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Characterization of the cellular damage induced by Aflatoxin B_1 in sea bream (*Sparus aurata* Linnaeus, 1758) hepatocytes

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ABSTRACT - Gilthead sea bream (*Sparus aurata* L.) is one of the most intensively farmed fish species in the Mediterranean, greatly studied for the relevant economic value, although its sensitivity to Aflatoxin B_1 (AFB_1) has to be investigated, yet. The aim of this study was to perform an *in vitro* evaluation of cytotoxic potential of AFB_1 on *S. aurata* hepatocytes in order to grade the range of AFB_1 toxicity, and the boundary between acute and long-term toxicity. Primary monolayer cultures of hepatocytes from *S. aurata* juveniles were treated with a wide range of concentrations from $5x10^3$ ng/ml to $2x10^{-5}$ ng/ml of AFB_1 for a different period of exposure (24, 48, 72 hours). The cytotoxic activity was characterized by MTT reduction assay. After each exposition hepatocytes were examined for morphologic alterations and apoptosis induction. AFB_1 exposure significantly reduced cell viability in a dose- and time-dependent manner. Dose-response curves obtained after 24, 48 and 72 hrs revealed that prolonged exposure times lead to a significant increase of the toxic potency of AFB_1. Our results demonstrate that *S. aurata* hepatocytes are highly sensitive to AFB_1 exposure. Such scientific findings could provide new insights to investigate the real impact of aflatoxin on marine farmed fish.

Key words: Sparus aurata hepatocytes, Aflatoxin B_1 cytoxicity, Feedstuff contamination.

Introduction - Aflatoxin B_l (AFB_1), a secondary metabolite of the fungus *Aspergillus flavus*, is the most potent food-born toxin known and frequently found in animal feedstuffs. Numerous studies demonstrated that AFB_1 has been implicated as a causative agent in sudden outbreaks of fish mortality due to acute aflatoxicosis (Cagauan *et al*., 2004). Previous experimental results indicate that AFB_1 has been found to produce hepatotoxic, carcinogenic, mutagenic and immunosuppressant effects in aquatic animals long-term exposed via diet administration (Eaton *et al.,* 1990; Ramsdell and Eaton, 1990; Eaton *et al*., 1995). Chronic aflatoxicosis was found to be implicated in a gradual decline of reared fish stock quality, thus representing a significant problem in aquaculture systems (Lovell, 1992; Murjani, 2003). The extent of the dangerous effects depends on the particular species susceptibility, which was found to be dissimilar among the farmed species (Stewart and Larson, 2002). The different fish AFB_1 sensitivity has been greatly investigated in freshwater species and seems to be correlated with interspecies variation in the biotransformation efficiency (Santacroce *et al*., 2008). However, AFB_1 susceptibility in typical Mediterranean rearing species as well as the adverse effects of AFB_1 on fish health, welfare and flesh quality have never been tested. The aim of this study was to perform an *in vitro* evaluation of AFB_1 cytotoxic potential on *S. aurata* hepatocytes, to screen its sensitivity and to evaluate the toxicity range, in order to provide new information on the exposure risk of *S. aurata* to contaminated feedstuffs.

Material and methods - To perform this *in vitro* study we established first primary hepatocyte culture from *S. aurata* juveniles (30 g±4) according to a new method recently developed in our labo-

ratory (Centoducati *et al*., 2008). Freshly isolated hepatocytes were seeded at a density of 30,000 cells/cm^2 in 96-well plates pre-coated with collagen I. The cells were grown in Leibovitz L-15 modified medium with 10% (v/v) inactivated fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin/100 µg/ml streptomycin/amphotericine, and supplemented with insulin, aminoacids, vitamins and growth factors. Cells were maintained and cultivated in refrigerate incubator at 18°C, in atmosphere with 3% CO_2/97% air. At 70–80% confluence, cells were exposed to various AFB_1 concentrations ranging from $5x10^3$ ng/ml to $2x10^{-5}$ ng/ml for a period of exposure of 24, 48, and 72 hrs. At the end of each exposure period hepatocytes were examined for morphological alterations, cytotoxic response and apoptosis induction. Cytotoxicity was characterized by measuring the mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) as substrate. For each treatment time the measure of cytotoxic effect was determined as the half-maximal inhibiting concentration (IC_{50}) resulting in 50% of reduction in cell viability. Viability response values normalized to control were plotted against the logarithm of AFB_1 concentration to produce the conventional dose-response curves. The IC_{50} values reflecting a 50% inhibition of cell viability were determined fitting data to a four-parameter logistic model by using a Hill function non-linear regression analysis. 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.

