UNIVERSITY OF TERAMO

FACULTY OF VETERINARY MEDICINE

PhD in
"Veterinary Medical Sciences, Public Health and Animal Welfare"

XXXII CYCLE

Effects of dietary exposure to Ochratoxin A (OTA) mycotoxin below/ around guidance values on embryo/fetal development and pregnancy success

SDS VET/10

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 713714

Co-funding institutions

University of Teramo

Abruzzo Region

ESR 5 Shafaq Asif

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgments, the work presented is entirely my own.

I confirm that this thesis presented for the degree of PhD, has
i) been composed entirely by myself
ii) been solely the result of my own work
iii) not been submitted for any other degree or professional qualification
I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or processional qualification except as specified.

Parts of this work have been published in.

i) 64^{O} Convegno GEI - Società Italiana di Biologia dello Sviluppo e della Cellula. L'Aquila,Italy 13-14 June, 2018
ii) 72^{O} Convegno SISVET - Società Italiana Delle Scienze Veterinarie Torino, Italy 20–22 June 2019 Page no.202
iii) First Symposium of the European College of Animal Reproduction (ECAR 2019) Vienna, Austria 4-6 July 2019 Page no.74
iv) The World Mycotoxin Forum IUPAC(2019) Belfast, Northern Ireland 4-6 July 2019
v) 73oConvegno SISVET - Società Italiana Delle Scienze Veterinarie Olbia Sardegna, Italy 19–22 June 2019
vi) Journal Mycotoxin Reserch Published on 29 September 2020

Shafaq Asif
10thNovember, 2020

Certificate by the Research Tutor – University of Bari

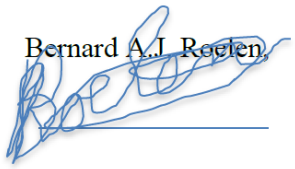
The undersigned M.E. Dell'Aquila, in the quality of supervisor at the University of Bari Aldo Moro,Italy, confirm that Shafaq Asif has carried out her activity as scheduled in her career development plan, at our institution from the 18th September 2017 until the 30th September 2018 then from the 1st January 2019 until the April 2019 and finally from the 1st February 2020 30th May 2020

M.E. Dell'Aquila,

Certificate by the Research Tutor –Utrecht University

The undersigned Bernard A.J. Roelen, in the quality of tutor at the Utrecht University, confirms that Shafaq Asif has carried out her activity as scheduled in her career development plan, at our institution from the 2nd May 2019 until the 9th December 2019.

Bernard A.J. Roelen



Certificate by the Research Tutor – ISPA-CNR

The undersigned Fiorenza Minervini, in the quality of supervisor at the Institute of Sciences of Food Production (ISPA – CNR) Italy, confirm that Shafaq Asif has carried out her activity as scheduled in her career development plan, at our institution from the 1st October 2018 until the 30th December 2018.

Fiorenza Minervini,

Acknowledgments

All praise to **Allah Almighty** the Omnipotent who is the most Merciful and the most Beneficent. He is the one who gave me the courage and determination to carry on when all seemed impossible.

My sincere gratitude to **University of Teramo** and **REP-EAT** Marie Skłodowska-Curie actions Cofund Project funded by the EC H2020 Programme, the Region Abruzzo ESF Operational Programme 2014-2020 and the University of Teramo for letting me be the part of this incredible network. Sincere thanks to project coordinator **Prof. Barbara Barboni** and other **REP-EAT team** members for making this jouney smooth for me.

I would like to express my sincere gratitude to my supervisor, internal and external tutors **Prof. Augusto Carluccio** (UniTe), **Prof. Domanico Robbe** (UniTe) **Dr. Bernard.A.J. Roelen** (UniU) for their consistent support and guidance during the reserch project.

I would like to pay special thanks to **Prof. M.E. Dell'Aquila** (UniBa) and **Dr. Fiorenza Minervini** (ISPA, CNR) for their dedicated support and guidance Prof. Dell'Aquila and Fiorenza Minervini continuously provided encouragement and were always willing and enthusiastic to assist, in any way throughout the research project. I would also like to thank Dr. Minervini and Dr. Bernard.A.J. Roelen providing advice regarding statistical analysis.

Finally, many thanks to all participants that took part in the study and enabled this research to be possible.

To conclude, I cannot forget to thank my family and friends for all the unconditional support in this very intense academic years.

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OTA	Ochratoxin A
3β-HSD	3β-hydroxysteroid dehydrogenase/isomerase
4(R)-OH-OTA	4(R)-4-hydroxyochratoxin A
4(S)-OH-OTA	4(S)-4-hydroxyochratoxin A
4-OH-OTA	4-hydroxyochratoxin A
8-OHdG	8-hydroxydeoxyguanosine
ATP	Adenosine triphosphate
AFB1	Aflatoxin B1
AFM1	Analyze aflatoxin M1
AP	Anterior pituitary gland
AR	Antibiotic resistance
ADU	Arbitrary densitometric units
As	Arsenic
BEN	Balkan endemic nephropathy
BEN	Balkan endemic nephropathy
BCB+	Blue-colored cytoplasm
BW	Body weight
BSA	Bovine Serum Albumin
BCRP	Breast Cancer Resistance Protein
BCB	Brilliant Cresyl Blue
Cd	Cadmium
LLC-PK1	Cells and pig kidney cell line
BCB-	Colourless oocyte cytoplasm
CTUP	Combined thickness of uterus and placenta
Cr	Cronobacter
CEG	Crude Equine Gonadotrophin
COCs	Cumulus-oocyte-complexes
Dd1	Day 1 of the diestrus
Ed1	Day 1 of the estrus
DON	Deoxynivalenol
DCF	Dichlorodihydrofluorescein
DDAH	Dimethyl arginine dimethyl amino hydrolase
DON3GlcA	DON-3- O-glucoside
ET	Embryo transfer
En	Enterobacte
ELISA	Enzyme linked immunosorbent assay
EGF	Epidermal growth factor
E	Escherichia
EBV	Estimated breeding values
ERK1-2	Extracellular Signal-Regulated Kinase
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
FF	Follicular Fluid
FB1	Fumonisin B1
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
G6PDH	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
GnRH	Gonadotropin releasing hormone
GH	Growth hormone
HPLC	High performance liquid chromotography
hCG	Human Chorionic Gonadotrophin
HPG-axis	Hypothalamic–pituitary–gonadal axis
IVF	In vitro fertilization
IVM	In vitro maturation
IVEP	In vivo embryo production
i NOS	Inducible nitrogen oxide synthase
IARC	International Agency for Research on Cancer
Fe^{3+}	Iron

JIVET	Juvenile in vitro Embryo Technology
KNDy	Kisspeptin/neurokinin B/dynorphin
LOPU	Laparoscopic ovum pick-up
Pb	Lead
LH	Luteinizing hormone
MF	Martina Franca
MPF	Maturation promoting factor
Hg	Mercury
MI	Metaphase
mETC	Mitochondrial electron transport chain
MAPKs	Mitogen-activated protein kinases
MRP-2	Multidrug Resistance Associated Protein-2
NO_2	Nitrogen dioxide
NO	Nitrogen monoxide
OTA-	Ochratoxin A mono anion
OTA^0	Ochratoxin A non-ionised
OSS	Oestrus Sheep Serum
1PN	One pronuclei
OATPs	Organic anion-transporting polypeptides
OGGI	Oxyguanosineglyosylase
ONOO-	Peroxynitrite
P-gp	P-glycoprotein
Phe	Phenylalanine
PEPCK	Phosphoenolpyruvate-carboxy kinase
PB	polar body
PL	Pregnancy length
PGF2a	Prostaglandin F2alpha
PT	Proximal tubule
Ps	Pseudomonas
ROS	Reactive oxidative species
ROS	Reactive oxygen species
MII	Second meiotic spindle
Nacl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
SH	Sperm head
S	Staphylococcus
Str	Streptococcus
SAPK	Stress-activated protein kinases
SOF	Synthetic Oviductal Fluid
SOFM	Synthetic Oviductal Fluid Medium
TI	Telophase I
2PB	Two polar bodies
2PN	Two pronuclei
ZAN	Zearalanone
ZEN	Zearalenone
ZAL	α-Zearalanol
ZEL	α-Zearalenol

Effects of dietary exposure to Ochratoxin A (OTA) mycotoxin below/ around guidance values on embryo/fetal development and pregnancy success.

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by some *Aspergillus* and *Penicillium* fungi and it induces several toxic effects. The present Ph.D. thesis investigated: 1) an *in vitro* pre-natal exposure model, by analyzing the effects of acute oocyte exposure to OTA on oocyte maturation and embryo development in the sheep; 2) an *in vivo* pre and post-natal exposure model, through examining OTA levels in feed, blood and milk samples of jennies and related foals. The sheep was used as a relevant model for human reproductive medicine. The donkey was used as suitable model to follow the route of natural exposure from feed to jennies during pregnancy and in foals after delivery. Moreover, donkey milk has high nutritional value in human diet.

