OCHRATOXIN A: A TOXICOLOGIC EVALUATION USING *IN VITRO* AND *IN VIVO* BIOASSAYS

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Abstract. Ochratoxin A (OTA) is a secondary fungal metabolite that enters the food chain by cereals, beer and other products. Its toxicity is an important aim regarding the human pathologies such as nephrotoxicity. This mechanism is intense studied because of the affinity for blood albumins and the renal accumulation by the organic anion transporter. Its serum half-life is different in humans (850 h) and chicken (4.1 h) after oral administration. These data could lead to the idea of analyzing the deep mechanism in contact with blood elements. An important protocol for observation of necrosis/ toxicity and angiogenesis is CAM (chorioallantoic membrane assay) developed on embryonated chicken eggs. This test could be correlated with the red blood cell test (RBC).

In this study the toxicological effect of Ochratoxin A was tested. The Ochratoxin A was disolved in corn oil, in the similar concentration used in test on rats. The lipophilic solvent assures an important penetrability for tested compound on vascular plexus. The evolution of embryo vessels was observed after 15 minutes, 1h and 1 day. Samples were collected for haematoxilin-eosin staining and imunohistochemical evaluation. The same corn oil solution was used for the tests on blood red cells to see the damages. The OTA was also administered to Sprague Dawley male rats and a detailed blood test was made. The main results indicated that OTA influences the blood vessels and blood quality *in vitro* and *in vivo*. The irritation created on blood vessels is moderate comparing to strong irritants but it is significant. It determines moderate changes on blood elements after a period of presence of a few weeks in sytemic ic circulation.

Keywords: Ochratoxin A, HET-CAM bioassay, Irritation score, in vivo toxicity.

INTRODUCTION

Ochratoxin A (OTA) 1(N-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]carbonyl}-3-phenyl-alanine), is a mycotoxin produced by several fungi of *Aspergillus* and *Penicillium* [4] (Fig. 1).

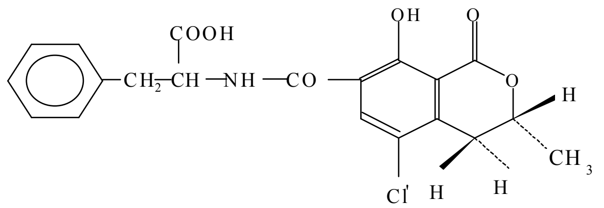

Figure 1. Molecular structure of Ochratoxin A.

OTA is important because of the contamination of agricultural products including cereals and Grains and influence chronic human exposure [1,2]. OTA is associated with Balkan endemic nephropathy disease that characterizes mainly the rural population in Romania, Bulgaria, Serbia and Croatia [13]. In Bulgaria, much higher prevalence of ochratoxin A (OTA), exceeding 2 microg/L, was observed in the blood of affected population [6]. OTA is potently nephrotoxic and carcinogenic, it is also teratogenic and immunotoxic. [14, 12]. In Croatia, previous studies shown that wheat and maize contamination with OTA came from endemic nephropathy region [17]. Ochratoxin A determines the carcinogenity dependent on sex, aspect observed on rodents and develope DNA adducts in renal tissue and tumours [8, 23, 24]. It also determine an important binding on albumins [10]. The detection of each sign of toxicity is a very important objective [7]. Male rats are most susceptible to OTA toxicity correlate with its dose between 70 and 210 mg/kg bw. First signs of carcinogenity appear after a long period of consumption (years). Lower doses didn't increased tumor rats. This mechanism is intense study because of the affinity for blood albumins and the renal accumulation by the organic anion transporter. Its serum half-life is different from humans (850 h) and chicken (4.1 h) after oral administration. These data could lead to the idea of analyzing the deep mechanism in contact with blood elements. An important protocol for observation of necrosis and angiogenesis is CAM (chorioallantoic membrane assay) developed on embryonated chicken eggs. This test could be correlated with the blood cell test (RBC).

The aim of our study was a preliminary test regarding the toxic effects of Ochratoxin on blood level on embryonated egg and rat blood. Evaluation of haematogram after OTA consumption could be an indicator of toxicity degree.

MATERIALS AND METHODS

Materials

OTA was obtained from Fluka BioChemika, lot code:1304356. OTA was dissolved in pure ethanol; the stock solution (100µg/ml) was diluted with distilled water just before the application onto the chorioallantoic membrane to a concentration of 0.01 mg/ml. For oral administration on rats was used as solvent corn oil and also 0,25mg/ml stock solution on corn oil dissolved by sonication and observed (OTA solubility) on a UV lamp. Animals were administred 0.5 mg/kg body weight.

