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Research Article

Ochratoxin A and Liver Damage: A Case-Control Study

Gian Maria Prati[1], Francesca Maria Cicognini[2], Filippo Rossi[2]*, Terenzio Bertuzzi[2], Amedeo Pietri[2], Milena Casali[2], Michele di Stasi[1], Bruna di Stasi[3] and Fabio Fornari[1]

[1]Division of Gastroenterology and Hepatology, "G. da Saliceto" Hospital, Piacenza, Italy

[2]Istituto di Scienze degli Alimenti e della Nutrizione, Facoltà di Scienze agrarie, alimentari e ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy

[3]Clinical Laboratory "G. da Saliceto" Hospital, Piacenza, Italy

***Corresponding Author**: Filippo Rossi, Istituto di Scienze degli Alimenti e della Nutrizione, Facoltà di Scienze agrarie, alimentari e ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.

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Abstract

Ochratoxin A (OTA) is a mycotoxin suspected to exert toxic effects on liver cell: in our research, we investigated the role of OTA on the genesis of liver disease and HCC in a case-control study.

43 subjects with chronic liver disease (case group) were matched for age and sex with 62 volunteers without liver disease (control group). In order to avoid any confounding effect of alcohol intake, all subjects consumed less than 20 g/day of alcohol for men and 10 g/day for woman. A blood sample was taken from each subject and analyzed for OTA, liver transaminases, ALP. Bilirubin, CRP and creatinine.

In our study, OTA intake was low (0.039 ng/kg b.w./day - 0.065 ng/kg b.w./day) and 49% (51/104) of subjects had plasma OTA levels lower than LOD (25 ng/l). Only 10 (%) subjects exceeded the value of 200 ng/l; no differences were found between serum OTA concentrations of control and case groups.

In the case group, the CRP levels were linearly related with bilirubin levels ($r = 0.298$; $P < 0.05$) and in subjects with liver disease and positive serum OTA concentration, this relationship improved ($r = 0.638$; $P < 0.01$).

Although OTA did not affect the prevalence of liver disease, among the 29 cirrhotic patients, the prevalence of HCC was higher in OTA positive subjects than in OTA negative ones ($P < 0.05$). Our results do not clearly support the role of OTA as a risk factor for HCC or cirrhosis, however the observed prevalence of HCC in OTA positive subjects requires further investigation.

Keywords: *OTA; Cirrhosis; ALT; AST; ALP; C Reactive Protein*

Abbreviations

OTA: Ochratoxin A; UTT: Urinary Tract Tumors; BEN: Balkan Endemic Nephropathy; HCC: Hepatocellular Carcinoma; GGT: Γ-Glutamyl Transpeptidase; AST: Aspartate Transaminase; ALT: Alanine Transaminase; ALP: Alkaline Phosphatase; CRP: C-Reactive Protein; LOD: Limit of Detection

Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species belonging to the *Penicillium* and *Aspergillus* genera, mainly *Aspergillus carbonarius, Aspergillus niger, Aspergillus westerijkiae, Penicillium verrucosum, Penicillium tubingensis* and *Penicillium nordicum* [1].

OTA can be found in several human foods, such as cereals and derivatives, grapes, cocoa, coffee, nuts and spices and some pork meat products, even after food processing [1-3].

OTA exhibited immunosuppressive, teratogenic and carcinogenic properties both in humans and animal models [5] and has been classified as possibly carcinogenic to humans (Group 2B) by IARC [4].

An association with renal cancer was suggested: human exposure to high levels of OTA in the diet has been linked to an increased incidence of urinary tract tumors (UTT) and to the pathogenesis of Balkan endemic nephropathy (BEN) [5-7].

Moreover, OTA has recently been considered for its hepatic toxicity [8]. Results from *in vivo* (animal model) and in vitro experiments, confirmed that the ingestion of OTA-contaminated foods negatively affected liver function [2,9] and induced tumors in the kidney as well as in the liver [10,11].

