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Evaluation of immunologic effect of Enniatin A and quantitative determination in feces, urine and serum on treated Wistar rats

Cristina Juan*, Lara Manyes, Guillermina Font, Ana Juan-García

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, València, Spain

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ABSTRACT

Study of dietary supplementation with ENN A mycotoxin during 28 days of exposure time on Wistar rats to determinate its levels in serum, urine and feces and, to evaluate the immunologic effect in peripheral blood lymphocytes (PBL) is presented. The first method for ENN A extraction, determination and detection by LC–MS/MS in serum, urine and feces samples is reported. ENN A food dose administrated was detected in serum samples and influenced lymphocyte phenotyping. Levels in serum were founded from the second week of the experiment; reaching values of 4.76 μg/ml on the fourth week, which corresponds to 3.24 μg/ml in blood. PBL as T helper ($CD4^+$) were presented in greater percentages compared to control ($p \leq 0.001$), while T cytotoxic ($CD8^+$) decreased significantly compared to control ($p \leq 0.001$). ENN A treatment significantly increased $CD4^+/CD3^+$ and $CD4^+/CD8^+$ ratios but significantly decreased $CD8^+/CD3^+$ ratio. $CD4^+/CD8^+$ ratio was 2.94:1, indicating that PBL surface antigen expression and immune status in Wistar rats treated were impaired by the ENN A mycotoxin.

1. Introduction

Enniatins (ENNs) are mycotoxins produced by *Fusarium* species, also known as fusarotoxins, but due to their recent finding they have been classified such as emerging mycotoxins. Fusarotoxins are commonly found in cereals and their products, stuff constituting an important part of human food and animal feed. There are four main mycotoxins belonging to ENNs group: ENN A, ENN A1, ENN B and ENN B1. Recent studies about ENNs occurrence in several countries, have been described as well as how frequent these contaminants are in cereals (wheat, barley, rye and oat) and cereals products (baby food and pasta), observing concentrations from 5.3 to 284.2 μg/kg and from 1 to 1100 μg/kg, respectively (Juan et al., 2013a; 2013b).

Regarding their biological activity, acting as enzyme inhibitors, antifungal and antibacterial agents, and also their toxicological effects, ENNs induce a wide range of effects *in vitro* and its action is mainly based on their ionophoric properties which are capable of transporting cations through the cell membrane, leading to toxic actions by an altered membrane potential (Ivanova et al., 2012; Jestoi et al., 2009). Furthermore, ENNs inhibit acyl-coenzymeA: cholesterolacyl transferase (ACAT) and 30,50-cyclo-nucleotide phosphodiesterase enzymes causing mitochondrial dysfunction, and the inhibition of multidrug resistance associated protein-1(ABCG2) and P-glycoprotein (ABCB1) efflux pumps (Tonshin et al., 2010; Dornetshuber et al., 2009a).

Many *in vitro* studies have reported cytotoxic effects of ENNs on several cell types, as reviewed by Jestoi (2008).

* Corresponding author. Tel.: +34 96 354 41 16; fax: +34 96 354 49 54.
E-mail address: cristina.juan@uv.es (C. Juan).

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Data on their *in vivo* toxicity are however lacking (Jestoi, 2008). Nevertheless, their possible subclinical effects are of greater importance as these may lead to reduced performance parameters in food producing animals and increased susceptibility to infectious disease. Opinions of the European Food Safety Authority (EFSA) on ENNs are currently being drafted on request by the European Commission. To evaluate their potential *in vivo* toxic effects, the knowledge of their toxicokinetic properties, namely absorption, distribution, metabolization and excretion (ADME) in livestock is crucial. Jestoi (2008, 2009) detected trace level residues of ENNs in Finnish eggs, poultry meat and liver samples. To date, only one paper carried out in our laboratory collects data on the tissue distribution of ENN A in Wistar rats (Manyes et al., 2014); however, its toxicokinetic properties in general have not been studied. This lack of data and studies could explain why there are no maximum guidance levels set for animal feed yet, in contrast to other mycotoxins (European Commission, 2006). It is obvious that for assessing animal exposure to ENNs and to investigate their toxicokinetics, the availability of sensitive and specific validated analytical methods is mandatory. In recent decades, many liquid chromatography–tandem mass spectrometric (LC–MS/MS) methods for the analysis of ENNs in food, feed and other matrices have been described (Jestoi, 2008; Uhlig and Ivanova, 2004; Uhlig et al., 2006; Sulyok et al., 2006; Juan et al., 2013a; 2013b). However, no methods for the analysis of these compounds in plasma, urine and feces have been reported. Their development and application in these fluids would allow evaluating and complete ADME studies in animals for ENNs mycotoxins.