Corn oil (Mazola Oil) and ethanol were purchased from Fluka (Germany) and Chimopar (Romania). *Ethanol.* A vehicle control solution was prepared for the application anto the CAM; the pure reagent was diluted with distilled water (1:10, v/v).

SDS. SDS was administered onto the CAM as positive control using a solution 1mg/ml in distilled water.

Animals: Sprague dawley rats were kindly offered by University of Medicine and Pharmacy „Victor Babeş" Timişoara (UMFVBT) biobase.

HET CAM Assay

The bioassay is used to evaluate the potential ocular irritancy of any test substance as measured by its ability to induce toxicity at the vascular level of the chorioallantoic membrane of the chicken [20].

The HET-CAM bioassay was performed following ICCVAM recommendations published in November 2006 in Appendix G and adapted to our laboratory conditions [11]. In brief, fertilized eggs were horizontally incubated 7 day prior to use, at 37^0C, in controlled wet atmosphere. On the 3^{rd} day of incubation, in order to detach the chorioallantoic membrane, 3 ml of albumen were aspired thorough a perforation at the more pointed end of the eggs. The hole was resealed and returned to the incubator. The next day, a big window was cut and resealed on the superior side of the shell. The eggs were returned to incubate until the 8^{th} day. 5 eggs were used for each test substance. After inspecting and recording the surface of the CAM by means of a stereomicroscope, 0.3 ml of the control and test solution diluted in NaCl 0.9% were applied to the CAM.

The reactions produced were observed over a period of 300 seconds. The time for the appearance of each selected endpoint was registered in seconds. The endpoints observed were: hemorrhage, H (bleeding from the vessels), vascular lysis, L (blood vessel disintegration), coagulation, C (intra- or extra-vascular protein denaturation). A variety of analysis methods may be used to assess irritancy potential of test substances. One analysis method that has been used extensively is an irritation score (IS). The formula used to generate an IS value is:

$$IS = 5 \cdot \frac{(301 - SecH)}{300} + 7 \cdot \frac{(301 - SecL)}{300} + 9 \cdot \frac{(301 - SecC)}{300}$$

Hemorrhage time (Sec H) = observed start (in seconds) of hemorrhage reactions on CAM; Lysis time (Sec L)= observed start (in seconds) of vessel lysis on CAM; Coagulation time (Sec C) = observed start (in seconds) of coagulation formation on CAM.

After the treatment time of 5 min, the main reaction was scored (either hemorrhage or lysis, or coagulation) according to the following scheme: 0 = no reaction; 1 = slight reaction; 2 = moderate reaction; 3 = severe reaction. Mean scores were determined.

Animals and treatment

Male Sprague dawley rats (8 weeks old, male: 240–300 g) were purchased from UMFVBT Biobase. The animals had free access to water and a standard diet (ad libitum) and all the standard conditions (12 hday/night cycle, temperature 22°C, humidity 45-55%). All animal experimentation was performed under permit of UMFT Bioethical Committee. Animals were administered OTA 0.5 mg/kg b.w. in the morning. The blood samples were collected after 15 days of daily administration. All animals were sacrificed by CO_2 asphyxiation and cervical dislocation. Animals were divided in 2 groups of 6 animals/group: A- witness and B administration of OTA as is presented in materials and method 0.5 mg/kg b.w. [23].

RESULTS

The experimental results regarding the toxicological evaluation for Ochratoxin A (OTA) on blood red cells are presented in Tables 1-2 and Fig. 2.

HET CAM Assay

The effects induced by the tested compounds as well as the selected controls were registered as mycrophotographs representing the en face surface of the chorioallantoic membranes before and after 5 minutes of substance contact (Fig. 2).

The results show a great difference between the positive control, SDS and the test compound, ochratoxin A. SDS induced major dammage at the vascular level of the chorioallantoic membrane (Fig. 2). After the application of 0.3 ml of solution (1mg/ml), a large area was affected by high number of mycrohemorrhages, the death of the specimen was registered after 20 minutes of contact with the solution. The ochratoxin A sample had somehow a better viability, until the next day. As observed in figure 2g, 1h, there are signs of microhemorrhage emerged in capillary isles. Compared to the positive controll, the reaction is weaker. The other endpoints were noted as well. Targeting imature vessels, there were spots where degradation of blood vessel wall was observed. A few areas where microcoagulation happened were noted. Even if all the endpoints were observed, for none of them severe reactions were registered, and the appearance time was longer, which explains the value of the irritation score 8.87 (Fig. 2h).