Ovine cumulus-oocyte complexes (COCs) were exposed to OTA during in vitro maturation (IVM) in a micro-to nanomolar range. After IVM, some oocytes were analyzed for Metaphase II (MII) rate, while others underwent in vitro fertilization (IVF) and embryo culture up to day7. Micromolar concentrations had adverse effects on cumulus expansion and viability, oocyte nuclear and cytoplasmic maturation and embryo development. At nanomolar concentrations, no adverse effects were found on COC viability and integrity whereas blastocyst had higher nuclear apoptosis.

In donkey feed, toxin concentration was up to 2.7 ng/g with an incidence of 32%, far below the guidance values of OTA in feed materials reported by EU Recommendation 2016/1319. In jennies, the incidence of positive blood samples was 73% (median value= 114 ng/L; range 51 to 6000 ng/L). In foals, the incidence of positive blood samples was 50% (median value= 136 ng/L; range 79 to 4030 ng/L). No placental transfer of OTA was observed in all tested jennies and no influence on

pregnancy length and health of foals was observed. In milk, the incidence of positive samples was 36% (range 17 to 82 ng/L).

In conclusion, in sheep, in vitro exposure to OTA affected oocyte function and embryo development; in jennies, no placental passage was observed but OTA passed from feed to milk through the blood. Effects on female fertility and newborn health need to be further investigated.

THESIS STRUCTURE AND ORGANIZATION

This Ph.D. thesis consists of three chapters:

Chapter 1 provides a general introduction on: 1) Ochratoxin A (OTA), particularly on its occurrence, mechanisms of action, different aspects of toxicity including reproductive toxicity in human and animals, use of biological fluids as potential biomarkers of OTA exposure; 2) reproductive physiology and biotechnologies in the sheep as a polygastric animal model; 3) reproductive physiology and lactation in donkey as a monogastric animal model;

Chapter 2 includes the experimental part of the thesis performed during the Ph.D. program and summarizes the obtained results. This chapter is divided into 2 subchapters.

- In **subchapter 2.1**, the effects of OTA on ovine cumulus-oocyte complex (COC) viability and maturation and mitochondrial dysfunction and embryo development are presented. It was shown, that OTA has a different effect on oocyte and embryo development depending on the used concentrations, either micromolar or nanomolar. Thus, micromolar concentrations induced adverse effects on cumulus expansion and viability, nuclear maturation, cytoplasmic maturity and oocyte fertilization ability. Arrest of embryo development at early stages was also observed. At nanomolar concentrations, no adverse effects were found apparently on COC integrity and viability whereas, on embryos which reached the blastocyst stage, higher nuclear apoptosis was found.

- In **subchapter 2.2**, the natural occurrence of OTA in blood and milk samples of jennies and their foals during late period of pregnancy and after delivery was presented. The possible transfer through the placenta, blood, and milk from mother to fetus was studied. The blood, as a potential biomarker, depicts the transfer of OTA from contaminated feed to jennies. Seasonal effect was also observed during the transfer. No placental transfer was observed, as the blood samples collected from foals after birth were found free of OTA presence. Milk showed OTA presence but no correlation was found in OTA levels between feed and milk, thus only between jennies blood and milk.

In **Chapter 3**, the conclusions and future perspectives of the thesis are summarized.

Wherever possible, the translational relevance of the two experimental studies in human reproductive and developmental toxicology has been indicated, as one of the purposes of the thesis is to provide model data of OTA toxicity on human reproductive medicine, in the female fertility area and fetal and newborn health area, as placenta-mediated fetal transfer and milk-mediated new-born transfer were analyzed.

Also, due to the high commercial value of donkey milk, as a healthy portion for the human infant diet, and as it is already known that donkey milk composition resembles that one of human milk, rather than that one of dairy animals, the safety of donkey milk needs to be monitored for minimizing hazards to human health.

CHAPTER 1 – INTRODUCTION

1.1 OCHRATOXIN A

1.1.1 Ochratoxin A (OTA) Synthesis

Ochratoxin A (OTA) is one of the most important and deleterious mycotoxin produced by several species of fungi, including *Aspergillus ochraceus, A. carbonarius, A. niger* and *Penicillium verrucosum* (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016).

The growth of these fungal species is particularly promoted in extreme conditions (humidity and temperature): in cool to temperate conditions, *Penicillium verrucosum* is produced, while in tropical regions *Aspergillus ochraceus* is the main source to affect commodities (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016).

1.1.2 Chemical characterization of Ochratoxins

Ochratoxin A is structurally composed of a *para*-chlorophenolic group containing a dihydro-isocoumarin moiety that is amide linked to *L*-phenylalanine with a molecular weight of 403.8. (Figure 1.1). Analogues of OTA, such as OTB and OTC and others with slight changes in the chemical structure are also known but OTA is the most toxic among them, with order of toxicity OTA>OTB>OTC (Gupta, Lasher Miller Mukherjee, Srivastavam & Lall 2017).

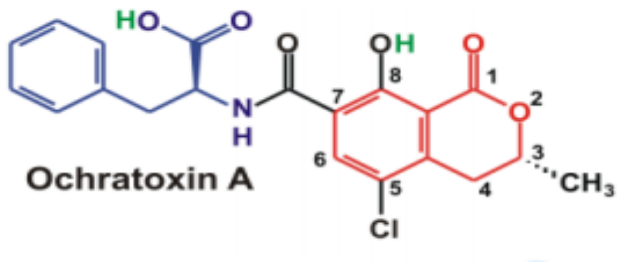

Figure 1.1 Chemical structure of Ochratoxin A

1.1.3 Occurrence of OTA

OTA has been reported as a ubiquitous natural contaminant of **food** and **feed**. Contamination generally occurs because of suboptimal conditions of storage of commodities and agricultural practices during the drying of foods (EFSA, 2020). OTA has been detected in foods of plant and animal origin. In foods of plant origin, OTA is frequently found in cereal products, beer, coffee,

cacao, chocolate, vegetables, green tea, raisins, grape juice, pistachios, figs, wine, liquorice, chestnuts and spices e.g. dried red pepper, chilli powder, black pepper, coriander, ginger, cayenne pepper, curcuma and nutmeg (EFSA, 2020). OTA frequently occurs in baby food and breakfast cereals. Rice, barley, oats and wheat are the most contaminated ingredients. Contamination of foods of animal origin such as pork meat, pork blood products, poultry kidney or liver with OTA results from consumption of OTA-contaminated feed. Meat products, such as raw ham muscle, cured meats, salami or dry-cured ham can also be directly contaminated during processing or storage (EFSA 2020). Over the last decade, several studies reported the possible occurrence of OTA in ripened cheese. the OTA occurrence in cheese is very likely due to environmental contamination leading to fungal growth on the cheese surface (EFSA, 2020).

OTA has been found in cereal grains (maize, barley, wheat, oats, rye), hay and mixed feed and its amount in animal feed varies from country to country. some authors have been conducting a global survey to monitor mycotoxin contamination of animal feeds (Gruber-Dorninger, Jenkins, & Schatzmayr, 2019). As observed in Table 1.1, global occurrence in different commodities, including finished feed, maize, maize dried distillers grains with soluble, maize silage, soybean grains, soybean meal, wheat, barley, and rice was analyzed. Finished feed was among the commodities showing the highest percentage of positive samples whereas, the prevalence of OTA (22% positive samples) was higher in maize dried distillers grains with soluble than in most of the other commodities. However, nearly all samples (99.6%) complied with the most stringent EU guidance value for OTA in feed. In addition, OTA was prevalent in South Asia, being detected in 60% of the samples (reported as, underlined value) and in Eastern Europe, OTA showed a relatively high prevalence (36.4%) of occurrence. The highest incidence rate and OTA levels in feed were underlined in Table 1.1.

