Study of zearalenone contaminated feedstuffs on the detoxification enzymes

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ABSTRACT
Zearalenone (ZEA), a Fusarium toxin, is frequently found in animal feed materials. It is a naturally occurring estrogenic contaminant of moldy feeds and is present in high concentrations in dairy products and cereals. Its mechanisms of toxicity involve the binding of both zearalenone and its metabolites on steroid receptors. A general detoxification process, leading to reduction of bioaccumulation of the potentially toxic substances in animal organism, is based on the bio-transformation reactions of xenobiotics catalyzed by Cytochromes P450 (CYP). CYPs are part of an enzymatic multigenetic superclass, involved in the oxidative metabolism of a great variety of molecules, such as xenobiotics (drugs, pesticides, toxins, carcinogens) and also endogenous substances (steroid hormones, fatty acids, vitamins). Our studies concern the elucidation of the effects of contaminated aliments (especially those containing Zearalenone) on the enzymes of detoxification (Cyt P450) within animal liver, particularly those involved in steroid metabolism. Another point of interest is the metabolic studies concerning the effects of zearalenone purity, alone or in natural mixtures, on CYPs expression and its animal or human pharmacokinetics. The metabolic effect of Zearalenone on the CYPs is not very well known and the previous experiments were limited to in vitro exposure. Our preliminary data are in favor of a liver CYP 1A induction upon rat i.p. treatments.

Keywords: mycotoxins, zearalenone, detoxification, rats

INTRODUCTION
Cytochromes P450 (CYP) are part of an enzymatic multigenetic superclass, involved in the oxidative metabolism of a great variety of molecules, like xenobiotics (drugs, pesticides, toxins, carcinogens) and also endogenous substances (steroid hormones, fatty acids, vitamins). The bio-transformation reactions of xenobiotics catalyzed by CYP’s are a part of the detoxification process that has as result the limitation of bioaccumulation of potential toxic substances in organism.
The liver is the organ directly involved in the detoxification process. Live organisms are exposed everyday, directly or indirectly to different substances, with different provenances, those are entitled xenobiotics. Those compounds include the natural products, the drugs and environmental pollutants, like: plants and animal toxins, derivatives of domestic and industrial combustibles, solvents, dyes, alimentary additives, pesticides, herbicides, etc…

The metabolism of xenobiotics does not always result in a detoxification process. In particularly cases, the oxidized or reduced products can exhibit a very high reactivity by electrophile or nucleophile attacks on the close molecules (lipids, proteins, nucleic acids). As an example, the reactions with DNA (oxidation, intercalation or adductsformation) have as consequences mutations and the initiation of teratogenesis or carcinogenesis. The protein additions can determine tissues necrosis and allergic reactions (Dansette et al. 1998).

**Zearalenone** (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone) is a secondary metabolite produced by *Fusarium graminearum* (teleomorph *Gibberella zeae*) and is classified as toxin due to its perturbation of steroid receptors. Structurally it resembles to 17-estradiol, the principal hormone produced by the human ovary, which allow it to bind to estrogen receptors in mammalian target cells. Zearalenone is better classified as a nonsteroidal estrogen or mycoestrogen and is largely named as phytoestrogen. Zearalenoneand its reduced analogs (Figure 1) are biosynthesized through a polyketide pathway by *Fusarium graminearum, F. culmorum, F. equiseti*, and *F. crookwellense*. All these species are regular contaminants of cereal crops worldwide (J.W. Bennett and M. Klich, 2003).

The studies of physically and chemically properties of Zearalenone implies that this compound present ideal characters for a large diffusion in the tissues (e.g. water solubility of 20 mg/l at 25°C). The important differences of sensibility of different species concerning the effects of Zearalenone could in part result from the difference in their metabolism levels. (J.L. Gaumy et al., 2001; S. Cavret and S. Lecoeur, 2006).

