Ochratoxin a in human breast milk, maternal and placental blood from Cochabamba-Bolivia

Ferrufino-Guardia E.[1]*; Chavez-Rico V.[1] and Larondelle Y.[2]

[1] Centro de Biotecnología-Facultad de Ciencias y Tecnología – Universidad Mayor de San Simón.
[2] Institute of Life Sciences, Université catholique de Louvain, Louvain-La-Neuve, Belgium

Abstract: The nephrotoxic and carcinogenic mycotoxin Ochratoxin A (OTA) is a contaminant in a wide variety of foods. Mothers who ingest food contaminated with OTA during the gestation and lactation period can transfer this mycotoxin to the fetus as to the infant, respectively. To quantify the transmission of Ochratoxin A from mother to newborn and to infant, samples were taken from maternal blood and umbilical cord of women at the moment of delivery and breast milk of ladies in lactation period in the city of Cochabamba-Bolivia. From a total of 31 samples of maternal plasma and its respective cord, 4 were positive in both sample types (incidence 13%, 12% allowable relative error to the population at 95% confidence). Maternal plasma samples had a range of 0.3-10.2 ng of Ochratoxin A/ml with a mean of 3.0 ng/ml (± SD=3,0). Similarly, in the umbilical cord plasma Ochratoxin A was also detected with values of 0.3 to 1.7 ng/ml and with a mean of 0.7± 0.65 ng/ml. In two samples the concentrations of ochratoxin A were higher in umbilical cord blood (0,5 and 1.7 ng/mL) than their corresponding pairs of maternal blood (0.3 and 1.3 ng /ml, respectively) with an average concentration ratio of 1.5 ng/ml. Two other samples presented positive in ochratoxin A in umbilical cord blood and negative in their corresponding pairs of maternal blood confirming the transfer of the mycotoxin through human placental barrier. Out of 29 samples the breast milk samples from the Bolivian mothers collected during the summer period, 13 (44.8%) were found positive in a ranged from 20.2 to 146.1 ng/l. Concerning the samples collected in the winter season, only one (2.4%) out of 42 had a measurable ochratoxin A concentration of 28.3 ng/1, and seven samples showed traces of the toxin, below the detection limit.

Keywords: mycotoxin, ochratoxin A, sang, umbilical cord, breast milk.

Resumen: ***Presencia de ocratoxina a en leche, placenta y sangre materna en Cochabamba-Bolivia***

La micotoxina nefrotóxica y cancerígena Ocratoxina A (OTA) es un contaminante en una amplia variedad de alimentos. Las madres que ingieren alimentos contaminados con OTA durante el período de gestación y lactancia pueden transferir esta micotoxina al feto y al bebé, respectivamente. Para cuantificar la transmisión de la ocratoxina A de la madre al feto y al niño, se tomaron muestras de sangre materna y cordón umbilical de mujeres en el momento del parto y de leche materna de madres en el período de lactancia en la ciudad de Cochabamba-Bolivia. De un total de 31 muestras de plasma materno y su respectivo cordón umbilical, 4 resultaron positivas en ambos tipos de muestra, (incidencia 13%, error admisible de 12% respecto a la población con 95% de confianza). Las muestras de plasma materno se encontraron en un rango de 0,3 a 10,2 ng/ml con una media de 3,0 ng/ml (± SD = 3,0). De igual manera, en el plasma de cordón umbilical se detectó también OTA en cuatro muestras, con valores de 0,3 a 1,7 ng/ml y con una media de 0,7 ng/ml (± SD=0.65). En dos muestras las concentraciones de ocratoxina A eran mayores en la sangre del cordón umbilical (0,5 y 1,7 ng/ml) que sus correspondientes pares de la sangre materna (0,3 y 1,3 ng/ml, respectivamente) con una relación de concentraciones promedio de 1,5. Otras dos muestras presentaron positivo en OTA en la sangre del cordón umbilical y negativo en sus correspondientes pares de sangre materna. Confirmándose así el traspaso de esta micotoxina a través de la barrera placentaria humana. De otra parte, de un total de 29 muestras de leche materna humana colectadas de madres bolivianas durante la estación de verano, 13 (44.8 %) se encontraron positivas en un rango de 20,2 a 146,1 ng/l. Respecto a las muestras de leche colectadas durante la estación de invierno solo 1 (2,4%) de 42 fueron positivas en ocratoxina a una concentración de 28,3 ng/l y siete muestras mostraron trazas de la toxina por debajo del límite de detección.

Palabras clave: micotoxina, ocratoxina A, sangre, cordón umbilical, leche materna.

Introduction

Ochratoxin A (OTA) is a nephrotoxic and nephrocarcinogenic mycotoxin. One of its most important mechanisms of toxicity is the inhibition of protein synthesis (WHO, 1990; IARC, 1993). This is due to competitive inhibition of phenylalanine-tRNA synthetase (PheRS) which catalyzes the aminoacylation reaction and stops the elongation of the peptide (Konrad and Röschenthaler, 1977; Bunge *et al*., 1978).

OTA is a low molecular weight molecule (403.83 daltons), which includes a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-(3R)-methyl isocoumarin linked through the carboxyl group to a L-β-phenylalanine. The pure crystalline toxin is: white, odorless and crystalline. Among some of its chemical characteristics are: a melting point between 168-173ºC; optically active at $[\alpha]_D$: -46.8º (C = 2650 µmol / liter in chloroform), UV absorptivity varies with pH and polar solvents (for example: in ethanol the maximum absorption is reached at 213 nm and 332 nm).