Table 1.1 Survey on Ochratoxin contamination in feed samples during ten years (Gruber-Dorninger, Jenkins, & Schatzmayr, 2019)

Countries (number of feed samples)		**Ochratoxin A incidence (mean concentration) µg/kg**
Northern Europe	(n=1,958)	8.1% (1.9)
Central Europe	(n=21,036)	11.9 % (2.8)
Southern Europe	(n=3,527)	21.2% (2.6)
Eastern Europe	(n=2,382)	36.4% (3.6)
North America	(n=5,471)	4.3% (2.4)
Central America	(n=367)	3.8% (2.5)
South America	(n=17,332)	4.9% (17)
Middle East / North Africa	(n=1,075)	20.3% (3.1)
Sub-Saharan Africa	(n=208)	31.9% (7.2)
South Africa	(n=1,077)	5.6% (2.2)
Oceania	(n=1,695)	7.5% (3.6)
South Asia	(n=1,136)	**60.4%** (4.6)
Southeast Asia	(n=4,310)	15.2% (3.0)
East Asia	(n=13,232)	14.1% (2.9)
Central Asia	(n=15)-	13.3% **(22)**

1.1.4 Toxicokinetics

1.1.4.1 In human

Regular exposure to OTA could be a threat to healthy human life. Frequently human exposure to chronic lower doses of OTA is more alarming instead of acute exposure to higher doses (Malir, Ostry, Pfohl-Leszkowicz, & Novotna. 2013). Ochatoxin A, after oral ingestion, is rapidly absorbed from the jejunum against a concentration gradient and appears to involve a transport by organic anion transporters (OAT) in addition to passive diffusion. In a male adult volunteer the OTA bioavailability is of 93% . The high binding to serum proteins facilitates absorption of OTA but delays its elimination from blood and its excretion and explains the long half-life (up to 35 days) assessed in one human individual (EFSA, 2020). The Table 1.2 reported the half-life values of OTA in different animal species.

Table 1.2 OTA half –life values in different animal species (Zepnik Völkel, & Dekant 2003, Dietrich, Heussner, & O'Brien. 2005).

Species	**Sex**	**Abs. (%)**	**$t_{1/2\alpha}$(h)**	**$t_{1/2\beta}$(h)**	**Clearance (ml $h^{-1}kg^{-1}$)**
Human	M	>85.5	20.13	853.2	0.0905
Monkey (M. mulata)	M	57	n.r.	510	0.18
Monkey (M. mulata)	F	57	n.r.	510	0.18
Rat (F344)	M	n.r.	n.r.	224	n.r.
Rat (F344)	F	n.r.	n.r.	231	n.r.
Rat (Wistar)	M	44	n.r.	120	0.91
Rat (Sprague-Dawley)	M	n.r.	2.1	57.8	n.r.
Mouse (NIH-Bethesda)	M	97	n.r.	39	6.1
Fish (C. carpio)	n.r	1.6	n.r	0.68	58
Quail (C. coturnix)	M	62	n.r.	6.7	57
Quail (C. coturnix)	f	62	n.r.	6.7	57

M: male; F: female; n.r.: not reported

The major OTA metabolite is OTalpha, which is formed when the amide bond between phenylalanine and dihydroisocoumaric acid is hydrolysed. OTalpha has first been found in the caecum and large intestine of rats and in the excreted faeces after oral administration of OTA. It is believed to be mostly generated by the intestinal microbiome in non-ruminants including humans. OTA and its metabolites are excreted with the urine and the faeces but both routes are slow, owing to the high binding of OTA to plasma proteins and the low rate of metabolism (EFSA, 2020).

Due to slow elimination from the body, OTA accumulates in tissues and fluids of both human and animals. OTA concentration in blood of patients, suffering from various kidney diseases, is higher as compared to the OTA level in blood of healthy individuals. In the healthy population, the reported OTA concentrations in blood serum ranges from few hundreds of pmol/L to few nmol/L whereas, in endemic areas, its concentrations might raise up to exceeded level, greater than hundred nmol/L approximately (Kőszegi & Poór. 2016).

OTA elimination is possible from human body through the kidney. Glomerular filtration of OTA is strongly limited due to its albumin binding affinity, the small filtrated and secreted fraction is partially reabsorbed which might help the accumulation of the toxin and damage in the kidney tubule cells (Ringot, Chango, Schneider & Larondelle. 2006). Renal excretion of OTA is of particular interest because the kidney is the major target organ of OTA toxicity. Because of its high plasma protein binding, OTA undergoes tubular secretion rather than glomerular filtration, followed by reabsorption at all segments of the nephron. The transport of OTA into the proximal tubule cells of human kidneys is mediated by Organic Anion Transporter 1 (OAT1) and 3 from the basolateral (blood) side and by OAT4 from the apical (proximal tubule) side, whereas multidrug-resistant protein (MRP) 2 and efflux transporter NPT4 mediate the secretion from the proximal tubule cell into the tubule. Because OAT1, 3 and 4 are more efficient than MRP2 and NPT4, high intracellular concentrations of OTA are reached . The reabsorption of secreted and filtered OTA not only leads to its accumulation in the renal tissue but also delays its excretion (EFSA, 2020). OTalpha does not accumulate in the kidney but is rapidly excreted with the urine, also as a glucuronide. In humans, OTalpha-glucuronide is a major urinary metabolite (EFSA, 2020).

Transplacental transfer is one of the major pathways of OTA toxicokinetics and it is not possible to neglect it in human. In order to determine placental transfer of OTA, the kinetics of tritium-labelled OTA was studied in human perfused placenta after delivery. Only minimal passage of OTA through the placenta was observed and it was only about 0.02 (EFSA, 2020). The OTA concentration reaches fetal serum and, within it, it is double than maternal concentration. The reason behind the fact is that placenta is actively involved in the rapid transfer of toxin to the fetus through the mother womb. The involvement of active placental transporters and the inhibition of the placental efflux transporters ABCG2 and ABCC2 are involved in the increased OTA fetal serum levels .Differences in fetal uptake of OTA were observed in mice after different durations of gestation, suggesting that the transfer is influenced by the developmental stage of the placenta (EFSA, 2020).

The excretion of OTA was found also through the milk. In human breast milk, presence of OTA was reported in previous studies, although its concentration is less (0.2%) than the concentration in the blood (Malir, Ostry, Pfohl-Leszkowicz, & Novotna. 2013). Studies from European countries report levels ranging between 10 and 400 ng OTA/L (EFSA, 2020). According to the literature, OTA levels are significantly higher in milk of habitual consumers of bread, bakery products, cured pork meat and sweets. In addition to OTA, the occurrence of OTalpha was also monitored in milk and blood from nine lactating women (EFSA, 2020). In a study carried out in Italy with 82 samples of breast milk, it was observed that there were differences in OTA levels related to food ingestion and drink consumption habits of women (Soto, Ruiz, Manyes & Juan-García. 2016).

Concentration of OTA depends upon the dietary habits of the mother and the geographical region of residence. In some countries, such as Turkey, Egypt and Sierra Leone, its concentration in breast milk is 100-fold higher than other European countries. Blood, milk and urine are major biomarkers for the detection and monitoring of OTA exposure to human (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

OTA and its metabolite OTα are excreted also in faeces. Faecal excretion is mainly due to biliary excretion (Ringot, Chango, Schneider & Larondelle. 2006)

1.1.4.2 In animals (monogastric & polygastric species)

Even though OTA has been detected in the air of farms, such as poultry houses (Wang, Chai, Lu, Quan, Duan, Yao, Zucker, & Schlenker. 2008) and cows sheds, the inhalation of air contaminated with OTA and fungal conidia represents a rare and not yet exhaustively investigated source of exposure to the mycotoxin. Instead, it is considered that contaminated feed is the main source of OTA exposure to farm animals (Duarte, Lino & Pena. 2011).

Cereals, such as oat, wheat, barley and their by-products, such as bran, are the feed materials that are the major cause of OTA exposure to animals. Conversely to monogastric animals, ruminants, such as sheep, goats and cows, are less susceptible to the adverse health effects associated with OTA exposure. The protozoan fraction of rumen is capable to degrade OTA to OTα, a less toxic OTA derivative by the cleavage of the peptide bond (Duarte, Lino, & Pena. 2011).

In rodents, this detoxification pathway appears mainly due to the caecum microbial flora. Carboxypeptidase A, trypsin,α -chymotrypsin and cathepsin C have been shown to be able to hydrolyze OTA to OTα in vitro (Ringot, Chango, Schneider & Larondelle. 2006).

Whereas monogastric species and immature (with rumen partially functioning) ruminants are at higher risk due to consumption of OTA contaminated feed (Minervini, Giannoccaro, Nicassio, Panzarini & Lacalandra. 2013)

Monogastric (non-ruminant) species, for example swine, are at higher risk to OTA damage as they don't have appropriate ruminal microbiota and microbes for the degradation of OTA toxin to less toxic compounds (Duarte, Lino & Pena. 2011) and because they have a long half-life OTA able to accumulate the mycotoxin in tissues of monogastric farm animals such as pigs and thus be present in meat and meat products (EFSA , 2020). After dietary intake, the 66% of OTA was adsorbed by intestine of swine and subsequently was transported via the portal system and distributed to different tissues and organs (Ringot, Chango, Schneider & Larondelle. 2006).

