Occupational Exposure to Aflatoxin B1 in a Portuguese Poultry Slaughterhouse

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ABSTRACT

Aflatoxin B1 (AFB1) is a secondary metabolite produced by the fungi Aspergillus flavus and is the most potent hepatocarcinogen known in mammals and has been classified by the International Agency of Research on Cancer as Group 1 carcinogen. Although dietary exposure to AFB1 has been extensively documented, there are still few studies dedicated to the problem of occupational exposure. Considering recent findings regarding AFB1 occupational exposure in poultry production, it was considered relevant to clarify if there is also exposure in poultry slaughterhouses. Occupational exposure assessment to AFB1 was done with a biomarker of internal dose that measures AFB1 in the serum by enzyme-linked immunosorbent assay. Thirty workers from a slaughterhouse were enrolled in this study. A control group (n = 30) was also considered in order to know AFB1 background levels for Portuguese population. Fourteen workers (47.0%) showed detectable levels of AFB1 with values from 1.06 to 4.03 ng ml⁻¹, with a mean value of 1.73 ng ml⁻¹. No AFB1 was detected in serum of individuals used as controls. Despite uncertainties regarding the exposure route that is contributing more to exposure (inhalation or dermal) is possible to state that exposure to AFB1 is occurring in the slaughterhouse studied. It seems that reducing AFB1 contamination in poultry production can have a positive result in this occupational setting.

KEY WORDS: aflatoxin B1; occupational exposure; poultry slaughterhouse

INTRODUCTION

Aflatoxins are secondary metabolites produced under certain environmental conditions (temperature, water activity, substrate composition, and pH or modified atmospheres) by species from Aspergillus section Flavi (flavus and parasiticus) (Bhatnagar et al., 2006). Among all aflatoxins, aflatoxin B1 (AFB1) is normally predominant in food cultures and products (example: peanuts, maize, rice, tree nuts, cotton seeds, spices, green tea, and milk) and is also the one with highest toxicity (Elshafie et al., 2011; Saini and Kaur, 2012). It is the most potent hepatocarcinogen known in mammals and due to that is classified by the International Agency of Research on Cancer as Group 1 carcinogen.
It also has mutagenic and teratogenic properties (IARC, 1993; Dash et al., 2007).

AFB₁ is metabolized by the mixed function oxidase system into a number of hydroxylated metabolites including the 8,9-epoxide. This metabolite has carcinogenic properties because it has the capacity to react with cellular deoxyribonucleic acid (DNA) and proteins to form covalent adducts (Autrup et al., 1991; Brera et al., 2002; Dash et al., 2007).

Although dietary exposure to AFB₁ has been extensively documented, there are still few studies dedicated to the problematic of occupational exposure. Moreover, there are some relevant aspects to consider in the specific case of occupational exposure, such as inexistence of health-based threshold limits for exposure through inhalation and by dermal intake and, also, the fact that mycotoxins in general are rarely monitored in occupational environments (Méheust et al., 2014; Viegas et al., 2015). In addition, most occupational studies that focus in fungal load disregard the burden by their metabolites, such as mycotoxins, and also their possible interactions, that is crucial for a proper risk assessment (Oppliger, 2014).

Occupational exposure by inhalation of AFB₁ can occur during tasks involving storing, loading, handling or milling contaminated materials such as grain, waste, feed, and others (Sorenson et al., 1981; Jargot and Melin, 2013; Mayer et al., 2012; Viegas et al., 2015). Additionally, there is also the possibility of exposure by dermal absorption since several mycotoxins are lipid soluble, having properties that enable occupational exposure by this route (Boonen et al., 2012). Therefore, exposure by dermal absorption can be a reality in workplaces where skin areas are exposed to particulate matter deposition or when in direct contact with contaminated products (Degen, 2008; Mayer et al., 2008). This last route of exposure is not ponder in most of the cases and justifies more attention in some specific occupational environments (Boonen et al., 2012; Taevernier et al., 2015).