OTA is usually produced by *Penicillum verrucosum* and *Aspergillus ochraceus*. The post-harvest conditions are the preponderant factors for OTA production and consequently the contamination of food and fodder. This problem has been reported in almost all cereals including corn, barley, wheat, sorghum, rye, oats and rice (Council for Agricultural Science and Technology Cast, 1989; Krogh, 1987)

OTA has received considerable attention since 1993 from the International Agency for Research on Cancer (IARC, 1993) who classified this toxin as a possible human carcinogen of group 2B (sufficient evidence from studies of carcinogenicity in experimental animals and inadequate evidence in humans) and it would not be inappropriate to consider an upgrade of its classification from Group 2B to Group 2A (Probably carcinogenic to humans)(Ostry *et al*., 2017). For that reason, exposure to OTA can increase the risk of getting cancer. Especially in Europe, several efforts have been made to manage this risk and establish strict regulatory standards, (Clark and Snedeker, 2006; Pfohl-Leszkowicz and Manderville, 2007).

Due to its frequency in a wide range of foods, the presence of OTA in human blood has been suggested as a direct indicator of oral exposure to contaminated food. Analysis of human serum samples in several countries in Europe, Asia, Africa and America revealed that the blood of healthy people frequently contained OTA, which could confirm a continuous and extensive exposure (Breitholtz-Emanuelsson *et al.*, 1994; Jonsyn *et al.*, 1995b; Rizzo *et al.*, 2002).

The human exposure to ochratoxin A can be surveyed by analysing human blood. Such analyses have been performed in different European countries such as Croatia (Peraica *et al*., 2001), Czechoslovakia (Fukal and Reisnerova, 1990; Ruprich and Ostrý, 1993a; Ruprich and Ostrý, 1993b), Czech Republic (Malir *et al*., 2001), Denmark (Hald, 1991), France (Creppy *et al*., 1991; Creppy *et al*., 1993), Germany (Bauer and Gareis, 1987; Hadlock, 1993; Rosner *et al*., 2000; Degen *et al*., 2007), Hungary (Kovács *et al*., 1995), Italy

*e-mail: nanferru@hotmail.com

(Breitholtz-Emanuelsson *et al*., 1994; Palli *et al*., 1999), Norway (Thuvander *et al*., 2001; Skaug, 2003), Poland (Golinski *et al*., 1991; Grajewski *et al*., 2007), Portugal (Jimenez *et al*., 1998), Spain (Lino *et al*., 2008; Burdaspal and Legarda, 1998; Jimenez *et al*., 1998), Sweden (Breitholtz *et al*., 1991;Thuvander *et al*., 2001), Switzerland (Zimmerli and Dick. 1995); UK (Gilbert *et al*., 2001) and ex-Yugoslavia (Hult *et al*., 1982); as well as in Canada in North America (Kuiper-Goodman *et al*., 1993); Japan (Kawamura *et al*., (1993; Ueno *et al*., 1998); Lebanon (Assaf *et al*., 2004) Pakistan (Aslam *et al*., 2005) and Turkey (Özçelik *et al*., 2001) in Asia, Algeria (Khalef *et al*., 1993), Ivory Coast (Sangare-Tigori *et al*., 2006), Morocco (Filali *et al*., 2002), Sierra Leone (Jonsyn. 1999) and Tunisia (Bacha *et al*., 1993; Maaroufi *et al*., 1995) in North Africa; Argentina (Pacin *et al*., 2008), Chile (Muñoz *et al*., 2006) and Costa Rica (Guzmán *et al*., 2007) in South America, (for reviews see: IARC-International Agency for Research on Cancer, 1991; INSERM colloque, 1993; Smith *et al*., 1994; European Commission, 1997; Mycotox 98, 1998; Coronel *et al*., 2010).

The occurrence of ochratoxin A has also been reported in human milk collected in some European countries such as Germany (Gareis *et al*., 1988; Ali *et al*., 2017), Hungary (Kovács *et al*., 1995), Italy (Micco *et al*., 1991; Micco *et al*., 1995; Miraglia *et al*., 1995; Turconi *et al*., 2004; Galvano *et al*., 2008; Biasucci *et al*., 2011), Norway (Skaug *et al*., 1998; Skaug *et al*., 2001); Poland (Karwowska *et al*., 2004; Postupolski *et al*., 2006); Slovakia (Dostal *et al*., 2007); Spain (Rubert *et al*., 2014); Sweden (Breitholtz-Emanuelsson *et al*., 1993b) and Switzerland (Zimmerli and Dick.1995). In North Africa such as Sierra Leone (Jonsyn *et al*., 1995a; Jonsyn, 1999); Egypt (El-Sayed *et al*., 2000; El-Sayed *et al*., 2002; Hassan *et al*., 2006). In Asia: Iran (Afshar *et al*., 2013; Dehghan *et al*., 2014) and Turkey (Gürbay *et al*., 2010; Uyar *et al*., 2014). In the case of South America the contamination by ochratoxin A in human milk was reported in Brazil (Iha *et al*., 2014; Navas *et al*., 2005; Andrade *et al*., 2013) and in Chile (Muñoz *et al*., 2010; Muñoz *et al*., 2014).

The human exposure to ochratoxin A can be surveyed by analysing human blood and human milk in European, North America, Asia, North Africa and South America countries, (for reviews see; IARC, 1991; INSERM colloque, 1993; Smith *et al*., 1994; European Commission, 1997; Mycotox 98; Coronel *et al*., 2010).