Ochratoxin A is bound at more than 99% to serum proteins, limiting the transfer from the blood to the hepatic and renal cells (Ringot, Chango, Schneider,& Larondelle. 2006). In pigs, only 0.1% remained unbound. The tissue distribution in pig follow the order kidney > liver > muscle > fat. Both faecal and urinary excretions play important roles in plasma clearance of the toxin. (Marin, Taranu, Tabu & Burgehelea. 2009). Ochratoxins are differentially metabolized in various animals depending on the metabolizing enzymes present in liver, kidneys and/or gut (Heussner & Bingle. 2015).

Several studies showed in utero transfer in mammalian species like mice, rat and swine. As an example, whole-body autoradiography studies in mice, using high doses of intravenous route of ^{14}C-labelled OTA showed that OTA crosses the placenta more readily at days 8 and 9 than at day 10 of gestation, with radioactive labelling appearing within 20 min in uterine wall and placental and fetal tissues. The differences observed in OTA fetal uptake after different durations of gestation suggest that the transfer is influenced by the developmental stage of the placenta, which is considered to be completely developed after day 9 of gestation (Ringot, Chango, Schneider & Larondelle. 2006).

Several studies have demonstrated transplacental transfer of OTA in swine although contrasting reports have been published: non-residues in piglets of sows fed with diets containing OTA through gestation or no placental transfer after ingestion of OTA by a pregnant sow have been described (Barnikol & Thalmann 1988). In contrast, some authors found OTA transmission to piglets in uterus with blood concentration ranged from 0.075-0.12 ng/ml, however, in the sow it was 0.12ng/ml (Barnikol & Thalmann 1988).

Both biliary and renal routes are involved in the excretion of OTA with the biliary route predominating (Dietrich, Heussner, & O'Brien. 2005).

In the horse, natural occurrence of OTA in serum samples and placental transfer was reported by some authors (Minervini, Giannoccaro, Nicassio, Panzarini & Lacalandra. 2013). OTA was found in 83% serum samples (with concentrations ranged from 52.8 to 705.4 pg/ml) collected from horses and 50% of the blood samples collected from foals (with concentrations ranged from 69.5 to 252.6 pg/ml). This study demonstrated the presence of OTA in blood samples and strongly supported the hypothesis of placental transfer of OTA from mother to fetus in this species.

Concerning polygastric animals, ruminants have the supportive rumen and ruminal microbiota for this purpose. Acute toxicity is observed in young ruminants with underdeveloped rumen because they have less developed microbiota of rumen that help in degradation and detoxification of OTA. Although in ruminants the OTA is degraded and detoxified to OTα still the traces of OTA are found in blood, milk and urine samples of ruminants (Mobashar, Hummel, Blank & Sudekum. 2010). Some authors reported that a part of OTA ingested escaped microbial degradation in the rumen and was absorbed. OTA disappeared quickly from cow plasma after the ingestion of OTA-free diet (Hashimoto, Katsunuma, Nunokawa, Minato & Yonemochi. 2016). The dietary OTA was not carried over into milk or edible tissue, such as liver, muscles and fat of cow (Hashimoto, Katsunuma, Nunokawa, Minato & Yonemochi. 2016)

Enzymatic degradation of OTA means cleavage of the amide bond into non-toxic phenylalanine and OTα. Proteolytic enzymes are the most likely to develop significant OTA degrading activity. Different groups of microbes (protozoa, bacteria or fungi) could be considered to contribute in metabolizing OTA (Mobashar, Hummel, Blank & Sudekum. 2010).

The main route of excretion of OTA in functional ruminants dosed orally is the urinary tract as its metabolite OTα is excreted via urine. Interestingly, in a study in young ruminants, most of the OTA dose was found to be excreted in the form of OTα. Biliary secretion has been postulated for OTA, explaining repeated peaks in blood concentrations of OTA and its metabolites and the occurrence of OTA in the faeces of animals intravenously administered (Ringot, Chango, Schneider & Larondelle. 2006).

The few data published on OTA placental transfer in ruminants showed a very low penetration of OTA through the placenta. After intravenous administration of a high dose of OTA (1 mg/kg body weight) to pregnant ewes, OTA was not detected in the amniotic fluid and fetal tissue levels were 400- to 1000- fold lower than in the maternal blood (Ringot, Chango, Schneider & Larondelle. 2006).

OTA concentration in milk obtained by ruminants/non-ruminants is 10 times less than the concentrations in the blood samples (Breitholtz-Emanuelsson, Olsen, Oskarsson, Palminger & Hult. 1993).

The presence of OTA in 14% of cow's milk samples collected in different regions of Sweden, while no OTα was detected. Unfortunately, OTA determination in feed was not performed in this study. The authors however suspected a high OTA contamination level in feed because of the long period of storage (September–June) (Ringot, Chango, Schneider & Larondelle. 2006)

1.1.5 Mechanisms of action

1.1.5.1 Oxidative stress and toxicity

Generation of ROS, oxidative stress and reactive nitrogen species

Both *in vitro* and *in vivo* OTA exposure results in the overproduction of free radicals and glutathione (GSH) can prevent the OTA damage induced by ROS. OTA exposure of primary rat proximal tubule (PT) cells and pig kidney cell line (LLC-PK1) cells results in a concentration-dependent elevation of ROS levels, depletion of cellular GSH levels and an increase in the formation of 8-oxoguanine, indicating that oxidative stress contributes to the tubular toxicity of OTA (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).
OTA facilitates the expression of inducible nitrogen oxide synthase (i NOS) enzyme and increases the expression and activity of dimethyl arginine dimethyl amino hydrolase (DDAH) with subsequent elevation of nitrogen monoxide (NO) synthesis and increased nitrite/nitrate concentrations. High levels of NO may cause nitrosative stress because it can react with O_2•- resulting in the formation of peroxynitrite (ONOO-), which in turn gives nitrogen dioxide (NO_2) and OH• (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

In conclusion, oxidative stress and nitrosative stress are related to OTA-induced DNA damage and provide additional evidence for a role of this mechanism in OTA carcinogenicity (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

The downregulation of nuclear factor erythroid 2–related factor 2 (Nrf2) associated with OTA decreased superoxide dismutase (SOD) and downregulated expression of glutathione S-transferase (GST) are major contributors to the induction of cellular oxidative stress induced by OTA (EFSA, 2020). Oxidative stress is evidences along with evidenced along with downregulation of gene

expression of markers of inflammations and these effects were correlated with renal toxicity (EFSA, 2020).

OTA-mediated oxidative damage

Damage to lipids

OTA enhances lipid peroxidation stimulating both NADPH-dependent and ascorbate-dependent lipid peroxidation in microsomes, iron ($Fe^{3+)}$ being the co-factor. An OTA–Fe^{3+} complex would be formed, which would facilitate the reduction of iron in the presence of the NADPH-cytochrome P450 reductase system. The resulting complex OTA–Fe^{2+} would then generate OH•, leading in turn to membrane lipid peroxidation. OTA-enhanced lipid peroxidation affects the permeability of the plasma membrane to Ca^{2+} and thus impairs calcium homeostasis by enhancing Ca^{2+} influx (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

Damage to proteins and DNA

A significant increase in the levels of protein nitro tyrosine residues was observed with OTA, and it was also found that Nrf2 activators could prevent OTA-induced protein nitration. OTA could lead to oxidative chromatin and DNA damage *in vitro*, resulting in growth inhibition of canine umbilical cord matrix mesenchymal stem cells (Rutigliano, Valentini, Martino, Pizzi, Zanghì, Dell'Aquila & Minervini. 2015). Oxidative DNA damage primarily includes DNA strand breaks (single- and double-strand breaks) and base lesions, such as the formation of 8-hydroxydeoxyguanosine (8-OHdG) 8-OHdG is commonly used as an in vitro biomarker for the assessment of OTA-induced oxidative damage (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

1.1.5.2 Inhibition of proteins and RNA synthesis

OTA is an inhibitor of protein synthesis in both *in vivo* and *in vitro* models. It was verified that OTA could inhibit the activity of phenylalanine-RNA synthase, phenylalanine (Phe) moiety has a significant impact on protein synthesis because modification of the structure can lead to inhibition of protein synthesis and toxicity (Kőszegi & Poór. 2016).

In fact the Phe-moiety of OTA can bind to both sites and impair the hydroxylation reaction so it is thought that the mechanism involved in inhibiting protein synthesis is the inhibition of peptide elongation through competition with Phe-t RNA synthetase (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018). Some authors have considered that the predominant downregulation of RNA and protein expression may be a result of protein synthesis inhibition (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang, & Yuan. 2018).