In particular, Ibrahim., *et al.* [12] reported that a chronic OTA exposure could increase the risk of hepatocarcinoma (HCC) in Egyptian population. Blood levels of this mycotoxin were higher in people with HCC than in healthy subjects. In addition, HCC was found 9.8 times as frequent in the group exposed to OTA than in the cohort of people not contaminated by the mycotoxin [12].

Recent studies demonstrated a hepatotoxicity of OTA in hepatic cells due to oxidative stress and DNA damage [13]. In fact, an inverse correlation between serum OTA and albumin levels, a relevant marker of liver function, has been found in animal models, proving an interference of OTA on hepatic cellular function [9]. The same authors reported also significantly higher levels of ALT and GGT in the higher quartile of OTA concentration in blood of healthy volunteers of the Moli-Sani Project [9].

OTA can be the main responsible of increase of some molecule such C-reactive protein (CRP) present in chronic inflammation state as well as in chronic liver disease. Indeed, Di Giuseppe (2012) demonstrated a linear correlation between OTA and CRP levels in a healthy cohort of Moli-Sani subjects [14].

Nonetheless, data from specifically designed clinical studies focused on the relationship between OTA and liver damage are still lacking. Then, this study was designed to investigate the relation between OTA and liver function in a non-alcoholic population of patients with chronic liver disease comparing to patients without liver disease.

Methods

Patients and study design

This study was conducted between April 2013 and July 2014 and included patients afferent to Gastroenterology and Hepatology Unit of the "G. da Saliceto" Hospital in Piacenza, Italy. The study was approved by the Ethical Committee of the "G. da Saliceto Hospital" (Protocol n° 16310 of 20th March 2013) and all the participants gave written informed consensus. Inclusion criteria were absence or consume less than 20 g/day of alcohol for men and 10 g/day for woman, to avoid any confounding effect of alcohol consumption on liver tests. Alcohol consumption has been evaluated using a FFQ.

43 subjects with chronic liver disease (case group) and 62 volunteers without liver disease (control group) having similar mean age and sex proportion, were enrolled in this study.

Serum analysis

A blood sample (5 ml) was obtained by all the subjects in order to estimate OTA, ALT, AST, GGT, ALP, bilirubin, CRP, creatinine levels in human plasma. The glomerular filtration rates (GFR) has been calculated with the Cockcroft-Gault formula.

The OTA analysis was performed in the laboratory of the Università Cattolica del Sacro Cuore, following a previously described method [14]. The limit of detection (LOD) and the limit of quantification (LOQ) were 25 and 50 ng/l respectively. The daily dietary intake (ng/kg

body weight/day) of OTA has been estimated as a function of plasma OTA using the following equation proposed by Klassen [15]:

OTA intake = Clp * Cp / A = 1.97 * Cp
where Clp is the plasma clearance (0.99 ml/Kg body weight/day);
Cp is the plasma concentration of OTA (ng/ml);
A is the toxin bioavailability, estimated at 50%.

The analyses on bio-humoral parameters were conducted at the laboratory of the Hospital "G. da Saliceto" in Piacenza, Italy, using a Au500 Beckman Coulter Analyzer. ALT, AST, GGT, ALP were analyzed with the colorimetric kinetic method proposed by IFCC [16,17], bilirubin was determined with the colorimetric photometric method (DPD 3,5 diclorophenil-diazonio-tetrafluoroborate), the C-reactive protein was determined with the immunoturbidimetric method [14]; creatinine levels were determined automatically with the Jaffe method.

Statistical analysis

The analysis was performed with the Statistical Analysis Systems version 6.11 (SAS Institute, Cary, NC, USA) [18].

Data were tested for normality with Shapiro-Wilk test, and where not normal, the non-parametric Wilcoxon test was applied.

In particular, the linear relationship between OTA levels in serum and hepatic and renal functional markers was studied with the Pearson's method through the PROC CORR procedure.

The comparison between OTA serum levels and hepatopatic incidence was performed using the t Student test except for non-normal distributed data, that were analysed using the non-parametric Wilcoxon test. A < P0.05 level of significance has been adopted.