OTA is a potent teratogen in mice, rats, hamsters and chickens (Fukui *et al*., 1987; O'Brien *et al*., 2005, Wangikar *et al*., 2005), but not in pigs (Shreeve *et al*., 1977). The differences in susceptibility between the species are attributed to the: type of placental transfer of OTA (Fukui *et al*., 1987), route of administration, and the time of exposure to the toxin during the gestation period.

Oral administration of a single-dose of 1.0 mg of OTA/kg of body weight during pregnancy causes malformations in the fetuses of mice and rats. An increase in prenatal mortality was reported (Kuiper-Goodman and Scott, 1989; WHO, 1990; Marquardt and Frohlich, 1992). There is no much information about the mechanism of transfer of OTA through the placenta. The presence of transporters, the immature metabolism and the low elimination capacity of the toxin by the fetus contributes to the bioaccumulation of OTA in the fetal circulation (Woo *et al*., 2012). The transfer of OTA is limited by its binding and high affinity to plasma proteins (Woo *et al*., 2012). Approximately 99% of circulating OTA binds to plasma proteins (mainly albumin, Chu, 1971 and 1974; Dai *et al*., 2004). In the perfused human placenta, the transfer of OTA does not depend on the concentration (Woo *et al*. , 2012). Because, only the unbound molecule is able to cross the placental barrier (Loebstein and Koren, 2002).

Several studies have been published on the placental transfer of OTA in animals (Patterson *et al*., 1976; Moré *et al*., 1974; Appelgreen and Arora, 1983; Mortensen *et al*., 1983, Ballinger *et al*., 1986; Fukui *et al*., 1987; Barnikol and Thalmann 1988; Hallén *et al*., 1998; Minervini *et al*., 2013). Barnikol and Thalmann (1988) studied the OTA transfer to uterus in sows that have ingested a naturally contaminated food. The blood concentrations in newborn piglets were 0.075-0.12 ng / ml, while in the sow was 0.20 ng / ml. Hallen *et al.* (1998) also observed a placental transfer in rats receiving 50 mg / kg of body weight by gastric intubation. Minervini *et al.* (2013) analyzed OTA in the blood (average 106.5 pg/ml) and in the umbilical cord (96.6 pg / ml) of 17 mares. Conversely, Patterson *et al*. (1976) and Mortensen *et al*. (1983) did not find transfer of OTA to pig's placenta. After the intraperitoneal and oral administration of 4 mg / kg of OTA in rats, Moré *et al*., (1974) detected superficial and deep hemorrhages in embryos. At the beginning of pregnancy, OTA passes rapidly through the mouse placenta (Appelgreen and Arora, 1983). After crossing the maternal placental barrier, ochratoxin A reaches the fetus and it interferes with organ development (Appelgreen and Arora, 1983).

The placenta plays an important role in the development of the human being from its conception. It is the main route of: communication, oxygen input and transfer of various nutrients between the mother and the fetus. In the placenta, hormones are produced to support pregnancy. It serves as a pathway for the excretion of several metabolites and carbon dioxide. However, it also plays a role in the exposition of the fetus to possible contaminants. Very little is known about the mechanism of OTA transfer through the human placenta. Studies conducted by perfusion on human placenta showed that OTA can cross the human placenta, particularly at the beginning of pregnancy and not at the end (Woo *et al*., 2012; Partanen, 2012).

In humans, Postupolski *et al*., (2006) found that the concentration of OTA in fetal serum was twice the concentration in maternal blood. Several studies in umbilical cord suggest an active placental transfer of OTA (Jonsyn *et al*., 1995b; Miraglia *et al*., 1998; Postupolski *et al*., 2006: Pfohl-Leszkowicz and Manderville, 2007; Biasucci *et al*., 2011).

This study aimed to determine the human placental transfer of OTA in samples of maternal and umbilical cord blood from patients hospitalized in "Hospital Obrero" Nº 2 of the "Caja Nacional de Salud" and the level of OTA in samples of breast milk from ladies in lactation period in Cochabamba-Bolivia.

Materials and Methods

Chemicals

Ochratoxin A was obtained from Sigma Chemical Co. (St Louis, MO, USA). A stock solution of ochratoxin A (10 mg/ml in toluene-acetic acid 99:1) was spectrophotometrically calibrated at 333 nm using the value 5440 l/mol/cm as the extinction coefficient (Wood *et al*., 1996).

Acetonitrile methanol and toluene of HPLC grade, as well as chloroform pro analysis, were supplied by Lab Scan (Dublin, Ireland). *Ortho*-phosphoric acid (85%) and acetic acid *pro analysis* were purchased from UCB (Brussels, Belgium). Phosphate Buffer Saline (PBS) solution (NaCl 120 mM, KCl 2.7 mM, phosphate buffer 10 mM, pH 7.4) was supplied by Sigma Chemical Co. (St Louis, MO, U SA). Ochratoxin A immunoafinity columns (OchraTest^{TM}) were purchased from VICAM (Water-town, MA, USA). All other chemicals were of analytical grade. Silica Cartridges Sep Pak was purchased from Waters (Milford, MA, USA). All water used was distilled and run through a Milli-Q System supplied by Millipore (Molsheim, France).