The effects of OTA on Phe-t-RNA synthase and Phe hydroxylase, were observed after the treatment with relatively high OTA doses. In addition to these non-specific ways of protein synthesis inhibition, OTA may influence the transcription of many proteins resulting in specific intracellular effects (Kőszegi & Poór. 2016). OTA alters the expression of proteins via transcriptional, microRNA and protein posttranslational modifications to modulate cell signalling systems responsible for apoptosis and also pathways related to stress responses, inflammation, fibrosis and the balance between cell survival, cell proliferation, cell division and cell death (EFSA, 2020)

OTA has a strong negative effect on cellular energy (ATP) production. Mitochondrial dysfunction is an early sign of toxicity resulting in an overall decrease in protein synthesis also proven that OTA interferes with the expression of phosphoenolpyruvate-carboxy kinase (PEPCK) at the mRNA level. Furthermore, the toxin can penetrate into the mitochondria and most probably binds to proteins involved in maintenance of the membrane potential and the oxidative phosphorylation by interfering with phosphate transport and by inhibition of electron transport as well as consequence to OTA-related mitochondrial toxicity, free radicals generate reactive oxidative species (ROS) in cell and results in cellular apoptosis (Kőszegi & Poór. 2016).

1.1.5.3 Stress-mediated biological response

Apoptosis and cell signaling

Cell death can generally be produced via necrosis or apoptosis. Studies show that p53-dependent cell cycle arrest, DNA repair and apoptosis are associated with oxidative stress. OTA has been shown to deregulate a variety of different signal transduction pathways while the Extracellular Signal-Regulated Kinase (ERK1-2) and Stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) are members of the Mitogen-activated protein kinase (MAPK) family and both are major targets. Knowledge about their role in OTA-mediated cell survival and death is fragmented. OTA activates oxidative stress and increases NADPH oxidase activity. ROS are highly reactive species formed by an incomplete one-electron reduction of oxygen. The major endogenous source of cellular ROS is the mitochondrial electron transport chain (mETC). ROS play an important role in the toxicity of OTA (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

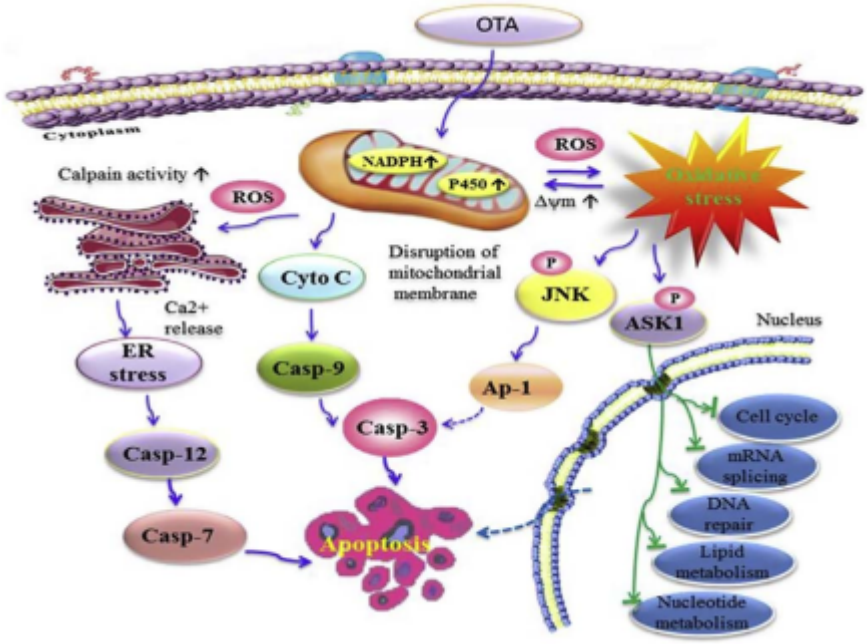

Figure 1.2 OTA mechanism of action (oxidative stress, inducing calcium release and inhibiting the cell cycle, mRNA splicing, DNA replication, lipid and nucleotide metabolism) (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang, & Yuan. 2018).

As observed in Figure 1.2, OTA induced the increase of NADPH and P450 enzyme, which activates the caspase signaling pathway and induces apoptosis. The increase of ROS caused mitochondria and endoplasmic reticulum oxidative stress, inducing calcium release and inhibiting the cell cycle, mRNA splicing, DNA replication, lipid and nucleotide metabolism. All of these could lead to cell apoptosis (Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

The association between oxidative stress and the loss of mitochondrial membrane potential associated with apoptosis produced by OTA was noted in a range of cell types in vitro (EFSA, 2020).

Caspase 3 activation associated with upregulation of the levels of p53 and p21 appeared responsible for apoptosis in oocytes and blastocysts treated with OTA (Huang and Chan, 2016).

OTA can cause apoptotic and necrotic death. Even at nanomolar concentration, apoptosis markers could be observed e.g. DNA fragmentation, chromatin condensation and increase caspase-3 activity (Kőszegi & Poór, 2016).

Altered expression of different genes could be a potential cause of apoptosis: marked transcriptional changes of many genes were observed which are involved in DNA damage response and apoptosis such as activation of MAPK, ERK, p38 and JNK were described in kidney and /or liver cells (Kőszegi & Poór. 2016).

Another study suggests the role of apoptosis signal-regulating kinase 1 ,which can activated JNK and p38 pathways, as well as it has a pivotal role in oxidative stress and in endoplasmic reticulum stress-induced cell death, as observed in Figure 1.2 (Kőszegi & Poór. 2016). Activation of PKC and MAPK-ERK signalling pathways play an important role in the stress response and cell proliferation (EFSA, 2020).

DNA adduct production

In the previous EFSA opinion on OTA (EFSA, 2006), the CONTAM Panel concluded that 'DNA damage and genotoxic effects of OTA were most likely attributable to cellular oxidative damage and within the limit of sensitivity of the analytical procedures, there was no clear evidence for the formation of specific OTA-containing DNA adducts. Subsequently the mechanism of action of DNA adduct formation can be approximately understood by the Figure 1.3. OTA is transported via transporters inside the cell and is bio-transformed to electrophilic products, such as OTHQ, OTB and OH-OTA which can covalently bind to DNA causing mutations and subsequent formation of malignant tumors (Kőszegi & Poór. 2016). Up to now, it remains unclear whether and to what extent these DNA adducts are formed in vivo, which metabolic pathway(s) are responsible for the formation and the molecular mechanism underlying OTA genotoxicity remains unclear. The formation of covalent OTA-DNA adducts remains controversial. The extremely low reported levels of DNA adducts ($10/10^9$ nucleotides) are difficult to reconcile with the genotoxic effects of OTA and the proposed OTA-DNA adducts are likely to be, at best, minor contributors (EFSA, 2020)

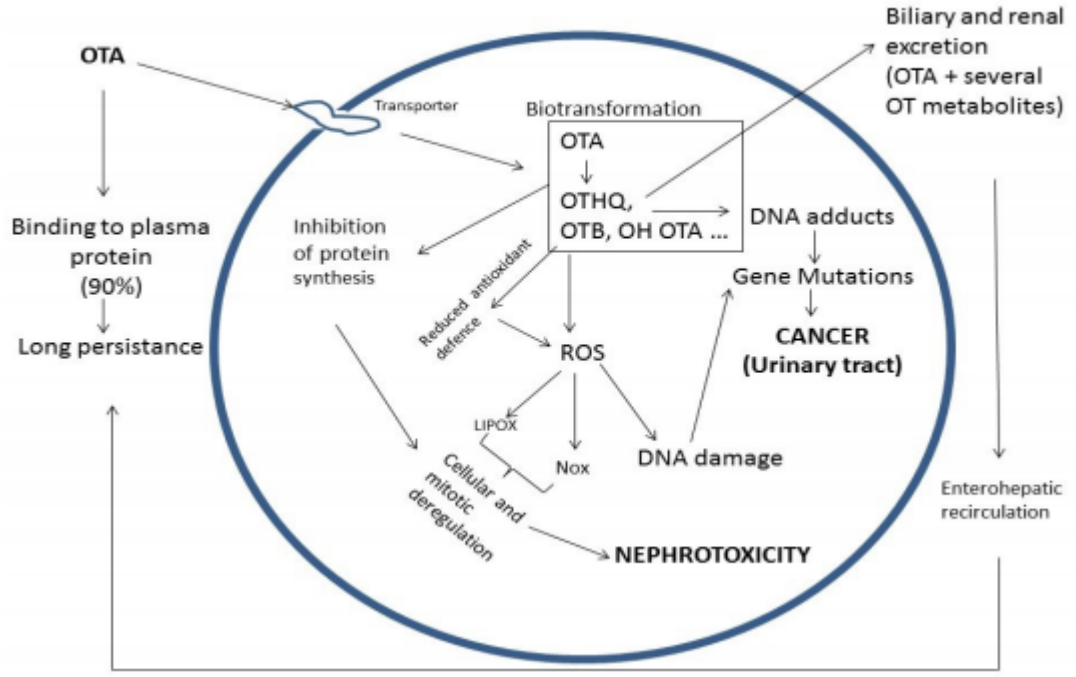

Figure 1.3 Mechanism of action and toxicity of OTA (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016).