Results and conclusions - A significant increase of cell lethality was assessed at doses ranging from 0.01 to $5x10^3$ ng/ml at all the exposure time (Figure 1). This lethality threshold dose was confirmed by the immunocytochemical analysis, with the Annexin V staining, revealing the loss of cell membrane integrity. After 72 hrs cell death remained constant in almost 75% of seabream hepatocytes from 78 ng/ml for up to $5x10^3$ ng/ml. Cytotoxic effects, and delayed secondary cell death, were registered at AFB_1 concentrations ranging from 10 ng/ml to 0.2 ng/ml, with signs of apoptosis. The threshold dose level at which lethality first appears was (LOEC) estimated to be 10 ng/ml. At concentrations approaching the no-observable-adverse-effect-concentration (NOEC, 5 ng/ml) and below it, however delayed mortality for apoptosis induction was observed. At this apparent safe level was registered the most sensitive sublethal response to AFB_1. Conversely, uncontrolled cell proliferation was registered at AFB_1 concentrations lower than 0.2 ng/ml. The formation of tumoral foci increased over time of exposure, and with the lowering of doses, as revealed by the mitotic index rising calculated by the N° of mitotic cells positives by immunofluorescent labeling of DNA (PCNA antigen +). High proliferation was registered after 72 hrs when AFB_1 was as low as $1x10^{-2}$ ng/ml – $2x10^{-5}$ ng/ml. The comparison of dose-response curves obtained after 24, 48 and 72 hrs revealed that prolonged exposure times lead to a significant increase of AFB_1 toxicity (Figure 1).

AFB_1 exhibited dose- and time-dependent cytotoxic effect, the IC_{50} being inversely related to the exposure time (MTT-IC_{50}-24h, $5x10^3$ ng/ml; MTT-IC_{50}-48h, $6x10^2$ ng/ml; MTT-IC_{50}-72h, 60 ng/ml). The longer the periods of exposure, the lower the IC_{50}, while toxic potency increased. The 72 hrs subacute exposure seems more sensitive to toxicity than the 24 hrs exposure, and more accurately reveals the cytotoxic potency and the kind of response triggered. Three separate experiments have been carried out and performed in triplicate. Statistical significance among the two parameters was calculated using a two-way analysis of variance (ANOVA) and a post hoc test (least squares difference [LSD]) with Bonferroni-correction. Statistical calculations revealed that the effect of time and dose was extremely significant ($P<0.0001$) as shown in Table 1.

Sublethal and subcytotoxic concentrations of AFB_1 trigger apoptosis prior to induce necrosis, as assessed by the occurrence of a damage which is not recoverable, but permanent, even if the toxic insult is removed. Our results indicate almost three distinct pathways of cytotoxic response in AFB_1-treated sea bream hepatocytes: necrotic cell death, apoptotic cell death, uncontrolled cell proliferation and tumoral foci formation. Accordingly, we can not define an *in vitro* threshold of safety dose for sea bream hepatocytes exposed to AFB_1, since sublethal and subcytotoxic doses, however, elicits

a response like damage to DNA and apoptosis. Finally, we established that *S. aurata* hepatocytes are highly sensitive to AFB_1 exposure, as adverse effects were found at all tested doses. Based on these preliminar results, further researches are needed to ascertain whether exist a possible limit value of no-toxicity for AFB_1 on *in vitro* cultures, and to hypothesize the *in vivo* safety dose for reared sea breams. Innovative scientific findings could provide new data to stem the real impact of aflatoxin on fish health and farming production.

Figure 1. Measure of cell viability by the MTT assay after exposure to different concentrations of Aflatoxin B1 for 24h, 48h, 72h. Data expressed as % of the unexposed controls (vitality relative to controls assumed as 100%).

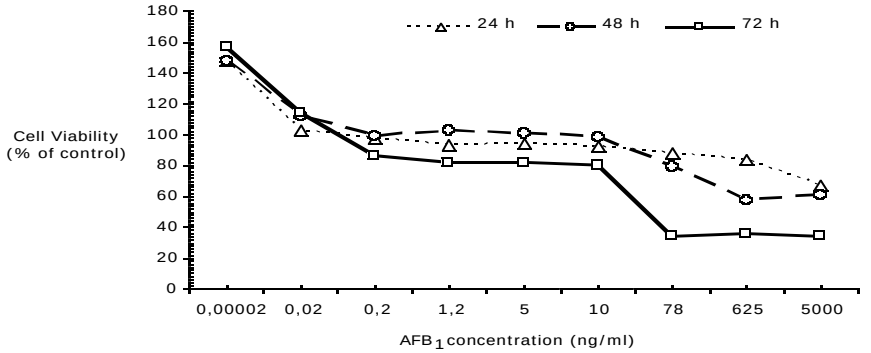

Table 1. Statistical Analysis of Variance.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	P- value
Interaction	1.14	44	2.328	0.05291	2.903	0.0001
Time	79.16	2	161.8	80.92	4440	0.0001
Dose	18.47	22	37.77	1.717	94.2	0.0001

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