HPLC Standards

A stock solution of ochratoxin A (10 µg/ml) was prepared in toluene-acetic acid (99+1, v+v) and calibrated by its absorption at 333 nm using the extinction coefficient 5440 l/mol/cm (Wood *et al.*, 1996). Ochratoxin A standards used for standard curve determinations, were prepared by diluting known amounts of the stock solution in the HPLC mobile phase, which was made of acetonitrile-water-acetic acid (450+540+10, v+v+v)

Biological Samples

Samples of maternal and umbilical cord blood were obtained from 31 volunteers at the Department of Gynecology and Obstetrics of Obrero Hospital Nº 2 of Cochabamba-Bolivia. Maternal blood samples were taken in the delivery room before delivery. Samples of cord blood were taken after cutting the cord. Both samples were collected in dry tubes heparinized and homogenized in a vortex shaker. Blood samples were centrifuged at 3000 rpm for 15 minutes and frozen at -18°C until their respective analysis.

A total of 71 human milk samples were collected at the "Hospital Albina Patino" of Cochabamba-Bolivia. A first set of 29 samples were collected in February and March (summer season), and a second set of 41 milk samples in June and July (winter season). The milk donors were all volunteer mothers who had reached at least 5 week of lactation. Upon collection, the samples were directly frozen at -22°C and were stored until analysis.

Ochratoxin A Extraction

Ten ml of milk sample were extensively mixed during 2 min with 10 ml of an acid solution (0.5 mM H_3PO_4, 2 M NaCl, adjusted at pH 1.6). Ten ml of chloroform were then thoroughly mixed with the preparation during 2 min, and the resulting mixture was centrifuged at 3,300g for 20 min at 4°C. The chloroformic phase was carefully withdrawn with a Pasteur pipette and transferred to another screw-cap tube. The extraction was repeated twice with 10 ml of chloroform. Ochratoxin A was then re-extracted from the pooled chloroform fractions by addition of 5 ml 1M $NaHCO_3$, in order to separate the toxin from lipophilic components. The extraction was repeated twice.

Immunoaftnity column clean-up

The immunoafinity columns were equipped with an adapter and a 10 ml syringe on the top, and with a stopcock at the bottom, to adjust the flow-rate at 1 ml/min for the first steps and at 0.5 ml/min for the elution. The pooled sample extract in sodium bicarbonate was poured on a column after equilibration with 20 ml PBS. Ochratoxin A was eluted with 2.0 ml of methanol and 2.0 ml of water; 20 ml air were passed through the column (syringe) to collect all the eluent. The eluate was evaporated to dryness at 40–45°C under a stream of nitrogen. Prior to HPLC analysis, the ochratoxin A was carefully dissolved in exactly 0.5 ml methanol and subsequently mixed with 0.1 ml Milli-Q water.

Derivatization of Ochratoxin A to its Methyl Ester

Two hundred µl of sample extracts and ochratoxin A standard solutions were evaporated to dryness, and the methyl-ester of ochratoxin A was prepared as described by Ferrufino-Guardia *et al.* (2000). This method involved a treatment with methanol in boron trifluoride. The ochratoxin A methyl-ester formed was subsequently extracted with chloroform. The chloroform phase was washed with water and thereafter evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 200 µl of the HPLC mobile phase and analysed by HPLC.

HPLC Analysis

The HPLC system consisted of one P1000 HPLC pump from Spectra System (Northporth, LW, USA), an 7125NS injection valve (50 µl) from Rheodyne (Cotati, CA, USA), a RF-535 fluorescence Spectrophotometer, equipped with a 150 W xenon lamp (excitation 332 nm and emission 462 nm) and a C-R3A chromato-integrator from Shimadzu (Kyoto, Japan). Separations were achieved on a 3-µm Hypersil BDS reversed-phase C18 (150 x 4.0 mm I.D.) analytical column supplied by Tracer analitical (Barcelone, Spain).

Purified sample extracts were dissolved in 500 µl of mobile phase. The solutions were then filtered through a 0.45 µm microfilter and 50 µl samples were injected into the chromatograph. The flow-rate was adjusted to 1 ml/min. The excitation wavelength of the fluorescence detector was set at 330 nm, and the emission wavelength at 460 nm. Under the conditions described, the retention times of ochratoxin A and its methyl ester were approximately 7 and 15 min, respectively. For quantitation, peak heights were measured and compared to those of appropriate standard solutions in the range of 0.5 to 10 ng ochratoxin A /ml. The column was washed during 10 min after each utilization with a solution made of acetonitrile and water (75+25, v+v).

Ochratoxin A-containing samples were dissolved in 500 ml of the mobile phase consisting of acetonitrile– water–acetic acid (450:540:10). The solutions were then filtered through a 0.45 mm microfilter and 50 ml samples were injected into the chromatograph. The column was left at ambient temperature and the flow-rate was adjusted to 1 ml/min. Under the conditions described, the retention time for ochratoxin A was approximately 7 min. For quantitation, peak heights were measured and compared with those of appropriate standard solutions in the range of 0.5–10 ng ochratoxin A/ml.

Detection Limits

The calibration curve was based on the analysis of ochratoxin A standards diluted in the mobile phase. The curves were calculated using the least-square method. The recovery of ochratoxin A was determined by adding known amounts of ochratoxin A to human milk samples. It reached 87.8%. The detection and quantitation limits were calculated by taking the average noise signal and adding 3 and 6 standard deviations of the noise, respectively (European Commission, 1997).

Results

Results of the OTA Level in the Samples

Sixty-two samples were collected (31 maternal plasma and 31 umbilical cord plasma). From 31 maternal plasma samples, four (13%) were positive for OTA. The concentration range of OTA in the positive samples was from 0.3 to 10.2 ng/ml with a mean of 3.0 ± 3.0 ng/ml. Similarly, in the umbilical cord plasma, OTA was also detected in twenty-seven samples (87%) but the concentration range in umbilical cord plasma was much lower than in maternal plasma, with values of 0.3 to 1.7 ng/ml and with a mean of 0.7 ± 0.65 ng/ml.