1.1.5.4 Mode of action of carcinogenesis

Concerning carcinogenesis several mode of actions should be considered i.e. DNA damage (genotoxic) and non DNA reactive mechanisms (non genotoxic mechanisms). The first mechanism induced by OTA includes the formation of DNA adducts, gene mutations with altered expression of genes involved in the DNA damage, cell cycle regulation , disturbance of mitosis and chromosomal segregation. In fact, post-traslational modification of proteins and the alteration of gene expression may contribute to modulation of the cell cyle and spindle function in association with the cell proliferative response, leading to genomic instability. Non-genotoxic mechanisms appear to contribute to carcinogenesis. These are based on a stress response (including an oxidative stress), altered cell signaling and cell proliferation, interference of intercellular communication and epigenetic mechanisms (EFSA, 2020). Up to now, the CONTAM Panel EFSA for neoplastic effects of OTA, emphasises, that it was not possible to make a clear distinction between a direct and an indirect mechanism of genotoxicity (EFSA, 2020).

1.1.6 Toxicity of OTA

1.1.6.1 Nephrotoxic effects

Ochratoxin A has been found to be nephrotoxic in all mammalian species treated although differences in toxicity have been found among species and sex by different mechanisms: induction of oxidative stress, disruption of transcriptional regulation via inhibition of the protein synthesis, interference of metabolic enzymes, disturbance of cell signalling [$Ca2^{+}$signalling homeostasis and MAPKs signalling], cell cycle arrest, induction of apoptosis, activation of autophagy and the interaction network among the above mechanisms (Tao, Xiao, Wenying, Xin, Jieyeqi, Yan, Yan & Cuilan. 2017).

OTA-induced porcine nephropathy in pigs and Balkan endemic nephropathy (BEN) in humans are supposed to be cause by OTA exposure. Epidemiologic studies showed that in areas where high OTA levels are reached in food and in the blood of the population, there is a high incidence of nephropathy and renal tumours. Occurrence of chronic kidney tumors is described in certain endemic regions of the Balkan Peninsula and chronic interstitial nephropathy is present in Tunisia and other North African countries (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

Kidneys are the principal organ in charge of filtration and any alteration in them can give rise to difficulties in the elimination process. BEN disease is due to the alteration and modification in the epithelial cell lining of the proximal tissues without any definite damage to the organ. The main nephrotoxic effect in humans is in the postproximal-nephron and proximal tubule, which have been reported as a self-enhancing effect. The nephropathy is characterised by polyuria, glucosuria, and proteinuria and decreased osmolality of the urine (Hope & Hope. 2012). After chronic exposure, kidneys are reduced and interstitial fibrosis is the most important picture. At the end stage, impairment of renal function leads to enzymuria (e.g., gamma glutamyl transferase, alkaline phosphatase, lactate dehydrogenase), polyuria accompanied with red tongue, thirst, and bitter taste. Neither edema nor hypertension can be observed. Other symptoms, such as headaches, lumbar pain, asthenia, and anemia (iron deficiency) were recorded (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

In a survey performed in Italy (Breitholtz-Emanuelsson, Minervini, Hult & Visconti. 1994) a group of humans without and with five different kinds of kidney, disorders (such as transplanted subjects, chronic glomerulonephritis, renal calculus or cyst, chronic renal failure and subjects treated by dialysis) were selected to assessed the OTA exposure through analysis of blood samples. The concentrations of OTA in the healthy group ranged from 0.12 to 2.0 ng/ml serum. The OTA concentrations in humans with diseases ranged from 0.0.5 to 14 ng/ml.

The highest mean concentration was found in the group of patients treated by dialysis, (mean concentration: 1.4 ng/ml of serum). A higher incidence of samples containing > 0.44 ng OTA/ml serum was found in the dialysis group, compared to the other groups (Breitholtz-Emanuelsson, Minervini, Hult, & Visconti. 1994), probably due to the failure kidney filtration.

In 2012 another study was carried out in Italy (the Molise region) on patients with wide kidney diseases. OTA incidence went from 49% in healthy patients to 76% in patients with chronic interstitial nephropathy and unknown aetiology. OTA serum levels in healthy patients ranged from 1.7 to 8.5 ng ml–1, while in patients with renal disease it went from 1.8 to 65 ng ml (Soto, Ruiz, Manyes & Juan-García. 2016).

OTA occurrence was reported by several studies in the North Eastern European countries such as Bulgaria, Romania, Serbia, Croatia, Bosnia, Herzegovina, Slovenia, Macedonia, Monte Negro and also in African countries, (such as Congo, South Africa, Tunisia, Morocco and Egypt). OTA in human serum ranged from 5 to 50 ng/mL and OTA was detected in 87% human blood samples collected from patients suffering from various renal diseases as e.g. interstitial nephritis or

mesangial proliferating glomerular nephritis. No significant difference between OTA concentrations of healthy individuals and patients with chronic glomerular nephritis whose sera showed OTA concentration greater than 1.5 nmol/L was reported. In contrast, no values greater than 1.5 nmol/L were present in the sera of patients with membranous or focal-sclerotic glomerular nephritis (Reddy & Bhoola. 2010).

In female pigs fed on diets containing 1 mg OTA/kg feed, OTA nephropathy, without renal failure was observed. Diets containing 0.2 mg OTA/kg feed for two years had no such effects. Degenerative changes affecting epithelial cells in some proximal tubules were observed in pigs given a diet containing OTA at 0.8 mg/kg for six months, as well as proliferative changes in the interstitium, which predominated after one year (Denli & Perez. 2010).

Egg laying hens fed on a diet contaminated with 2 mg OTA/kg significantly reduced daily feed consumption, egg mass production, and serum triglyceride concentrations. Weight losses, diarrhea, excessive urine excretion and renal lesions have been noted in chickens fed a diet contaminated with 2 mg OTA/kg (Denli & Perez. 2010).

1.1.6.2 Immunotoxic effects

OTA displays a multiple toxicity, including immune toxicity. OTA modulated the immune response even at levels far below the toxicity threshold. It is not always clear from these studies whether OTA exerted directly its immunotoxic effects or if the acute toxic effects had also an indirect influence on the immune system. It was suggested that the immunotoxic effects of OTA could be related to its interference with the cell metabolism (Marin & Taranu, 2015). After exposure of animals to various doses of toxin OTA was shown to cause a significant reduction of the total serum immunoglobulin levels in animals fed with OTA contaminated diet. This decrease was associated with a decrease in the immunoglobulin-containing cells in all lymphoid tissues of animals (Marin & Taranu, 2015)

Decrease of the humoral immunity, as a consequence of the exposure to OTA, leads to the increase of the frequency of the infections. For example, salmonellosis arose spontaneously in pigs fed with OTA naturally contaminated diet (3 ppm) and *Salmonella cholera suis* was isolated from their faeces and liver (Marin & Taranu. 2015).

Several studies showed that OTA is a suppressor of the lymphocytes blastogenesis in not only healthy animals and humans but also in patients with severe disease. The decrease of the blastogenesis was correlated with the impairment of the IL-2 production and of the IL-2 receptor

expression of activated T lymphocytes. The lymphoid tissues seem to be very sensitive to OTA, where the toxin was able to induce necrosis (Marin & Taranu. 2015).

Contradictory effects were observed on the capacity of OTA to affect phagocytosis. Neutrophils and monocytes from mice intraperitoneally exposed to OTA showed a significantly lower phagocytosis of *E. coli* while, simultaneously, blood leucocytes showed an increasing production of oxygen radicals (Marin & Taranu. 2015).

Exposure to OTA during early stages of the development of the embryo resulted in a prenatal modulation of the offspring's immune system. OTA was described as modulator of humoral and cellular immunity, inflammation, nitrosative stress and gut immunity. In the literature, there are few studies concerning OTA immune toxicity and the results are sometimes contrasting. However, all these studies showed undoubtedly that OTA is able to modulate the immune response, with important implications for human and animal health (Marin & Taranu. 2015)

1.1.6.3 Carcinogenic effects

OTA, it was classified into Group 2B based on a great amount of evidence of carcinogenicity revealed in recent human and animal studies. When the studies were evaluated by International Agency for Research on Cancer (IARC) it was found that the evidence for carcinogenicity was either inconclusive or limited (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman, 2016). Concerning the OTA exposure in rats, the first renal tumour was discovered at 75 wk and 20% of the rats developed renal carcinoma and renal parenchyma. The severity and extent of various gross, skeletal, and visceral anomalies were proportional to the doses applied (Pfohl-Leszkowicz & Manderville. 2007).