For evaluate toxicological effects *in vivo*, it is important to expose the animals to a single substance because previous *in vitro* studies have demonstrated synergic and antagonism effect between ENNs (Lu et al., 2013). Furthermore, the IC_{50} values for ENNs studied in CHO-K1, Caco-2, Hep-G2 and HT-29 cells, are different and depending on the exposed cell line. The highest values were obtained for ENN A and ENN B as reported by Juan-García et al. (2013a) and Lu et al. (2013).

The goal of this study was to design a preliminary study of dietary supplementation with ENN A, as one of most toxicologically potential of enniatins mycotoxin group. ENN A mycotoxin (Fig. 1) was administrated during 28 days of exposure time *in vivo* (on Wistar rats) to determinate its levels in serum, urine and feces and to evaluate the immunologic effect, being the first time that ENN A immunotoxicity is evaluated. ENNs may have higher toxicity in hematopoietic progenitors than in mature blood cells. In this sense, none study with ENN A *in vivo* has been done at the moment and this study attempted to answer if ENN A doses and exposure time of Wistar rats altered peripheral blood lymphocytes (PBL).

2. Materials and methods

2.1. Chemical and reagents

Ethyl acetate, acetonitrile, and methanol for LC mobile phase and organic solvents were HPLC grade from Merck (Darmstadt, Germany), while acetic acid was obtained from Fluka (Milan, Italy). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

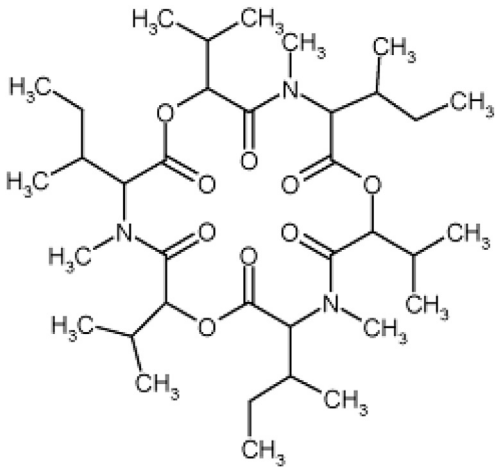

Fig. 1. Chemical structure of ENN A mycotoxin.

Enniatin A (ENN A) was purchased from Sigma Aldrich (Madrid, Spain). The stock solution and standard solution of ENN A were prepared in acetonitrile at concentration of 0.5 mg/ml and 100 μg/ml, respectively. These solutions were kept in safety conditions at −20 °C. For working standard solution, it was prepared immediately before use by diluting the stock solution with methanol.

Syringe filters PTFE (Polytetrafluoroethylene membrane, 15 mm, diameter 0.2 μm) were provided by Phenomenex® (Castel Maggiore, Italy).

2.2. Diets, animals, and experiment design

The control diet was Autoclaved Harlan lab blocks (Spain). All components were purchased from Harlan Laboratories Inc. (Madison, WI, USA). After mixing the basal diet, ENN A was added at 465 mg/kg. Dose assayed was obtained based on reproducing experimentally the natural presence of ENN A in the food matrix after grown of *Fusarium triticum* strain during 30 days in our laboratory as described in Manyes et al. (2014). The food was mixed with 20% water to form a dough and then cut into 10–15 g/bar, which were air dried for 2 days until the bar weight showed no further change. Diets were done at the beginning of the experiment and stored in sealed plastic bags at −20 °C. When making diets, disposable masks and gloves were used as protection. All items contaminated with ENN A were rinsed in 10% bleach before washing.

Ten 6–7 week-old female Wistar Rats (≈250 g) were purchased from Pharmacy animal facility (University of Valencia, Spain). Rats (5 control and 5 test animals) were housed in two plastic cages at 20 °C and a 12 h light/dark cycle with bars of food in the cage top. The study room where cages were stored was maintained under controlled conditions of (23 ± 2)°C and relative humidity (55 ± 10)%. Low dust absorptive bedding was changed twice per week.