In two samples, maternal plasma and their respective umbilical cord plasma were both positive. In these cases, it was observed that OTA concentration found in the umbilical cord plasma (0,5 and 1.7 ng/ml) was greater than that of the maternal plasma (0.3 and 1.3 ng/ml, respectively).

The results obtained from the human milk samples collected in Cochabamba during the summer and the winter seasons are shown in Table 1.

Table 1. *Occurrence of ochratoxin A in human breast milk collected during two periods in Cochabamba.*

	Summer				**Winter**	
	< DL	**DL-QL**	**QL-100**	**>100**	**< DL**	**DL-QL**
Ochratoxin A (ng/1)		20.2 28.1 28.7 31.2 32.7	40.9 48.3 49.1 51.4 55.0 55.7	101.9 146.1		28.3
N° of samples analysed	29				42	
N° of positive samples	2	5	6	2	7	1

DL = Detection limit (18 ng//l)
QL = Quantitation limit (36 ng/l)

Out of 29 samples the breast milk samples from the Bolivian mothers collected during the summer period, 13 (44.8%) were found positive and that contain an ochratoxin A concentration above the fixed detection limit and the ochratoxin A concentration for positive

samples ranged from 20.2 to 146.1 ng/l The majority of the positive samples (84.6%) contained less than 100 ng/1; five of these samples (38.5%) had an ochratoxin A concentration between the detection limit and the quantitation limit, and eight samples contained a higher concentration than the quantitation limit. Only two samples (15.4%) with concentrations greater than 100 ng/1, specifically 101 and 145 ng/1 human milk were found. Two additional samples presented traces of ochratoxin A, lying under the fixed detection limit and could not be considered as positive samples. The mean ochratoxin A concentration in breast milk was 53.02 ± 33.3 ng/l, median 48.3 ng/l.

Concerning the samples collected in the winter season, only one (2.4%) out of 42 had a measurable ochratoxin A concentration of 28.3 ng/1, and seven samples showed traces of the toxin, below the detection limit.

The presence of ochratoxin A in all positive samples was confirmed by the formation of the ochratoxin A methyl ester.

Discussion

In umbilical cord blood samples two samples were found to be positive along with the corresponding plasmas of the mothers, resulting in higher values to the latter (see Figure 4). The average ratio of blood concentrations of umbilical cord/maternal blood was 1.5 with a range of 1.3 to 1.7. These findings could be due to different reasons, the exposure of the mother to the toxin may be for continuous periods and at low concentrations, which would determine a constant but low level in her bloodstream, in other words, the concentration of OTA depends on the duration of exposure and the amount of toxin ingested. Similar results were published by Postupolski *et al*., (2006) in a study conducted in Poland. 85.7% umbilical cord samples have higher concentrations of ochratoxin A than their respective pairs of maternal blood at an average umbilical cord blood/maternal blood ratio equal to 1.96 with a range of 0.6 to 4.0.

Unexpectedly, the toxin was found in umbilical cord blood samples whose mothers did not have a detectable level of OTA, this result could be due to several causes: the mother could be in contact with a contaminated food during the gestation period, and that surely acquired a detectable level in its plasma during this period, which was transmitted directly to the pregnant woman. Although the mother has a high capacity to eliminate the toxin, the fetus has not yet developed its system for decontaminating certain metabolites or toxins, which suggests that they remain in the mother's organism for longer periods and that the they are eliminated progressively via the umbilical cord so that the mother can definitively eliminate them. This result is similar to a work done by Maxwell *et al.* (1989) where aflatoxins were determined in the mother and the umbilical cord, also obtaining positive results in umbilical cord blood whose mothers did not have an Aflatoxin level in plasma, these authors concluded that the level found was due to an accumulation of Aflatoxin in the fetus In another experience in rabbits made by Ferrufino-Guardia *et al.* (2000), an increased difference was found in the ability to accumulate the toxin in the body of the offspring with respect to the mother, which could be done in a similar way in other mammals and man.

The European Commission (European Commission. 1997) suggested that the main food contributing to the intake of Ochratoxin A are cereals and by-products and it has been determined that Ochratoxin A is one of the main contaminants in corn (Shotwell *et al.,* 1969). Corn is one of the most consumed foods by the Cochabamba population; the harvest of this grain begins in mid-December and continues according to the variety until April and May; and it is stored for more than a year, where the product can suffer serious damages due to bad storage conditions, high temperatures and humidity in the summer season (November-February). During this last period the optimal conditions for the development of the fungus are created, which would be a factor that increases the frequency of Ochratoxin A in the months of February and March. Of another part, in April we have the culmination of the new crop which would have a lower level of toxin, so that the levels in human blood could decrease significantly, this last could be a reason for the low incidence found in the analyzes of the samples of the present study whose sampling was carried out between the months of March and April.

On the other hand the origin of the samples is of great importance because it goes according to eating habits, most of the samples taken depended on chance and came from different areas of the city of Cochabamba, especially the majority of the samples positive came from peri-urban where eating habits of the population in these areas is based heavily on the consumption of cereals and cereal products. Human exposure to Ochratoxin A depends on the feeding habits of the mother and the seasonal period of her environment.