DISCUSSIONS

Ochratoxin A is a micotoxin with an important toxicological activity. It influences the blood vessels and blood quality *in vitro* and *in vivo*. The irritation created on blood vessels is moderate comparing to strong irritants but it is significant. It determines moderate changes on blood elements after a period of presence of a few days systemic circulation. Changes in „red line" of blood samples indicated the blood vessel toxicity in vivo that can be correlated with the results on embryonated egg model.HET-CAM assay is a well accepted test for evaluation of irritative and corrosive effects even on blood vessels [9, 11]. The intervention of such compounds with impact on agricultural area was observed on blood vessel quality by application of similar tests especially for pesticides [11]. For mycotoxins the blood toxicity impact was related to protein content, blood albumins binding and general noxiousness aspects on blood quality and quantity [10, 11, 15, 22]. The evolution of blood elements indicates toxicity on this level. The changes are not dramatic but are still detectable. OTA Is well absorbable from gastrointestinal tract [25]. The presence of OTA in human blood samples is a relatively frequent report, more specific for countries

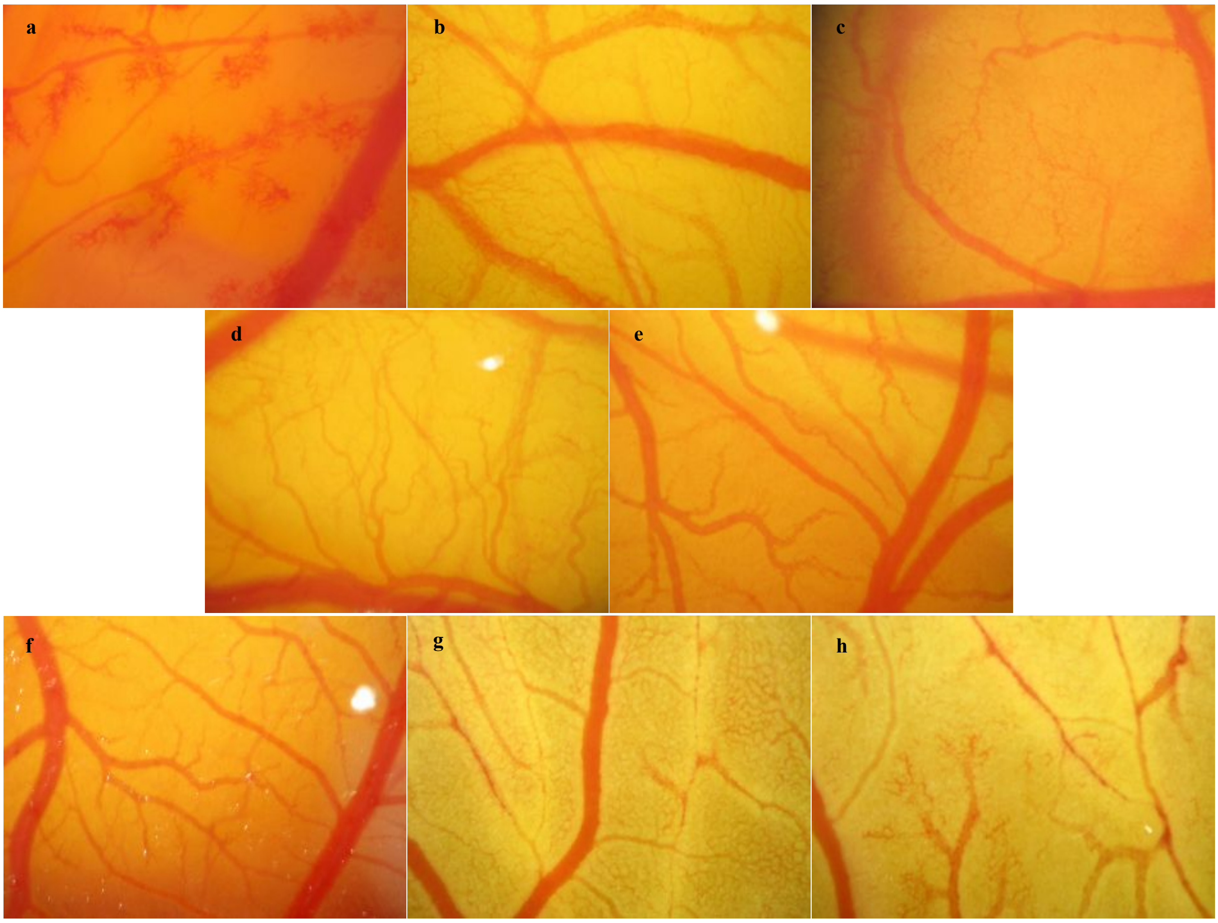

Fig. 2. HET CAM bioassay, en face images of chorioallantoic membrane before and after 5 minutes of contact with the tested compounds, Stereomicroscope mycrographs x20 : **a.** SDS – before; **b.** SDS – after; **c.** NaCl 0.9% – before; **d.** NaCl 0.9% – after; **e.** Ethanol: H_20 (1:10) – berfore; **f.** Ethanol : H_20 (1:10) – after; **g.** Ochratoxin A – before; **h.** Ochratoxin A – after.

Table 1. Irritation score, severity and effect classification.

Test compound and controls	Irritation score (mean)	Irritation severity (mean)	Classification of the effect
SDS (1 mg/ml) Positive control	17.99	3	Severe reaction
Distilled water Negative control	0	0	No reaction
Ethanol : H_20 (1:10) Vehicle control	0	0	No reaction
Ochratoxin A (0.01 mg/ml)	8.87	2	Moderate reaction

Table 2. Medium values for blood elements on the witness group and tested one (Ochratoxin A).

Determination type	Values of ochratoxin group	Values on witness group
(WBC) Leucocytes	5.50 x 10^3/mm	6.65 x 10^3/mm
(RBC) Hematies	7.25 x 10^6/mm	7.40 x 10^6/mm
(HGB) Haemoglobin	12.6 g/dl	13.1 g/dl
(HCT) Haematocryt	39.5 %	40.5%
(PLT) Trombocytes	1038 x 10^3/mm	1030 x 10^3/mm
(MCV) Erytrocytes medium volume	56.5 fl	54.2 fl
(MCH) Medium erytrocytar haemoglobin	17.8 pg	17.2 pg