Using a rat model, the aim of the present study was to analyze the effect of contaminated aliments (especially with Zearalenone) on the enzymes of detoxification (Cyt P450). This is why our work started with studies concerning the:

a)- interactions of mycotoxins with enzymes, effects on the metabolism of reference compounds

b) in vivo effects induction or repression of enzymes expression (total P450, mRNA, activities)

Another interest point is the metabolic studies concerning the effects of mycotoxins, pure or in natural mixture, on the animal or human pharmacokinetics. For the mycotoxins pharmacokinetics studies we will use $^{13}$C-enrichment.
Our final interest is to know which form or forms of cytochromes are modified (induced or repressed) by zearalenone treatment of rat. To answer to this question we have made several measurements on the microsomal preparations performed on the treated rat livers.

**MATERIALS AND METHODS:**

**Animals**
For our study we used 24 Sprague-Dawley albino rats, 3 rats per treatment.

**Experimental design for in vivo studies.**
Rats of initial average body weight of 200 g were studied for 3 days. They were fed on a common diet ad libitum. Every day of the experiment, animals were treated as followed (table 1).

<table>
<thead>
<tr>
<th>Lot name</th>
<th>intra peritoneal treatment</th>
<th>induced CYP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Na Cl 9‰ - 1ml/day</td>
<td>------------</td>
</tr>
<tr>
<td>Oil</td>
<td>Corn oil - 0.5 ml/day</td>
<td>------------</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>80 mg/Kg rat i.p. Na Cl 9‰ /day</td>
<td>CYP 2A, B, C; 3A</td>
</tr>
<tr>
<td>Natrium Salt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>100 mg/Kg rat i.p. corn oil/ day</td>
<td>CYP 3A, 2C</td>
</tr>
<tr>
<td>β – Naphtoflavone</td>
<td>100 mg/Kg rat i.p. corn oil/ day</td>
<td>CYP 1A</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>100 mg/Kg rat i.p. corn oil / day</td>
<td>CYP 4</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>25 mg/ Kg rat i.p. corn oil/ day</td>
<td>??????</td>
</tr>
<tr>
<td>Zearalenone PO:</td>
<td>25 mg/ Kg rat p.o.. oil / day</td>
<td>??????</td>
</tr>
</tbody>
</table>

Animals were killed 24 hours after the last treatment and liver microsomes were immediately prepared.

**Preparation of liver microsomes.**
Microsomes were prepared from thawed liver tissue using ultracentrifugation according to the method described by Guengerich (1989). The resultant microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) with 20% glycerol, frozen in liquid nitrogen and stored at –80°C until use.

**Measurements**
* Assessment of total P450, b5, protein amounts
* Enzymatic activities:
  - directs: metabolism of X-ROD (resorufine derivates), 7-BFC (7-Benzyloxy-4-(trifluoromethyl)-coumarin, testosterone or estradiol in the presence of zearalenone
For CYP assessment we used the next techniques:
- Total CYP – Spectrophotometric assessment based on the method of Omura and Sato (1964)
- Total b5 – Spectrophotometric assessment based on the method of Omura and Sato (1964)
- Proteins – Total Protein Kit Micro Lowry (Sigma Aldrich) according to the manufacturer’s instructions.

Cytochromes P450 metabolic activity was determined by fluorimetric method based on the quantification of fluorescence changes.