We have that the maximum concentrations of ochratoxin A in maternal blood found in Bolivia are similar to the ranges found in Germany, Denmark and Canada (Bauer and Gareis, 1987; Hald, 1991b; Kuiper-Goodman *et al*., 1993). However, the frequency obtained of 13% is lower than in these other studies. Conversely, the average concentration of ochratoxin A is higher in our studies. This may be due to the detection limits used in each study and the seasonal time of sampling. This result has a 12% admissible error with respect to the population, which was determined with 95% confidence and that may be due to the number of samples taken; through the results found in cord blood, the transfer of Ochratoxin A through the placental barrier is confirmed.

In developed countries the need to have regulations on the limits in the level of mycotoxins in food and feed is known, most of them have specific regulations; while, other developing countries like Bolivia do not have regulations. The limits set depend on several factors such as the availability of toxicological data, the frequency of Ochratoxin A in food and methods of sampling and analysis (FAO, 1997). During the last years, different tolerable levels have been proposed. The toxicology evaluation of Ochratoxin A was carried out between 1991 and 1995 by JECFA (FAO, 1997), establishing a provisional weekly tolerable entry of 100 ng / kg of body weight, corresponding to a TDI (Tolerable Daily Intake) of 14.3 ng / kg body weight / day, this without taking into account the production of carcinogenicity and only the renal damage produced. Kuiper-Goodman and Scott (1989) calculated the TDI of Ochratoxin A taking into account the carcinogenicity, the TDI was 4.2ng/kg body weight/day in humans. If we assume that the percentage of plasma in the blood for a newborn is 44%, this would represent a volume of 27.4 ml of plasma for 1 Kg of body weight, this would correspond based on the measurement of the results found in this study, at a level of Ochratoxin A of 19.18 ng/kg of body weight. In contrast, an adult woman who has a plasma percentage of 55% and a volume of 36.6 ml of grass for 1 kg of weight, the level of Ochratoxin A in the body would be 109.8 ng/kg of body weight. Both results are well above the value of the TDI, considering the carcinogenic effect as well as the value established by the FAO. Analyzing these results, the value found in umbilical cord plasma is close to the TDI level of 14.2 ng OTA/kg of body weight. However, this value is considered in general terms for adults, therefore, it is not ruled out that the effects of Ochratoxin A on the fetus are much greater than those of an adult.

In other studies it was determined that the average intake of Ochratoxin A of 0.7 ng OTA/kg of body weight corresponds to a concentration in blood serum of 0.25 ng/ml of Ochratoxin A (Zimmerli and Dick, 1995), additionally, studies based on the analysis of blood serum, the prenatal exposure of Ochratoxin A is around twice that of the mother (0.5 ng/ml), at least immediately before birth (Zimmerli and Dick, 1995), Taking into account the results determined in the present In this study, most of the positive samples would be found above these values, which, adding the effect of the possible accumulation in tissues and the seasonal variation, we would find before a result that could have toxicological consequences in the future.

In Sierra Leone, Jonsyn *et al*. (1995a) detected OTA in 16 (25%) umbilical cord serum samples in a concentration range of 0.2-3.5 ng/ml and only in 1 (12, 5%) in maternal blood at a level of 0.2 ng/ml. They did not detect OTA in the umbilical cord sample corresponding to their maternal blood pair. In Poland, 30 (100%) samples of maternal blood and 28 (93 %) of umbilical cord blood were positive in ochratoxin A. The mean concentration of ochratoxin A in maternal blood serum was 1.14 ng/ml and in serum Cord blood count was 1.96 ng/ml (Postupolski *et al*., 2006). Another study conducted in Italy by Biasucci *et al*., (2011) detected ochratoxin A in 129 (99%) umbilical cord blood samples in a concentration range 0.084 to 4.83 ng/ml; 55% of the positive samples showed levels of ochratoxin A less than 0.4 ng/ml.

Exposure of humans to ochratoxin A can be determined by a combination of food analyses and food intake studies or by monitoring levels of ochratoxin A in biological fluids, the second possibility being a much more direct and reliable indicator of actual exposure. Controls made in blood are undeniably the most frequent. The monitoring of breast milk may however present some specific interests: on one hand, breast milk can easily be obtained during breast feeding, and on the other hand, such an analysis directly permits the assessment of the exposure of infants to ochratoxin A.

In the present study, we have analyzed two sets of milk samples collected in Cochabamba, Bolivia. To our knowledge, it is the first report concerning the level of ochratoxin A contamination in human milk in Bolivia. The results of this study show that infants in Cochabamba are exposed to ochratoxin A at a level that may in some cases exceed the tolerated levels (4.2 ng/kg body weight/day for humans, as proposed by Kuiper-Goodman and Scott (1989)). A comparison with the data reported in studies made in others countries may certainly help to evaluate the problem.

The occurrence of ochratoxin A in human milk has been investigated in some Africa, Asia, European and South-American countries (Table 2). In Germany, ochratoxin A was found in 4 (11%) out of 36 analysed samples. The samples contained ochratoxin A in the range 17-30 ng/l milk (Gareis *et al.,* 1988). In Italy, two surveys made on 50 and 111 samples respectively (Micco *et al.,* 1991; Micco *et al.,* 1995) have revealed much higher concentrations of ochratoxin A (100-12000 ng/l) in about 20% of the samples. In Sweden ochratoxin A was found in 23 (58%) out 40 of samples. The concentration range in the positive samples (>10ng/l) was 10-40 ng ochratoxin A/l milk (Breitholtz-Emanuelsson *et al.,* 1993b). In a Swiss study, 4 (10%) out of 40 samples were positive but contained ochratoxin A at a very low level: 5-14 ng/1 (Zimmerli and Dick, 1995). In a Hungarian investigation, 38 (41.3%) out of 92 colostrum samples were found to be positive in a concentration range of 220-7630 ng/1 (Kovacs *et al.,* 1995). In the case of Sierra Leone, 40 out of 113 samples were heavily contaminated with concentrations ranging from 200 up to 337,000 ng/1 (Jonsyn *et al.,* 1995a).