The linear correlation between markers of inflammation and hepatic and renal functional blood parameters have been measured through the PROC CORR procedure and the r Pearson correlation coefficient was calculated.

Results

Anthropometric and clinical characteristics of subjects enrolled in the experiment are reported in table 1. The mean value of BMI fall in the normal range for both groups and sex.

Serum OTA levels and liver function

In our study 49% (51/104) of subjects showed serum OTA levels lower than LOD (25 ng/l) and only 10 % of subjects exceeded the value of 200 ng/l (Figure 1).

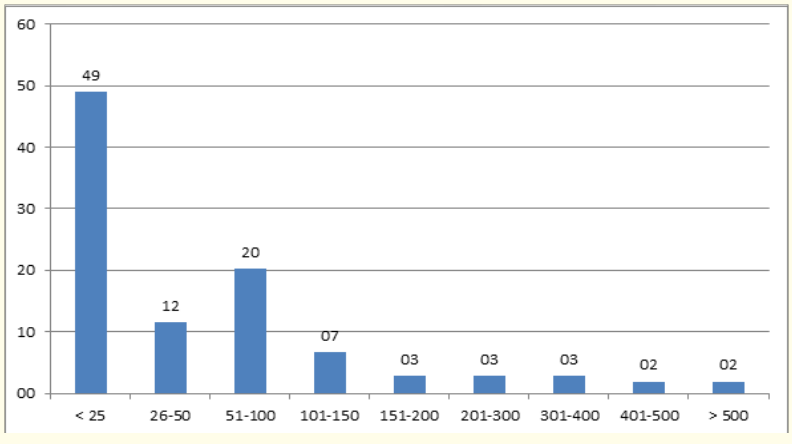

Figure 1: *Distribution (% of whole subjects) of blood samples according to their OTA content.*

	Sex	BMI	serum OTA	OTA intake	ALT	AST	GGT	ALP	Bilirubin	CRP	Creatinine	GFR
Normal range					5-52 U/L	14-36 U/L	< 55 U/L	90-280 U/L	0.2-1.1 mg/dL	0.1-8.0 mg/L	0.44-1.02 mg/dL	0.52-1.04 mg/dL
Control	F	22.5 ± 3.7	25.1 ± 38.2	0.049	27.5 ± 26.1	24.3 ± 22.3	79.2 ± 138.5	251.0 ± 128.3	0.8 ± 0.7	2.7 ± 3.8	0.7 ± 0.2	0.92 ± 0.53
	M	24.4 ± 3.0	28.3 ± 49.7	0.056	34.8 ± 30.3	28.9 ± 17.8	106.7 ± 226.9	254.9 ± 223.1	1.1 ± 0.9	1.1 ± 1.8	0.9 ± 0.2	0.93 ± 0.33
	Tot	23.3 ± 3.5	26.3 ± 42.4	0.052	30.3 ± 27.6	26.0 ± 20.6	89.6 ± 175.2	249.1 ± 168.0	0.90 ± 0.8	2.2 ± 3.3	0.8 ± 0.2	1.11 ± 0.61
Case	F	22.3 ± 2.8	20 ± 33.7	0.039	91.1 ± 61.3	88.8 ± 88.3	91.5 ± 99.2	357.2 ± 206.3	2.7 ± 3.1	2.0 ± 3.5	0.71 ± 0.2	0.80 ± 0.35
	M	24.2 ± 2.2	33.0 ± 55.5	0.065	78.4 ± 61.3	63.2 ± 48.7	102.6 ± 81.9	290.9 ± 132.2	1.9 ± 1.7	0.8 ± 0.8	0.9 ± 0.2	0.87 ± 0.33
	Tot	23.4 ± 3.6	27.5 ± 47.3	0.054	84.0 ± 68.1	74.5 ± 69.1	97.7 ± 88.7	320.1 ± 169.4	2.3 ± 2.4	1.4 ± 2.4	0.8 ± 0.2	0.97 ± 0.42
P	F	ns	ns	-	0.0005	<0.0001	0.0512	0.0497	0.0004	ns	ns	ns
	M	ns	ns	-	0.0058	0.0021	0.0265	ns	ns	ns	ns	ns
	Tot	ns	ns	-	<0.0001	<0.0001	0.0017	0.0153	0.0001	ns	ns	ns