(MCHC) Medium conc. of haemoglobin eritr.	31.4 g/dl	31.1 g/dl
(RDW) Indice of eritr. distribution	13.7%	12.5%
(MPV) Medium volume of plachetets	6.6 fl	6.65fl
(PDW) Indice of the plach. distribution	6.3%	6.2%

where is reported Endemic Nephropathy (Balkan Endemic Nephropathy) [3]. This aspect is important for detection of detailed changes of blood constituent and determines our global analysis on experimental model. Choosing of rats was determined because of previous reports in other publications and applications on male rats were influenced by other previous data [8]. Changes in leucocytes values are correlated to immunosuppressive potential of OTA. It can also produce inhibition of lipids and proteins [15]. OTA have a higher bioavailability on animals comparing with humans. The activity of OTA was studied intense on animals and also on cell cultures and the conclusion was that the resistance of cells is dependent on cell type. OTA determine an influence on fibroblasts also [16, 22]. OTA at low nanomolar and even subnanomolar concentrations administered for a long time led to cellular hypertrophy. Doses of 70-210 μg/kg after 4 weeks of administration on male rats determine decreases number of reticulocytes and leucocytes decreased after 4 weeks in a dose related manner aspects that are observed in our study after a short period of exposure. Other studies suggested also a decreasing of neutrophils and eosinophils also. These aspects as was also suggested in previous reports could become standard haematological analysis [19]. Importance of OTA analysis including all aspects of toxicity could be related to the aspect that our country is included on reports that present OTA toxicity and tolerated dosage and also on reports for Balkan Endemic Nephropathy [5, 18].

OTA influenced blood status and vessels integrity. It is a compound with a well known toxicity but it could very detail evaluated even on short term application. Haematological investigations are investigations to be performed even on humans and the influence on OTA on blood elements could be characterised [7]. The detection using blood investigations or tests on blood vessel quality are helpful in reducing its chronic consumes by early detections [7,21]. These test correlated to other specific evaluations can contribute to a complete image of OTA toxicity. OTA can be considered a n irritating agent for blood vessels, an immunosuppressant and a slow reducing compound on red elements of blood.

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