Recently, a published work described occupational exposure to AFB₁ in Portuguese poultry production (Viegas et al., 2012c, 2013b). In this research, 18 poultry workers from a group of 31 workers had detectable levels of AFB₁, in contrast to all individuals used as controls (n = 30) who showed no detectable values. Those findings corroborate the hypothesis that occupational exposure to AFB₁ is occurring in poultry production, probably during the handling of feed or the material that covers the pavilions floor (Viegas et al., 2012c). In this case, high dust contamination found in the workplace environment contributed for the exposure results (Viegas et al., 2012a, 2013a). Moreover, although in some of the assessed poultries were not found toxigenic strains from section Flavi (Viegas et al., 2014) the workers were indeed exposed to AFB₁, proving that biomarker data is a more accurate reflection of exposure assessment (Watson and Mutti, 2004).

Considering the information above and taking in account also the recent findings regarding AFB₁ occupational exposure in poultry production, it was considered relevant to clarify if there is also exposure in poultry slaughterhouses.

**MATERIALS AND METHODS**

**Slaughterhouse studied**

The slaughterhouse company is located in Portugal, Coimbra district. It has 400 workers distributed by several production phases. Main activities are slaughtering (8500 chickens h⁻¹), evisceration (6000 chickens h⁻¹) and meat preparation for storage and selling. This unit has Portuguese and International quality certification regarding food safety.

No respiratory protection devices were used by workers, aside from the workplace that involves chickens hanging in the beginning of the slaughter process.

**Detection of AFB₁ in serum of slaughterhouse workers**

After tasks observation in each workplace, individuals from the workplaces with higher contact with the chickens and, therefore, with higher risk of exposure, such as, the chickens slaughtering (pear for chicken reception and chickens hanging for slaughtering) and evisceration (removing liver, kidney and other organs from chicken inside; inspection and bleeding) were invited to participate in this study.

Occupational exposure assessment to AFB₁ was done with a biomarker of internal dose that measures AFB₁ in serum. Method used measure free AFB₁ and AFB₁ bound to albumin. Values obtained include recent exposure to AFB₁ and the exposure that could happen until 1–2 months earlier (Leong et al., 2012). The main objective was to obtain data regarding recent
exposure to AFB1 and also its level of intensity. This approach is useful for rapid screening for acute exposures and also reflects chronic exposure (Viegas et al., 2012c, 2013a, b, 2015; Leong et al., 2012; Mo et al., 2014).

Thirty workers from the slaughterhouse were enrolled in this study. The samples collection was done in two days from January and February 2015. A control group (n = 30) was also considered in order to know AFB1 background levels for Portuguese population and to discard exposure by ingestion of contaminated food in the workers group. Control group was composed of subjects who conducted administrative tasks in an educational institution without any type of activity known to involve exposure to AFB1. All participants were informed about scope and aim of this study and signed a consent form. The same approach was followed in Viegas et al., 2012c, 2013a, b), 2015 and is recommended by other authors (Mayer et al., 2003; Degen, 2011). It was not possible to investigate differences in the diet of both groups (workers and controls) which can influence AFB1 levels.

During a personal interview the workers answered a questionnaire to collect personal data, such as age, detailed current and previous occupational history, tasks performed in the two previous days, activities developed outside the company (such as agriculture or animal production).

Blood sample preparation
Blood was collected and allowed to clot, after centrifugation serum was aliquotted and stored at −20°C until analysis. Five hundred microliters of serum was incubated for 18 h at 37°C with pronase (Calbiochem®, 50 U per 5 mg protein) before application to pre-wet C18 column (RIDA C18 column, R-Biopharm®). Column was washed with 5 ml 5% methanol to remove small peptides and amino acids. Fraction containing aflatoxin was eluted with 80% methanol, which was posteriorly evaporated under a nitrogen stream and diluted to reach a 10% methanol solution. Eluate was then applied to an immunoaffinity aflatoxin column (Easi-Extract Aflatoxin; R-Biopharm®) and aflatoxin-containing fraction was eluted with 1 ml methanol in phosphate buffer 0.1 M, pH 7.4 (1:1), after rinsing the column with phosphate-buffered saline (PBS).