To compare the microsomes metabolic activity, we have used the next non fluorescent substrates:

Table 2 Used Non fluorescent substrates and fluorescent metabolites

<table>
<thead>
<tr>
<th>Substrate (non fluorescent)</th>
<th>Metabolite (fluorescent)</th>
<th>Wavelenght of Excitation/ Emission</th>
<th>Involved Implicated CYP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyloxyresorufin</td>
<td>Resorufin</td>
<td>530 nm/590 nm</td>
<td>CYP 3A</td>
</tr>
<tr>
<td>Methoxyresorufin</td>
<td>Resorufin</td>
<td>530 nm/590 nm</td>
<td>CYP 1A; 1B</td>
</tr>
<tr>
<td>Ethoxyresorufin</td>
<td>Resorufin</td>
<td>530 nm/590 nm</td>
<td>CYP 2B</td>
</tr>
<tr>
<td>Pentoxyresorufin</td>
<td>Resorufin</td>
<td>530 nm/590 nm</td>
<td></td>
</tr>
<tr>
<td>7-BFC</td>
<td>HFC</td>
<td>410 nm/510 nm</td>
<td></td>
</tr>
</tbody>
</table>

The metabolic profiles of testosterone or Estradiol were assessed using \textit{in vitro} incubation of different types of microsomes and adding a known quantity of testosterone or estradiol.

\textit{Incubation protocol:}

Reagents:
- System generator: NADP at 20mM+G6P at 200mM. (Diluted 20 times or 25µl in 500µl incubation)
- G6PDH: 1 of 2 UI / ml, with 1UI = 5
- 200 µl buffer PO4 100 mM with EDTA 0.1mM
- MgCl2 10 mM : 5 µl
- Microsomes: 1nmole in solution (200µl at 5µM in 500µl)
Zearalenone and its metabolites.

Table 3 shows the best known derivates of zearalenone:

<table>
<thead>
<tr>
<th>Name</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
<th>R7</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-Z 1640-1</td>
<td>-CH3</td>
<td>-H</td>
<td>-H</td>
<td>-OH</td>
<td>-OH</td>
<td>=O</td>
<td>-H</td>
</tr>
<tr>
<td>LL-Z 1640-3</td>
<td>-CH3</td>
<td>-H</td>
<td>-H</td>
<td>-OH</td>
<td>-OH</td>
<td>-OH</td>
<td>-H</td>
</tr>
</tbody>
</table>

The incubation were made at 37°C for 30 minutes and were stopped with 500 µl acetonitrile, and injected in a HPLC with a C18 column in order to identify the formed metabolites.

Using the same method and reagents we performed incubation in the presence of 20 µM Zearalenone to measure the effects of Zearalenone on the Estradiol metabolism. The used microsomes derived from the rats treated as previously described.

RESULTS AND DISCUSSIONS

Rats were treated with known P450s inducers in order to compare the liver induction profiles of zearalenone given orally or intraperitonrealy. The concentration of total cytochrome P450 are presented in table 4.

<table>
<thead>
<tr>
<th>Name</th>
<th>Conc P450 (µM)</th>
<th>Prot. Conc mg/ml</th>
<th>nmol P450 / mg Prot</th>
<th>Conc b5 µM</th>
<th>P450/b5 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>8.96</td>
<td>1.34</td>
<td>6</td>
<td>2.00</td>
</tr>
<tr>
<td>Oil</td>
<td>4</td>
<td>2.78</td>
<td>1.44</td>
<td>3</td>
<td>1.33</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>28</td>
<td>13.50</td>
<td>2.07</td>
<td>9</td>
<td>3.11</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>15</td>
<td>5.86</td>
<td>2.56</td>
<td>5</td>
<td>3.00</td>
</tr>
<tr>
<td>β-Naphtoflavone</td>
<td>18</td>
<td>9.64</td>
<td>1.87</td>
<td>9</td>
<td>2.00</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>17</td>
<td>11.17</td>
<td>1.52</td>
<td>9</td>
<td>1.89</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>16</td>
<td>11.67</td>
<td>1.37</td>
<td>9</td>
<td>1.78</td>
</tr>
<tr>
<td>Zearalenone PO</td>
<td>21</td>
<td>17.62</td>
<td>1.19</td>
<td>11</td>
<td>1.91</td>
</tr>
</tbody>
</table>

As compared to the well known P450 inducers (phenobarbitone, dexamethasone, β-Naphtoflavone) zearalenone treatments exert only a minor effect on P450 total amount.
Liver microsomal metabolic activities of different rat treatments

In order to assess the metabolic activities, we used fluorescence to measure the dealkylation of more or less specific substrates of each P450 family: Benzyloxyresorufin for 3A and 2 family, Ethoxyresorufin and Methoxyresorufin for 1A family, Pentoxyresorufin for 3A family and 7-Benzylxy-4-((trifluoromethyl)-coumarin for 3A. In figure 1 is presented the metabolic profile of resorufin derivates (table 2).

