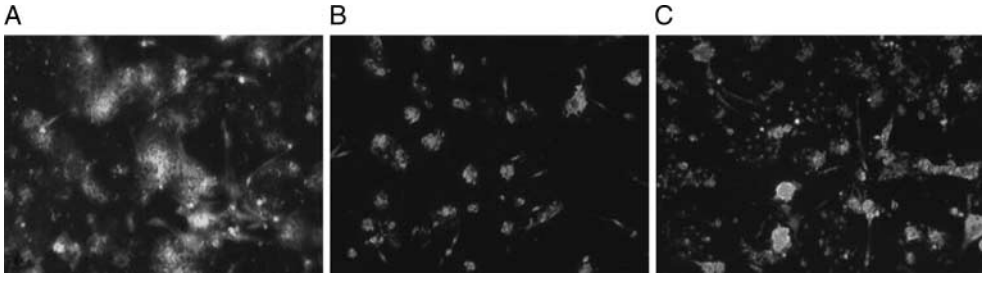

Figure 3. Detection of apoptosis in AFB_1-exposed hepatocytes.

visualised as marked inside (∗); and at $0.02\,\mu g{\cdot}mL^{-1}$ (Figure 3C) (annexin V+, 6-CFDA+) early apoptotic cells are orange, visualised as marked inside (Δ), respectively. Figure 3B and C were obtained using merged photos from Fluorescein Isothiocyanate and Tetramethylrhodamine Isothiocyanate filter channels acquired separately as digital images (original magnification ×200).

Statistical calculations revealed that the effect of time and dose was extremely significant ($p < 0.0001$). In particular, the toxic potency was found to be inversely related to the exposure time. Differences were considered to be significant at $p < 0.005$.

4. Discussion

Comparison of dose–response curves obtained after 24, 48 and 72 h for hepatocytes and after 5, 15 30 min and 3.5 h for Microtox® revealed that the increase in concentrations of AFB_1 led to rising mortality events, and prolonged exposure times led to a considerable increase in AFB_1 toxicity (Figures 1 and 2). The time-dependent cytotoxic effect was extremely significant in *S. aurata*, IC_{50} being inversely related to the exposure time. The longer the exposure, the lower the IC_{50} and EC_{50} values for all endpoints, while toxic potency increased. The cytotoxicity of AFB_1 *in vitro* has been studied previously in mammals, in the hepatocytes of rats and cattle [46,47], but rarely in fish, and overall exhibited concentration- and time-dependent increases in cytotoxicity. Fish aflatoxicosis has been investigated intensively *in vivo* and acute experiments, although long-term trials are still poorly studied. Recently, two *in vivo* surveys performed in tilapia [48] and sea bass [12] indicated that long-term AFB_1 exposure up to 20 weeks can cause similar adverse effects depending on both dose and time. Along with the few studies carried out *in vitro*, the cytotoxic response to AFB_1 was investigated in a cell line derived from the normal liver of a mature rainbow trout (*Oncorhynchus mykiss*) (RTL-W1), in non-liver-derived cell lines of rainbow trout gonad (RTG-2) and in chinook salmon embryo (CHSE-214). In these studies, AFB_1 altered cell morphology and inhibited cell proliferation and DNA synthesis. The effective concentration required for 50% inhibition of DNA synthesis in RTL-W1 was of $0.04\,\mu g{\cdot}mL^{-1}$. By comparing the dose–response between salmonid cells, RTG-2 cells were shown to be as sensitive as RTL-W1 cells (EC_{50} for inhibition of DNA synthesis $= 0.05\,\mu g{\cdot}mL^{-1}$), while CHSE-214 cells were unresponsive to AFB_1 at concentrations as high as $2\,\mu g{\cdot}mL^{-1}$. Moreover, after a single AFB_1 exposure, RTL-W1 sublines showed phenotypic changes typical of malignant transformation: increased growth rate, reduced contact inhibition of growth and altered cellular morphology [49].

The release of LDH is a more sensitive marker for cytotoxicity than the Trypan Blue exclusion method and has been used as an indicator of necrosis [50,51]. Other studies have reported that cellular LDH release and NR retention levels play an important role in determining the type of cell death, either necrotic membrane rupture or cytotoxic metabolic sufferance, that will result from a toxic insult [52].