ELISA assay
For AFB1 quantification, RIDASCREEN Aflatoxin B1 30/15 enzyme-linked immunosorbent assay (ELISA; R Biopharm®) was used, and was calibrated with aflatoxin standards from 1 to 50 ng ml⁻¹. Interference from other aflatoxins is negligible since this method use monoclonal antibodies for AFB1. Application of this kit for the determination of aflatoxins in human blood was optimized by the research team. The amount of sample used is 150 μl and the final AFB1 concentration is obtained taking into account all factors of dilution/concentration. Values below 1 ng ml⁻¹ were considered nondetectable since these are below the detection limit. For testing, samples or standards were added into wells coated with capture antibodies directed against anti-aflatoxin antibodies. Prior to the addition of AFB1-antibody solution, AFB1–enzyme conjugate was added. After 30 min of incubation, wells were washed three times. Substrate/chromogen is added to the wells, bound enzyme conjugate converts the chromogen into a blue product and the reaction was stopped after 15 min with a stop solution. Absorbance was measured at 450 nm and results were assessed with Ridasol Win software version 1.73 (R Biopharm®).

Statistical analysis
Mann–Whitney test was applied to compare the workers genders. SPSS (Statistical Package for Social Sciences) 21.0 was used to perform all the statistical analysis. The criterion for significance was set at P < .05.

RESULTS
Blood samples were collected from a total of 30 workers (13 from chicken hanging for slaughter; 3 from the pear where the reception of the chicken is made; 14 from evisceration) and 30 controls. Characteristics of both groups are summarized in Table 1.

Fourteen workers (47.0%) showed detectable levels of AFB1 with values from 1.06 to 4.03 ng ml⁻¹, with a mean value of 1.73 ng ml⁻¹. No AFB1 was detected in serum of individuals used as controls. Concentrations less than 1 ng ml⁻¹ (limit of detection, LOD) were considered no detectable (Table 2).

Significantly higher concentrations of AFB1 were found in slaughterhouse workers compared to controls (P < 0.0001). In the slaughterhouse workers group, there were not found significant differences between
subjects of different gender \((U = 99.50; P = 0.72)\). Moreover, 44% of the females and 50% of the males displayed detectable levels of AFB1.

Distribution of detectable values between the three areas of work was the following: 6 (46%) of 13 from chicken hanging for slaughtering; 1 (33%) of 3 from the pear where the reception of the chicken is made and 8 (57%) of 14 from evisceration.

In more detail, there were four values higher than 2 ng ml\(^{-1}\), three of them (2.29, 2.32, 4.03 ng ml\(^{-1}\)) in workers from evisceration area (removing chicken organs). The other higher value (2.24 ng ml\(^{-1}\)) was obtained in a worker from the pear.

Considering previous results obtained in poultry production (Viegas et al., 2012c) and when comparing with the results obtained in this slaughterhouse, it was detected statistically significant differences between poultry and slaughterhouse on the AFB1 concentration \((U = 281, P = 0.047)\). It is noted that poultry houses have significantly higher values \((U = 281, P = 0.024\), median poultry = 1.29, median slaughterhouse = 0.88) (Table 3).

**DISCUSSION**

This is the first study developed with the propose of assessing occupational exposure to AFB\(_1\) in the slaughterhouse setting. Most common scenarios studied before regarding occupational exposure to mycotoxins, were agricultural sector, where grain handling has some expression (Halstensen et al., 2007; Mayer et al., 2007; Dimich-Ward et al., 2011; Malik et al., 2014) and industrial sectors like waste management (Mayer et al., 2012; Viegas et al., 2015). In what concerns this subject there are several published studies in animal production but this work is the first one claiming attention for this occupational problem in the slaughterhouses and, for the fact that can also be a food safety concern. These two aspects will be presented and discussed in the following paragraphs.

Decision of studying occupational exposure to AFB\(_1\) in this setting was based in the previous work published (Viegas et al., 2012c) related with exposure to AFB1 in poultry production and with interest in understand if exposure was also a reality in the process after and what is the more critical scenario regarding AFB\(_1\) occupational exposure in poultry food chain.