Table 1: *Serum OTA concentration (ng/l) and daily intake (ng/kg b.w) estimated through the Klaussen equation in case and control groups in relation to the levels of hepatic and renal functional markers (average ± SD).*

ALT: alanine-aminotransaminase; AST: aspartate-transaminase; GGT: γ-glutammil-transferasi; ALP: alkaline phosphatase; CRP: C-reactive protein; GFR: Glomerular Filtration Rate; ns: not significant.

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No significant differences were found between serum OTA concentrations of control and case group (Table 1).

The OTA intake of our cohort ranged from 0.039 ng/kg b.w./day in females belonging to the case group, to 0.065 ng/kg b.w./day in males of the same group (Table 1).

Subjects in case group showed higher levels of the hepatic functional markers ALT, AST, GGT than subjects of control. ALP and bilirubin levels were higher in case group compared to control one, but only in females.

In contrast, the CRP, a marker of acute inflammation, did not show any difference between the two groups (Table 1).

No difference between the two groups was found about creatinine and GFR. The prevalence of OTA positive subjects was not different in case and control groups (Table 2).

	OTA negative	OTA positive
control group	28	34
case group	23	19

Table 2: *Subjects OTA positive and negative belonging to the two group.*

$\chi^2 = 0.925$, not significant.

Correlation between OTA and liver function

In table 3 the relations between serum OTA levels and the hepatic functional markers were reported for the whole cohort of OTA positive volunteers. There was no evidence of OTA effect on hepatic damage, but a positive relationship with CRP was found ($P < 0.05$).

	OTA	ALT	AST	GGT	ALP	BILIRUBIN	CRP
OTA	.	-0,137	-0,115	-0,133	-0,150	-0,155	0,571**
ALT		.	0,859**	0,122	0,378**	0,203	-0,176
AST			.	0,059	0,412**	0,342*	-0,124
GGT				.	0,398**	0,294*	-0,027
ALP					.	0,248†	0,261
BILIRUBIN						.	-0,054
CRP							.

Table 3: *r Pearson coefficient describing the relations among serum OTA concentration, C-reactive protein and hepatic functional markers in subjects from both the groups with serum OTA concentration > 0 (n = 53).*

** P < 0.05** P < 0.01† P < 0.07*

ALT: Alanine-Aminotransaminase; AST: Aspartate-Transaminase; GGT: Γ-Glutammil-Transferasi; ALP: Alkaline Phosphatase; CRP: C-Reactive Protein.

As expected, ALT was positively related to AST ($P < 0.01$) in the whole cohort as well as in the control and case group. Moreover, in hepatopathic patients both ALT and AST showed a positive relationship with ALP ($P < 0.05$, Table 4).

ALP was also linearly related to GGT in both groups ($P < 0.01$; Table 4).

In the case group, the CRP levels were linearly related to bilirubin levels ($r = 0.298$; $P < 0.05$) (Table 4) and when the analysis was limited to patients with liver disease and positive serum OTA concentration, the relationship between CRP and bilirubin improved ($r = 0.638$; $P < 0.01$).

			Control group (n=62)			
	ALT	AST	GGT	ALP	Bilirubin	CRP
ALT	.	0.892**	0.586**	0.263*	0.339	- 0.186
AST		.	0.503**	0.272*	0.289*	- 0.132
GGT			.	0.367**	0.373**	0.038
ALP				.	0.023	0.061
Bilirubin					.	- 0.126
CRP						.
			Case group (n = 43)			
	ALT	AST	GGT	ALP	Bilirubin	CRP
ALT	.	0.667**	0.284†	0.185	0.039	- 0.086
AST		.	- 0.032	0.282†	0.170	0.021
GGT			.	0.367*	0.182	0.052
ALP				.	0.446**	0.189
Bilirubin					.	0.298*
CRP						.