![Fig. 1 Metabolic activity of resorufin derivates (X-ROD)](image1)

Figure 2 shows the metabolic profile of 7-Benzylxy-4-((trifluoromethyl)-coumarin.

![Fig. 2 Metabolic activity using 7-Benzylxy-4-((trifluoromethyl)-coumarin (7-BFC)](image2)

Liver microsomes of rat treated by Zea IP are showing an increase of the ethoxyresorufin and methoxyresorufin dealkylation activities as compare to control animals and similar to those of rat treated with β-Naphtoflavone. The
microsomes Zea IP and Zea PO exhibit same metabolic activities on the 7-BFC than Control microsomes. These results are suggesting the idea that the zearalenone may be inductor of P450 1A family without modification of Cyp 3A.

**Metabolism of Estradiol**

Estradiol metabolism was assessed using in vitro incubation of different types of microsomes and adding a known quantity of estradiol.

**Estradiol incubation**

The profile of estradiol metabolism is shown in figure 3. The presented results are the mean of 3 manipulations, with a coefficient of variability that is not exciding 10%.

![Fig.3. Estradiol metabolic profile](image)

Figure 4 shows the profile of estradiol metabolism in presence of 20 µM Zearalenone. The presented results are the mean of 3 manipulations, with a coefficient of variability that is not exciding 10%.

In the case of 100 µM (according to the table 3, the concentration of E2 was 200 µM) Estradiol incubation, there are no essential differences between the metabolites formed by the Control microsomes and Zearalenone microsomes. In the presence of 20 µM Zearalenone an important decrease of metabolism is observed, mainly on the 2OH-E2 formation. The formation of the 4OH-E2 seems increased in comparison to the incubations performed in the absence of zearalenone. This 4OH-E2 formation is particularly important using microsomes PB (CYP 2, 3 inductor), DM (Cyp 3A inductor) and slightly on the Zea IP.
CONCLUSIONS

The X-ROD metabolism implies the idea that the Zearalenone could induced in the rat Cyt 1A.

The estradiol metabolism did not revealed special differences between Control and Zearalenone microsomes but is confirming the zearalenone effect on the forms 1A involved in 2OH-E2 formation. These reinforce the idea that Zearalenone on the estradiol can bind on similar enzymatic active sites.

The increase of the 4OH-E2 formation may reinforce the potential carcinogenic effect associated to Zearalenone exposition. The mechanism of 4OH-E2 increase needs to be elucidated.

The 7-BFC metabolism is showing the fact that the Zearalenone is not a specific activator of the forms 3A.

According to these results, one can conclude that Zearalenone may be P450 1A inductor.

Further work to be done:
- Confirmations of the results and explanation of the 4OH-E2 formation
- Use of purified enzymes in order to precise the P450 isoform involved in Zearalenone metabolism
- Precise the Zearalenone metabolism in vitro and in vivo using several types of animals (chickens, pigs)
- Precise its pharmacokinetic parameters: transport, binding, body accumulation using enriched Zearalenone
- In vitro effects on Estradiol or Testosterone metabolism
- Effects of Zearalenone and its metabolites on steroid receptors
- Effect of crude extract or contaminated feed on detoxification enzymes
REFERENCES
Cavret S. and Lecoeur S., 2006 – Fusariotoxin transfer in animal, Food and Chemical Toxicology, 44, p. 444-453