Nowadays, European regulations force employers to assess the risk resulting from workers exposure to biological agents and possible outcomes such as infections, sensitization and also toxicological threats. However, mycotoxins are not recognized as a risk factor in most of occupational settings that have already been assessed for fungi exposure. Is well known that fungi can disappear due to unsuitable environment conditions for their survival but mycotoxins can still be present since they are much more resistant. This aspect can result in some cases in an underestimation of the mycotoxin exposure (Halstensen, 2008; Alborch et al., 2011; Viegas et al., 2012c).

Besides, as in the study already mentioned that were assessed seven poultries farms (Viegas et al.,

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**Table 1. Characteristics of subjects studied**

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th>Age (mean; SD)</th>
<th>Years of activity (mean; SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Workers ((n = 30))</td>
<td>18</td>
<td>12</td>
<td>45.3; 9.0</td>
<td>7.9; 7.7</td>
</tr>
<tr>
<td>Controls ((n = 30))</td>
<td>16</td>
<td>14</td>
<td>36.3; 7.6</td>
<td>—</td>
</tr>
</tbody>
</table>

**Table 2. Aflatoxin B1 results in both groups (ng ml\(^{-1}\))**

<table>
<thead>
<tr>
<th></th>
<th>Female (mean(^a); range; SD(^a))</th>
<th>Male (mean(^a); range; SD(^a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Workers ((n = 30))</td>
<td>1.11; &lt;LOD –4.03; 0.93 (44% &gt;LOD)</td>
<td>0.96; &lt;LOD –2.32; 0.80 (50% &gt;LOD)</td>
</tr>
<tr>
<td>Controls ((n = 30))</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

\(^a\)Considered only results >LOD.
Table 3. Differences between poultry units and slaughterhouse

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>N</th>
<th>Mean rank</th>
<th>Sum of ranks</th>
<th>Mann–Whitney U</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>Poultry</td>
<td>27</td>
<td>33.59</td>
<td>907.00</td>
<td>281.000</td>
<td>0.047*</td>
</tr>
<tr>
<td></td>
<td>Slaughterhouse</td>
<td>30</td>
<td>24.87</td>
<td>746.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant differences at a 5% significance level.

2014), toxigenic strains from *Flavi* section were also targeted in the assessed slaughterhouse but were not able to amplify (data published elsewhere). This situation may occur due to several aspects, such as only one sample from each sampling site was taken, and variations in fungal contamination are expected, and that only air samples were analyzed by molecular methods might account for lack of detection of this section (Viegas et al., 2015 to be published). Both facts point out for the importance to use exposure biomarkers for exposure assessment purposes.

Regarding the biomarker used in this study, serum AFB₁ measure, we have to consider that it is a good exposure indicator but it is not related with intake. On the other hand, urinary AFM1 and urinary Aflatoxin-N7-guanine give quantitative relationships providing confidence for the use of these metabolites as exposure assessment tools (Kensler et al., 2011; Turner, 2013). Urinary AFM1 used as biomarker of internal dose and urinary Aflatoxin-N7-guanine as biomarker of biologically effective dose because formation of this adduct lies on the casual pathway to aflatoxin-induced hepatocellular carcinoma (Kensler et al., 2011). Additionally, these biomarkers are less invasive because are collect in the urine and not in the blood, being more suitable for future occupational exposure assessment studies.

Data obtained leads to the conclusion that occupational exposure to AFB₁ is occurring. This is supported by two different aspects: first, 14 workers (47%) presented AFB₁ measurable values and, second, controls have shown absence of positive results (below the LOD). This is of concern because a possible causative relation between occupational exposure to AFB₁ and cancer has already being presented in different occupational settings (McLaughlin et al., 1987; Olsen et al., 1988; Autrup et al., 1993).