Table 4: *r Pearson coefficient describing the relations among C-reactive protein (CRP) and hepatic functional markers in the two groups.*

** P<0.05 **P<0.01; † P<0.07*

ALT: alanine-aminotransaminase; AST: aspartate-transaminase; GGT: γ-glutammil-transferasi; ALP: alkaline phosphatase; CRP: C-reactive protein.

Correlation among OTA, HCC and cirrhosis

The prevalence of liver disease and cirrhosis were not affected by OTA presence in the case group (data not shown).

However, among the 29 cirrhotic patients, the prevalence of HCC was higher in OTA positive than in OTA negative subjects ($p < 0.05$) (Table 5).

	OTA negative	OTA positive
No HCC	10	4
With HCC	5	10

Table 5: *Prevalence of HCC in OTA positive or negative cirrhotic subjects (n = 29).*

$\chi^2 = 4.21$ *(P < 0.05)*

Discussion

Serum OTA concentration

In the whole cohort 51% of subjects showed serum OTA levels higher than 25 ng/l and a few (8.6 %) exceeded the value of 200 ng/l, differently than what has been reported in Italy by Palli., *et al.* [19] and Di Giuseppe., *et al*. [14]. In the first study [19] 97% of subjects were found positive to OTA; while in the second one [14], 38% of volunteers reported OTA serum levels exceeding 200 ng/l and only 0.9% showed OTA levels lower than LOD.

The difference between these studies is that in the cited papers wine consumers were included in the cohort; it must be taken into account that wine is the most contaminated food for OTA in Italy and one the most important foods contributing to OTA intake in EU member states [20].

Thus, the discrepancies between our study and other Italian studies could be due to the exclusion of wine consumers from our cohort.

For ALP and bilirubin a sex effect has been observed in our study, since these enzymes were significantly higher in case compared to control cohorts only in females, while in males the levels in pathologic subjects were higher but not in a significative manner. A different response between males and females to liver damage, has been already reported by Agarwal., *et al* [21], Alatalo., *et al.* [22] and Stranges., *et al* [23].

Serum OTA concentrations of control and case group in our cohort were similar (Table 1); this result is probably related to the low OTA levels found in serum, due to the exclusion of wine. As a matter of fact, the daily OTA intake estimated from the serum level through the Klaassen equation was lower than the average OTA intake in Italy suggested by EFSA [1]. Moreover, the OTA weekly intake of our cohort, estimated around 0.37 ng/kg b.w., was very far from the 120 ng/kg b.w. reported by EFSA as the tolerable weekly OTA intake for humans [1].

As depicted in table 2, the prevalence of liver disease was not affected by the presence of OTA in blood. This result supports the idea that OTA, per se, is not a causal factor of liver disease.

Correlation between serum OTA levels and renal function

No subjects in our cohort suffered of chronic kidney disease and no damage in relation to OTA was found in the study. This result is in accordance with some indications that reported 500 ng/l as the minimum OTA concentration in blood needed to express renal damage [21].

Correlation between serum OTA levels and liver function

Subjects of the case group showed higher levels of the hepatic functional markers ALT, AST, GGT, ALP and bilirubin than subjects of the control group. Moreover, these markers were significantly higher also within sex, except for ALP and bilirubin levels, where only females from the case group showed higher values, differently than males (Table 1).

As expected, ALT is positively related to AST ($P < 0.01$). This result is clearly due to transaminases, indicators of liver function. As a matter of fact, ALT and AST are both markers of liver damage.

Moreover, ALT showed a positive relation with ALP ($P < 0.05$): the two enzymes are both related to hepatic damage and cholestasis. Indeed, ALP is a specific marker for biliary obstruction. The levels of ALP increased also with AST and GGT ones, for the well know relationship between these markers of liver damage (Table 3).