Our data corroborate and confirm what has already been demonstrated for other species of vertebrates, including humans: there is not a dose of AFB_1 for which no toxic effects occur, because even sublethal doses induced apoptosis and tumorigenesis. Specifically, sublethal and subcytotoxic concentrations of AFB_1 activate apoptosis in sea bream hepatocytes prior to necrosis due to membrane disruption, whereas the Microtox® system revealed a balance between no toxicity and a biostimulatory effect (hormesis). After exposure to sublethal doses of AFB_1, seemingly viable hepatocytes were irreparably damaged at the gene level and tended to die because of the subsequent apoptotic process. By contrast, cells that managed to evade apoptosis continued to replicate in an uncontrolled tumour phenotype. This confirms that the damage to hepatocytes in culture is not recoverable but permanent, as the cellular repair systems are unable to remedy the induced toxic insult or presumably repair damaged DNA. Our data indicate that exposure to sublethal and subcytotoxic doses of AFB_1 induced cytotoxic cell death due to secondary necrosis for apoptosis induction. This pathway of secondary cell death triggered by AFB_1, via an apoptotic process, was previously suggested by other authors who have shown that AFB_1 caused DNA strand breaks and activated caspase 3 [53,54]. Moreover, Golli-Bennour and colleagues [55] reported an increase in DNA fragmentation in cultured monkey kidney Vero cells (Vero cell line) exposed to AFB_1, as well as an augmented expression of p53 protein levels, which was activated in response to DNA damage, indicating induction of the apoptotic process. In this study, p53 levels increased significantly after treatment with AFB_1 at concentrations ranging from 5 to 30 μM, whereas at higher doses of AFB_1 (40 μM) p53 levels decreased due to the severe cell death observed (40% cell mortality). The extent of apoptotic damage appeared to be dose- and time dependent, as also shown in cattle liver cells [56]. Several investigations have demonstrated that the induction of apoptosis and necrosis is highly dependent on the intensity of the initial exposure [50,57]. Similarly, our results suggest that differences exist in the type of cell death induced by AFB_1 in relation to dosage. Necrosis and apoptosis resulted from high and low concentrations of AFB_1, respectively, and the extent of the cellular damage may be dependent upon the exposure time. In fact, exposure of sea bream hepatocytes to subacute AFB_1 for 72 h revealed toxicity more than 24 h exposure, and more accurately highlights the cytotoxic potency and the type of response triggered. These results indicate distinct pathways for the cytotoxic response in AFB_1-treated hepatocytes: necrotic cell death, as confirmed by the LDH release (from 1.9 to 10 $\mu g \cdot mL^{-1}$), and apoptotic cell death (from 0.02 to 0.005 $\mu g \cdot mL^{-1}$). It is likely that the different exposure intensity of the toxic insult determines the type of hepatotoxic pathway triggered. The relationship between hepatocyte apoptosis and *in vivo* vacuolar degeneration, a sign of hepatocyte apoptosis, has been investigated in the liver of tilapia fed an AFB_1-contaminated diet [48]. Specifically, in tilapia exposed to high doses of AFB_1 for a long time, altered hepatocyte membrane permeability was observed, as well as an increase in the plasmatic release of the hepatic enzymes Aspartate Aminotransferase and Alanine Aminotransferase, suggestive of strong cytotoxicity and liver necrosis [48]. The induction of apoptosis by AFB_1 has been investigated previously in a human hepatoma cell line (HepG2) [52]. Morphologically, the authors observed extensive cell shrinkage, nuclear condensation and the budding off of small membrane-bound apoptotic bodies when cells were treated with AFB_1. In human cells, AFB_1 induced a concentration-dependent increase in apoptosis. The time dependence of LDH release was most evident for higher concentrations of AFB_1 (31–156 $\mu g \cdot mL^{-1}$), which increased significantly between 12 and 24 h. [52]. These results were consistent with visual inspection of our *S. aurata* AFB_1-treated cells. In the concentration range between the NOEC and the increase in the LDH release, AFB_1 induced the greatest degree of apoptosis.

Taking all the previous results into account, we can conclude that the *in vitro* susceptibility of sea bream hepatocytes to AFB_1 parallels the susceptibility and type of response triggered by AFB_1 in mammal cells, with the exception that *S. aurata* hepatocytes appear to be more sensitive. Finally, we established that *S. aurata* hepatocytes are highly sensitive to AFB_1 exposure, because several different adverse effects were found at all the doses tested.

5. Conclusions

At high concentrations of AFB_1 (1–10 $\mu g \cdot mL^{-1}$), cell swelling and loss of membrane integrity were noted as signs of primary necrotic cell death. This is the lethality threshold dose also identified by LDH_{50} (mean value 3 $\mu g \cdot mL^{-1}$). In the Microtox® system, the toxicity evaluation ranged from low to very high values proceeding from 5 min to 3.5 h. Specifically, at 5 $\mu g \cdot mL^{-1}$ the effect on bioluminescence reduction was directly of 90.54%.

At medium concentrations of AFB_1 (0.005–1 $\mu g \cdot mL^{-1}$), cytotoxic effects and apoptotic delayed death were recorded as signs of secondary necrotic cell death. Apoptosis induction represents an early response to DNA damage. This is the cytotoxic threshold dose identified by NR_{50} (NR_{50}–24 h, 2.98 $\mu g \cdot mL^{-1}$; NR_{50}–48 h, 0.31 $\mu g \cdot mL^{-1}$; NR_{50}–72 h, 0.03 $\mu g \cdot mL^{-1}$). In the Microtox® system, the toxicity evaluation ranged from no toxicity to a biostimulatory effect.

Sublethal and subcytotoxic concentrations of AFB_1 activate apoptosis in sea bream hepatocytes prior to inducing necrosis due to membrane disruption.

The results reported here show equivalent and overlapping toxic responses: at shorter exposure times EC_{50}/IC_{50} values overlapped in both test systems; at lower exposure doses, equivalent NOEC values were found in both test systems; at values corresponding to LOEC in hepatocytes, hormesis responses were detected by Microtox®. Hence, these *in vitro* systems could be considered as a useful starting point in the design of a new battery for evaluating the toxicity of potentially dangerous feed-borne substances. Further studies are required to better understand the hepatotoxic pathway triggered at concentrations below the NOEL and approaching the hormesis range.

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