A high rate of metastases from renal cell carcinomas mainly to lung, liver and lymph nodes was also observed in both males and females. In view of these results, IARC evaluated the experimental evidence for carcinogenicity as sufficient, and classified OTA as, possibly carcinogenic to humans (Pfohl-Leszkowicz & Manderville. 2007).

OTA causes many different tumors such as renal and hepatic cancers. In mice and rats, different type of carcinogenicities has been reported and DNA adducts formation is also the major cause of carcinogenic effect induced by OTA in urinary tract. After oral administration, tumours were induced in the kidney of mice and rats and hepatoma in the trout. (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

At 210 µg/kg body weight (bw) renal tubular adenomas and carcinomas were observed (72% for males and 16% for females) after 2 years. At 70 µg/kg bw, 39% of the males and 4% of the females developed renal adenomas or carcinomas. The females were less susceptible than the males to OTA carcinogenicity (Pfohl-Leszkowicz & Manderville. 2007).

Some large tumours were associated with metastatic nodules located extensively along the abdominal mesenteries, and occasionally carcinoma extended to the lungs. Other histopathological changes in kidney were observed, such as karyomegalic nuclei in tubular epithelia, predominantly in the cortico-medullary region (Pfohl-Leszkowicz & Manderville, 2007). Studies in animals have confirmed a causal connection between OTA exposure and cancers of the urinary tract, liver and mammary glands (Hope & Hope. 2012).

OTA also was supposed to have a possible etiologic role in testicular tumors in men because several areas of the Balkans, with high OTA contamination, showed high incidence of testicular tumors in men (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013).

1.1.6.4 Reprotoxic effects

During *in vivo* studies, OTA can cross the placenta and has been found to be embryotoxic in rats and mice. Studies with radiolabelled OTA in mice showed OTA to cross the placenta, preferentially at specific times during gestation (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013). Additionally OTA has been found in breast milk, which could represent a significant source of exposure for infants (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013). OTA, at different concentrations (2–4 mg/kg bw) and different gestations days (6–15), causes variable developmental defects (external hydrocephaly, incomplete closure of skull, and omphalocele) in foetuses (Hope & Hope. 2012). In fact, OTA is a potent teratogen in mice, rats, and hamsters, chickens and rabbit but not in pigs (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013). Maternal OTA can cross the placenta, accumulate in foetal tissues and induce malformation. Both OTA and OTB (the analog of OTA) cause craniofacial malformations, while OTA also causes reduced embryo growth. As expected, OTA is by far more potent in inducing these effects than OTB (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013).

Human placenta plays an important role in the development of the fetus, being the major interface between mother and fetus. Placenta provides a gateway to oxygen and nutrients from the mother to the fetus. Transport processes across the placenta (passive diffusion, active transport, facilitated

diffusion, filtration, and pinocytosis) and metabolism determine the exposure of the fetus to xenobiotics including OTA. After crossing the maternal placental barrier, OTA reaches the fetus and interferes with the development of the organs. A study on OTA in human perfused placenta shows that OTA is able to cross the placenta, more particularly, at early gestation rather than at late gestation (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013).

The most common developmental toxicities are spontaneous abortion, congenital malformations, intrauterine growth retardation, mental retardation, carcinogenesis, and mutagenesis. The reduced birth weight and craniofacial abnormalities are the most frequent signs observed. OTA also causes reduced embryo growth (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013).

In *in vitro* studies, (Huang & Chan. 2016) reported the deleterious effects of OTA on mouse oocyte maturation, in vitro fertilization (IVF), and subsequent pre and post implantation development *in-vitro.* OTA significantly impaired mouse oocyte maturation, decrease IVF rates, and inhibited subsequent embryonic *in-vitro* development (Huang & Chan. 2016).

A comparison study showed that 5μM OTA affected porcine oocyte maturation instead of 300 μM OTA in mouse oocytes (Lu, Zhang, Liu, Zou, Jia, Su, Sun & Sun. 2018).The average polar body extrusion rate of the control group was 80.23 ± 3.87% (n = 169) compared mouse oocytes treated with 300 μM OTA treatment in which a significant decrease (49.86 ± 4.29%) was observed. However, the rate of polar body extrusion in porcine oocytes was significantly decreased up to 5μM OTA treatment (53.83 ± 0.34 %,) indicating that porcine oocytes were more sensitive to OTA (Lu, Zhang, Liu, Zou, Jia, Su, Sun & Sun. 2018).

In vitro and *in vivo* studies on mouse reported that 10μM OTA induced apoptosis and impaired preimplantation and postimplantation embryonic development (Huang, Wang & Chan, 2019). OTA-induced embryotoxic effects on mouse were mediated through ROS generation that, in turn, triggered sequent mitochondrion-dependent apoptotic signalling cascades, exerting injurious effects on preimplantation and post implantation embryo development. In addition, significant impairment was observed in mouse oocyte maturation, in vitro fertilization (IVF) and early embryonic developmental injury in the presence of 10 μM OTA (Huang, Wang, & Chan. 2019).

In the mouse, as in vivo animal model, provision of 1-10 μM OTA in drinking water or intravenous injection of 1 or 2 mg/kg body weight OTA decreased oocyte maturation and IVF and had harmful effects on early embryonic development (Huang, Wang & Chan. 2019)

In another *in vitro* study, mouse blastocysts treated with 10 μM OTA displayed a significantly increased level of apoptosis secondary to ROS generation (Hsuuw, Chan & Yu. 2013).

OTA was potently toxic to zebrafish embryo development used as in vitro model. Developmental dysfunction of zebrafish exposed to OTA was characterized by discernible deformities, reduced growth and hatching rates, and lethality. At the highest concentrations (≥0.5 μM), OTA was generally lethal by 2nd development post fertilization and development of surviving embryos was severely retarded (Haq, Gonzalez, Mintz, Jaja-Chimedza, De Jesus, Lydon, Welch & Berry. 2016).

Besides its classical known toxicities, it is also associated with the impairment of steroidogenesis in rats. It is hypothesized that OTA may act as an endocrine disruptor by intervening 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD). To address this hypothesis, human placental cells JEG-3 were used in vitro to examine the effects of short- and long-term OTA exposures on expression levels of 3β-HSD1 and progesterone secretion at 24-96h. Results showed that both cytotoxic and non-cytotoxic levels of OTA induced 3β-HSD1 mRNA expression by 281-378% at 72 and 96h. A significant induction (43-316%) of 3β-HSD1 protein expression was observed at 48, 72 and 96h, and the progesterone production with the involvement of 3β-HSD1 was significantly increased by 22-89% after 48-96h (Koszegi & Poor. 2016).

Concerning OTA toxicity in male, testicular abnormalities and cancers are also major aspect of OTA exposure. It can affect the quality of sperm leading to the infertility in man (Gupta, Lasher, Miller Mukherjee, Srivastavam & Lall 2017). OTA was also shown to decrease testosterone secretion in testicular interstitial cells of gerbils (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013).

1.1.6.5 Neurotoxic effects

The developing brain appears to be very susceptible to the deleterious effects of OTA. OTA has been shown to affect proliferation and migration of neurons and reduce DNA content in the developing rodent brain (Doi & Uetsuka. 2011).

OTA is also reported to be neurotoxic to adult male rats. Neurotoxicity is more pronounced in the ventral mesencephalon, hippocampus, and striatum than in the cerebellum (Doi & Uetsuka. 2011). Recent animal and cellular studies have suggested that OTA may contribute to the development of human and animal systemic problems, including neurodegenerative diseases and brain dysfunction (Doi & Uetsuka. 2011).

Acute effects of OTA in the context of DNA damage, DNA repair and global oxidative stress across six brain regions, cerebellum, cortex, hippocampus, midbrain, caudate/putamn and pons/medulla, in male mice have been reported. Background fact is globally increased oxidative stress evidenced by significant increases in lipid peroxidation and oxidative DNA damage, and transient inhibition of oxidative DNA repair activity (oxyguanosineglyosylase, OGG1) across six brain regions, accompanying evidence for apoptosis in the substantia nigra, striatum and hippocampus or other regions (Sava, Reunova, Velasquez, Harbison & Sánchez-Ramos. 2006).

The adult brain retains a reservoir of stem-progenitor cells in the hippocampal "neurogenic zone" capable of proliferative activity throughout life, and it is known that injury, irradiation, drugs and endogenous factors such as hormones and trophic factors influence neurogenesis. Therefore, OTA may also affect neurogenesis in adult hippocampus (Doi & Uetsuka, 2011).