In the present study, the levels of ochratoxin A found in breast milk from Cochabamba were much lower than those reported in Italy, Hungary, Turkey, Egypt and Sierra Leone and were in the range of the levels found in the German and Swedish studies. In terms of frequency of contamination, our results are somewhat lower than those reported in Sweden. This may, however, be due to the difference in detection limit between the two studies.

The question of the relationship between the level of ochratoxin A in breast milk and the dietary exposure of the mother to the toxin still remains open. The finding of high ochratoxin A levels in the milk of relatively few individual mothers may suggest individual dietary habits that may result in the exposure of the infants even after weaning.

Table 2. *OTA concentrations in human breast milk reported for some countries worldwide*

Country	Positive samples /Total (%)	Mean±SD (ng/l)	Range (ng/l)	Reference
European countries				
Germany	4/36 (11)		17–30	Gareis *et al*., 1988
Germany	55/90 (60)		10–100	Muñoz et al. (2013)
Hungary	38/92 (41)		0.2–7.63(ng/ml)	Kovacs *et al.*, 1995
Italy	9/50 (18)	4.19	1.77-6.6(ng/ml)	Micco *et al.*, 1991
Italy	22/111 (20)		0.1–12(ng/ml)	Micco *et al.*, 1995;
Italy	13/31 (42)		80-540	Miraglia *et al*., 1995;
Italy	198/231 (86)	6.01±8.31	1–57	Turconi *et al*., 2004,
Italy	61/82 (74)	30±66.9	5–405	Galvano *et al*., 2008;
Italy	45/57 (78.9)		1–75	Biasucci *et al*., 2011
Norway	38/115 (33)		10–130	Skaug *et al*., 1998;
Norway	17/80 (21)	30	10–182	Skaug *et al*., 2001
Poland	40/78 (51)	18.7±7.9	22-26.4	Karwowska *et al*., 2004,
Poland	5/13 (38)	5.6±4	5.3–17	Postupolski *et al*., 2006
Slovakia	23/76 (30)		2.3–60.3	Dostal *et al*., 2007
Spain	35 (0)			Rubert et al., 2014
Swede	23/40 (58)		10–40	Breitholtz-Emanuelsson *et al.*, 1993b
Switzerland	4/40 (10)		5–14	Zimmerli and Dick, 1995
North Africa countries				
Egypt	3/10 (30)	8.87	3-15	El-Sayed *et al*., 2000
Egypt	43/120 (35.8)	21.1±13.7	5–45,01(ng/ml)	El-Sayed *et al*., 2002,
Egypt	36/50 (72)	1.89±0.98	(ng/ml)	Hassan *et al*., 2006
Sierra Leone	40/113 (35)	7.9±5.2	0.2–337(ng/ml)	Jonsyn *et al.*, 1995
Asia countries				
Iran	2/136 (2.72)		90-140	Afshar *et al*., 2013,
Iran	84/87 (96.6)	24.6±13.6	1.6-60	Dehghan *et al*., 2014
Turkey	75/75 (100)		620–13111	Gurbay *et al*., 2009;
Turkey	34/70 (48.6)	140±30		Uyar *et al*., 2014
South America countries				
Bolivia	13/29 (44.8)	53.2±33.3	20.2-146.1	This Study
Brazil	66/100 (66)		0.3-21	Iha *et al*., 2014,
Brazil	2/50 (4)		11–24	Navas *et al*., 2005,
Brazil	224 (0)			Andrade *et al*. 2013
Chile	11/11 (100)	106±45	44-184	Muñoz *et al*., 2010
Chile	40/50 (80)	52±46	10–186	Muñoz *et al*., 2014

In an Italian study (Miraglia *et al.,* 1993) six not hospitalized mothers were followed during six days. Each day, a sample of milk coming from a single suck was drawn at the same time. No correlation could be established between the diet and the contamination of human milk. Particularly in two cases a very high peak of concentration (21,900 and 8,500 ng/l) were preceded and followed by very low levels of about 100 ng/l. By contrast, the data that we have obtained in rabbit does fed a naturally-contaminated diet (10-20 µg/kg body weight/day) show a relative constancy of the ochratoxin A plasma and milk levels throughout a lactation period of 17 days (Ferrufino-Guardia *et al.,* 2000). The difference observed could be due to the difference in binding capacity to plasma proteins in different species (Galtier *et al.,* 1977; Mortensen *et al.,* 1983a; Holmberg *et al.,* 1991).

In Sweden, blood samples were collected at the same time as the milk samples. All blood samples contained ochratoxin A above the quantitation limit (Breitholtz-Emanuelsson *et al.,* 1993b). However, no fair concentration dependence could be estimated between blood and milk since the ochratoxin A positive samples of human milk and blood fell into narrow concentration ranges. In average, the ochratoxin A concentration in human milk is about 10 times lower than the blood concentration. In contrast to this, a linear relationship was found between the concentration of ochratoxin A in the blood and milk of rats administered a single oral dose of ochratoxin A (10, 50 or 250 µg/kg body weight)(Breitholtz-Emanuelsson *et al.,* 1993a). A milk/plasma concentration ratio of 0.2-0.5 was found in that study. The transfer of ochratoxin A into mammalian milk was also studied by Galtier *et al.* (1977) in rabbits. These authors found a milk/plasma concentration ratio of 0.1-0.2 after a single intravenous administration of ochratoxin A (1,000-4,000 (µg/kg body weight). Ferrufino-Guardia et al., (2000) found a lower milk/plasma concentration ratio (0.015) in rabbits nourished on a regular basis with ochratoxin A- contaminated diets at a dose of 42.1-71.7 µg/day.