If we consider the two groups, independently of OTA presence in the blood (Table 4), the linear positive relationship between ALT and AST, and of these transaminases with GGT and ALP, is explained by the release of these enzymes into the blood stream as a consequence of liver cells catabolism. In case group the absence of correlation between ALP and AST could be explained by the low specificity of these enzyme for the liver. As a matter of fact, AST can be released by myocardium, muscle, kidney and erythrocytes. AST levels sharply increased in viral hepatitis but this illness was not present in our case group. As clearly demonstrated by Roblez-Diaz., *et al*. [25], ALT was related to cytolysis while ALP to cholestasis and this could explain the different trend of the two enzymes.

Bilirubin showed positive trends with ALP in the case group. This result could arise from a damage of biliary duct related to the illness or it could be explained with the known relationship between this transaminase and a marker of cholestasis like bilirubin.

In the control group bilirubin was correlated with different parameters as AST and GGT, and this result was in agreement with Agarwal., *et al.* [21] which found the same positive correlation in alcohol consumers (although not alcohol addicted) compared to abstainers.

In our study, we enrolled only abstainers subjects or with a very low alcohol intake and perhaps the control group had a lower percentage of teetotal compared to case group.

CRP, a marker of acute inflammation, did not show any difference between case and control group, this result can be due to the low OTA levels found in our cohort, since OTA and CRP are related [14]. The positive direct relationship between OTA and CRP (Table 3) could be due to the inflammatory role played by the mycotoxin and confirm the findings of di Giuseppe., *et al*. [14].

CRP showed positive trends with bilirubin in the case group ($r- = 0.298$; $P < 0.05$). This result is in accordance with other Authors that reported an inverse relation between these molecules in healthy subjects and a direct relation in subjects having blood CRP levels higher than 10 mg/100 mL [22-24]. High CRP levels suggest the presence of an inflammation, thus the positive relation found between CRP and bilirubin in case group of our study could be justified by the inflammatory stress related to hepatic disease, even if the CRP plasma levels were lower than 10 mg/dl.

Our data have been obtained in a cohort of abstainers or low alcohol drinkers and for this reason, OTA intake could not be very high. Therefore, we cannot point out a threshold level of OTA intake related to liver damage. By contrast, much more researches have been carried out on the relationship between OTA intake and kidney diseases, for this topic Grosso., *et al*. [29] proposed the level of 500 ng/L as threshold of OTA intake related to the development of kidney disease. But this level of OTA in blood is much higher than ones detected in our research.

Correlation among OTA, HCC and cirrhosis

Data from our research do not clearly support the hypothesis that OTA is a risk factor for HCC or cirrhosis, even if a prevalence of HCC is reported in OTA positive subjects.

Therefore, the presence of low serum OTA levels as those reported in our study is not a sufficient condition to trigger liver disease, but in cirrhotic subjects, where liver is already injured, OTA can be a risk factor for HCC, due to its potential to induce DNA damage [13,30].

Conclusions

The choice of a cohort of teetotal subjects or consuming very low quantity of alcohol, has determined the low percentage of OTA positive subjects and thus no differences were found in OTA blood levels between case and control groups.

Although OTA levels were not different between case and control subjects, the linear relationship between OTA and CRP, confirms the inflammatory effect of this mycotoxin previously observed [14].

The linear relationship bilirubin-CRP in subjects with damaged liver, with a r-value of OTA-positive subject higher than that of the whole cohort of people with liver disease, confirmed the results of other studies that reported a similar relationship in people having CRP levels higher than 10 mg/100 ml.

Data from our research do not clearly support the hypothesis that OTA is a risk factor for HCC or cirrhosis, even if a prevalence of HCC is reported in OTA positive subject.

Therefore, the presence of OTA is not a sufficient condition to trigger liver disease, but in cirrhotic subjects, where liver is already injured, OTA can be a risk factor for HCC, due to its potential to induce DNA damage.

Conflict of interest

The authors declared no conflict of interest.

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