However, and contrary to previous published articles (Viegas et al., 2012c, 2013a, b, 2015; Mo et al., 2014) we cannot state that exposure is occurring only by inhalation since the workers enrolled in this study are from different workplaces conditions in the same slaughterhouse. Regarding the specific case of evisceration workplace, where eight workers obtained results higher than the LOD, and since there is a low particle contamination in the air (data published elsewhere), exposure can be occurring by dermal absorption contrary to other situations already reported in different settings (Viegas et al., 2013a, b, 2015; Mayer et al., 2012). In those cases, there was a high contamination by particles and probably the dust was acting as a carrier of AFB₁ to the breathing zone and mouth has previous discussed (Autrup et al., 1991; Brera et al., 2002; Jargot and Melin, 2013; Viegas et al., 2015). Additionally, and as reported by Herzallah et al. (2014) in a study where broiler chickens were fed with diet contaminated with AFB₁, liver and kidney presented the highest AFB₁ residue levels. So, bearing in mind that in evisceration most of the workers engaged in this study were from the workplace where liver and kidney were removed and protection gloves were not use, due to the risk of hands dragging to the belt, is possible to speculate dermal contact and possible absorption. Supporting this possibility, Boonen et al. (2012) developed a study intending to study skin penetration for specific mycotoxins and AFB₁ was included. The results showed that AFB₁ can penetrate into and through skin and should be avoid the contact with solutions containing this mycotoxin (Boonen et al., 2012).

Moreover, penetration of substances through the skin surface depends upon different factors; one
of them is the hydration of skin since penetration is higher in hydrated skin than dry skin. Water is an effective penetration enhancer (Abraham et al., 1989). Therefore, there is an increased potential for percutaneous absorption of environmental pollutants in scenarios such as bathing, swimming, or showering where the skin is well hydrated (EPA, 1992). This is an important aspect in evisceration since is done with water running and with workers hands always in contact with water, besides chicken organs. In the future, it would be of interest to perform AFB$_1$ analysis in liver and kidney from chickens to confirm this hypothesis and to guarantee consumers health.

In the case of the workplace where the chicken are hang for slaughtering, where 6 from 13 workers presented AFB$_1$ values higher than the method LOD, the route of exposure is probably the inhalation since this workplace is characterize by high particle contamination due to the number of chicken and their constant movement. The same is probably occurring in the pear, since also here was found higher particles contamination. Particles are probably contaminated with AFB$_1$, coming from many possible sources, such as feed, material that is use to cover the units floors (Viegas et al., 2012a, c) and, also, from the poultry environment that has the conditions for fungi dissemination and, consequently, mycotoxins can be produced. Considering this, we have to bear in mind that some studies indicate that exposure by inhalation to mycotoxins is more relevant regarding health effects than exposure by ingestion (Creasia et al., 1990; Amuzie et al., 2008; Degen, 2011).

Differences obtained in the workers results can be due to the fact that several variables can influence exposure, some related with possible chicken contamination and other with work practices that are normally different even between individuals doing the same task in the same workplace.

When comparing the results obtained in both settings (Table 3) we can conclude that in poultry production exposure is higher. This can be related with the fact that in the slaughterhouse not all workplaces have conditions for exposure occurring since chickens are killed in the beginning of the process and after that, in most of the workplaces, the workers use gloves during the handling of the birds. Additionally, the strict hygienic conditions applied in this setting eliminate and avoid fungi contamination and dissemination, something impossible to guarantee in the poultry production (HSE, 2009; Viegas et al., 2012b; c; Greco et al., 2014).

Moreover, possibly we are in the presence of a long-term exposure to low levels of AFB$_1$ by dermal and respiratory routes since these workers are doing the same tasks in the same conditions during the entire work shift. Regarding the expected health effects that can result from this kind of exposure there is still a severe lack of information and lot to do to clarify the possible outcomes.

**CONCLUSIONS**

Despite the uncertainties regarding the exposure route that is contributing more to exposure is possible to state that exposure to AFB$_1$ is occurring in the slaughterhouse setting. It seems that reducing AFB$_1$ contamination in poultry production can have a positive impact in reducing occupational exposure to AFB$_1$ in this setting. Future work should be developed to include more slaughterhouses and a higher number of workers and controls to increase the results statistical power. Additionally, and besides an occupational health problem we have to consider also a food safety issue and further research work is needed to clarify this.

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