In the present investigation, a seasonal variation of ochratoxin A in breast milk was clearly found in Cochabamba. The levels and frequency of ochratoxin A detection during summer time (February-March) were higher then those in winter time (June-July)(Table 3). Such seasonal variations may be related with observations made in other studies. Holmberg *et al.* (1991) studied the annual variation in ochratoxin A contamination of swine blood in Sweden over a period of five years. This survey was used as an indirect method to study the ochratoxin A contamination of the cereal grain. Blood samples corresponding to a long storage of the fed grains (August-September) showed a significantly higher ochratoxin A contamination than samples collected in January-February, when the grain storage is shorter. In the case of breast milk, no reports have been published so far, on ochratoxin A seasonal variations. Interestingly however, Lamplugh *et al.* (1988) and Maxwell *et al.* (1989) reported seasonal variation in the frequency of detection and levels of aflatoxins in breast milk. This may be related with the observation made in Australia, where the aflatoxin contamination of plant products was shown to be greater in warmer and more humid conditions (El-Nezami *et al.,* 1995).

The European Commission (1997) has suggested that the main contributors to the dietary intake of ochratoxin A are the cereals and cereal products, because almost all cereals seem to have the possibility to contain ochratoxin A. Moreover, the consumption of cereals in Bolivia is generally high. Ochratoxin A was first encountered as a natural contaminant in maize (Shotwell *et al.,* 1969). The maize grain is one of the major consumed foods by the "cochabambina" population. The harvest period of this grain is classically from April to May (Autumn) and storage may last over the whole year. The product may then suffer from bad climatic conditions, in terms of high temperature and humidity, especially during the months November to January. These optimal conditions for fungi development can be the origin of the increased frequency and concentration of ochratoxin A in human milk collected during the months of February to March in Cochabamba. The seasonal differences observed in our data suggest that the critical point for ochratoxin A contamination may be the grain storage conditions rather than the climatic conditions at harvest time.

Breast milk is the first, and to a very large extent, the only food (for at least six months) that babies receive in most Latin American countries. The duration of breast feeding varies, but in many countries up to two years is normal. This prolonged breast feeding may have the disadvantage of continuous or intermittent exposure of infants to the ochratoxin A that may be present in breast milk. No recommendations have been made with respect to tolerable intake levels of ochratoxin A in infants (estimation of the amount of toxin that can be ingested daily, over a lifetime, without appreciable health risks - expressed on a body weight basis). Milk is a major nutrient for the rapidly growing young who thereby is theoretically at its most vulnerable stage of development as far as induction of carcinogenesis and susceptibility to other toxic effects is concerned.

The need for regulations imposing limits to the concentrations of mycotoxins in foods and feeds is generally recognized in developed countries, and many of them have specific mycotoxin regulations. In contrast, many developing countries, e.g. Bolivia, have not established regulations for mycotoxins. The limits to be fixed for ochratoxin A contamination depend on several factors, such as availability of toxicological data, data on the occurrence of ochratoxin A in various commodities, and methods of sampling and analysis (FAO, 1997).

During the last years, several risk assessments on ochratoxin A have been performed and different tolerable daily intakes (TDI) have been proposed. The toxicological evaluation of ochratoxin A carried out between 1991 and 1995 by JECFA (FAO, 1997) established a Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg body weight, corresponding to TDI about 14.3 ng/kg body weight/day. In principle, the evaluation is based on the determination of a No-Observed-Effect-Level (NOEL) in toxicological studies, and the application of a safety factor. The safety factor means that the lowest NOEL in animal studies is divided by 100 (10 for extrapolation from animals to humans and 10 for variation between individuals). However, the calculated PTWI accounts for renal damage only and does not take carcinogenicity into consideration.

Regarding carcinogenicity data, Kuiper-Goodman and Scott (1989) calculated a TDI of ochratoxin A either with a NOEL/safety factor approach or with a mathematical low-dose extrapolation. With an experimentally observed NOEL for tumours at 21 µg/kg body weight/day and a safety factor of 5,000 they calculated an estimated tolerable intake of 4.2 ng/kg body weight/day for humans.

Assuming that the body weight of infant is set at 5 kg and that the mean daily intake of milk is set at 1 kg/day, the mean concentration of ochratoxin A not to be exceeded in the milk is of 71.5 ng/l, on the basis of JECFA data, and of 21 ng/l, if the recommendations of Kuiper-Goodman and Scott (1989) are considered. The values obtained in the bolivian milk, but also those found in other countries (Gareis *et al.,* 1988; Breitholtz-Emanuelsson *et al.* 1993b; Micco *et al.,* 1991 and 1995, Jonsyn *et al.,* 1995a, Kovacs *et al.,* 1995), should thus be a matter of concern.

Ferrufino-Guardia *et al.* (2000) has observed that the young rabbits present an extremely high capacity to accumulate ochratoxin A in their body, as compared to rabbit does. If this high capacity of young rabbits to retain ochratoxin A is shared by other mammals and by the human, the problem of breast-fed infant contamination by mother's milk may be much more alarming than presently thought.

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