Environmental exposure to OTA has potential harmful effects on neuronal cell viability and proteome profiles using mouse hippocampal HT22and human neuroblastoma SH-SY5Y cells. Generation of ROS was detected in OTA-treated SH-SY5Yand HT22 cells, however, caspase activation and an increase in p53 phosphorylation were only detected in HT22 cells, even though OTA treatment caused oxidative stress in both two cell lines. The expressions of several proteins which have been suggested to be implicated in the pathogenesis of neurodegenerative disorders, were up-regulated only in HT22 cells after treatment with OTA, which was interesting because OTA induced the apoptosis of HT22 cells but not of SH-SY5Y cells (Doi & Uetsuka. 2011).

Besides the toxic effects of OTA on neuronal cells, the relationship between OTA toxicity and glial reactivity in serum-free aggregating rat brain cell cultures has been investigated. In addition, OTA affects the cytoskeletal integrity of astrocytes as well as the expression of genes pertaining to the brain inflammatory response system, and suggested that a relationship exists between the inflammatory events and the cytoskeletal changes induced by OTA (Doi & Uetsuka. 2011)

1.1.7 OTA biomarkers

Biomarkers have been defined as the compounds (e.g., toxins and/or a metabolite) or the products of their interaction with target molecules (e.g., protein or DNA adducts and glucuronide conjugates) that can be measured in body fluids or tissues and can be correlated with ingested mycotoxins. Good biomarkers should be quantitative, sensitive, non-invasive, specific, and easily measurable, and should be related to OTA biochemical mechanisms at realistic doses (Arce-López, Lizarraga, Vettorazzi, & González-Peñas. 2020).

Biomarkers are useful to detect the toxin concentration in body as the biological fluids allow a homogeneous distribution of toxin respect to food matrix. Analysis of biomarkers is useful for diagnostic, clinical aspects, for the assessment of exposure in short time, and it is more convenient because the same extraction protocol could be used in all species. Main biomarkers help to detect the OTA concentration in human and animal of blood, milk, colostrum, and urine samples (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013). Levels of OTA concentration can be determined during assessment of human and animal OTA exposure.

1.1.7.1 OTA exposure biomarker in blood

The contamination incidence raises in the wide range of analysed samples (from 28% to 100%), it might be associated with the different methodologies used in each study for sample analysis (serum or plasma). Several studies have looked for a correlation of OTA levels in blood with other factors such as the region where the population was living, the season when the samples were collected, or gender and age (Soto, Ruiz, Manyes & Juan-García. 2016).

OTA exposure is determined in serum blood samples collected from both animal and human. The presence of OTA in the blood persists for long time because OTA has very high protein binding affinity at high percentage (99%) (Malir, Ostry, Pfohl-Leszkowicz & Novotna. 2013). Blood serum or plasma is considered as more reliable source especially for humans. OTA concentration found in blood serum/plasma are more reliable and trust worthy rather than the OTA concentration found in urine due to its high-affinity binding to serum albumin or to other small proteins, which should result in higher serum OTA levels and long persistence of OTA in blood serum. The evaluation of OTA concentrations in the blood will allow to evaluate exposures realized long time ago especially in humans with only one collection of blood, contrary to the urine that must be collected throughout the day with daily excretion fluctuations (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

In Italy, a survey on humans reported OTA plasma concentration in the range between 120 and 2,840 ng/L (n = 138), with significantly higher means in men (640 ng/L) than women (500 ng/L) (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri & Rossi. 2011).

In a survey carried out in Norway and Sweden found slightly lower mean OTA values in plasma: 180 ng/L in Oslo (n = 206) and 210 ng/L in Visby (n = 200). Furthermore, a strong correlation was found in the female population between the consumption of beer and whole bread and the serum levels of OTA. On the contrary, in a study carried out in Poland, the mean OTA concentration in

serum from the umbilical cord was 1,940 ng/L, markedly higher than that found in this research (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri & Rossi. 2011).

Finally, in Morocco only 56% of plasma samples (n = 96) were positive and the mean concentration was 260 ng/L (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri & Rossi. 2011).

Several studies carried out by analysis of blood samples collected from umbilical cord suggest an active placental transfer of OTA. To quantify the transmission of OTA in humans, from mother to new born and to infant, study was conducted in Bolivia (Ferrufino-Guardia, Chavez-Rico & Larondelle. 2019). Blood samples were collected from maternal blood and umbilical cord at time of child birth. OTA was detected in 87% umbilical cord blood plasma samples. The OTA concentration in blood samples from umbilical cord was twice to the OTA concentration in maternal blood, with a range much lower than in maternal plasma (values of 0.3 to 1.7 ng/ml) and a mean of 0.7 ± 0.65 ng/ml. These discordant findings could be due to different reasons such as the analytical methods used. In addition, 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. The concentration of OTA depends on the duration of exposure and the amount of toxin ingested (Ferrufino-Guardia, Chavez-Rico & Larondelle. 2019).

Among farm animals, pigs are particularly sensitive to OTA accumulation. Analysis of blood samples in Northwest Italy reported that OTA was detected in all serum samples at levels ranging from 0.03 to 0.87 ng mL-1 and from 0.15 to 6.24 ng mL-1 for conventional and organic farms respectively. (Pozzo, Cavallarin, Nucera, Antoniazzi & Schiavone. 2010).

In Japan a survey was performed in order to investigate the influence of repeated ingestion of OTA at different levels (from 5 to 100 μg OTA/kg of dry matter of feed) on milk production of lactating Holstein cows. OTA (0.1μg/kg) was detected in the blood plasma of one sample in the group exposed to OTA at 50 μg /kg of dry matter of feed and multiple samples in the group exposed to 100μg OTA/kg of dry matter of feed, indicating that blood could be as potential biomarker for the assessment of OTA exposure to animals (Hashimoto, Katsunuma, Nunokawa, Minato & Yonemochi. 2016).

1.1.7.2 OTA exposure biomarker in milk/colostrum

European Food Safety Authority (EFSA) has already pointed out that infants and children consume more dairy foods than adults do; hence, they are more exposed to compounds that could be present in milk. At least some mycotoxins such as OTA, were secreted into the milk, so that relatively high levels can be achieved. After birth, breast milk or infant formulas constitute an important or often sole food source for infants during their first months of life. It is evident that breast milk as well as infant formula diets represent a relevant source of mycotoxins for neonates and infants, since their presence in samples collected in several European countries has been documented. OTA is the most abundant mycotoxin in milk (Hof. 2016).

OTA is excreted in milk/colostrum excreted from mammary glands and it could be considered among potential biomarkers for the assessment of the exposure through the analysis of OTA concentrations in body. However, the OTA concentration in milk and colostrum is less than its concentration in the blood; in fact, the amount of OTA is 10 times less in milk as compared to the blood of same mother. This condition seems to be less alarming but both fetus and baby are at higher risk of OTA exposure both before and after birth (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

In a study carried out in Italy on 82 samples of breast milk, it was observed that there were differences in OTA levels related to food ingestion and drink consumption habits of women (Galvano, Pietri, Bertuzzi, Gagliardi, Ciotti, Luisi, Bognanno, La Fauci, Iacopino, Nigro, Li Volti, Vanella, Giammanco, Tina & Gazzolo. 2008). Interestingly, when comparing OTA levels in serum and those in breast milk, no correlation has been observed. Breast milk samples were collected for different periods of time and OTA values were found to be higher at the beginning of the breast feeding (days 1–6 of life) (0.086 ng ml–1) than at the end of the period of breast feeding (6 months) (0.044 ng ml–1) (Soto, Ruiz, Manyes & Juan-García. 2016).

Another study in Italy carried out on Italian and non-Italian women, reported the occurrence of OTA was detected in 45 (78.9%) maternal milk samples and the OTA concentration for positive samples ranged from 1.1 to 75.1 ng/L; the OTA level was lower than 5 ng/L in 58% of the positive samples. No significant difference between Italian and non-Italian women (73.0 vs. 85.0% of positive samples) was observed (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri & Rossi. 2011).

In another study performed in Egypt, 72% of breast milk samples resulted OTA positive, indicating that those women had been in contact with OTA and afterwards their babies. The OTA median level in serum from these women was 4.28 ng ml^{-1} and in milk 1.89 ng ml^{-1}, whilst for their babies the OTA median level in serum was 1.26 ng ml^{-1}. (Soto, Ruiz, Manyes & Juan-García. 2016).

Studies from European countries report levels ranging between 10 and 400 ng OTA/L. According to the literature, OTA levels are significantly higher in milk of habitual consumers of bread, bakery products, cured pork meat and sweets. In addition to OTA, the occurrence of OTalpha was also monitored in milk and blood from nine lactating women (EFSA,2020). The concentration levels of unconjugated OTalpha in breast milk were significantly lower than those observed for OTA (40 ± 30 ng/L vs. 106 ± 45ng/L). However, OTalpha concentrations were almost eight times higher (840 ± 256 ng/L) after an enzymatic treatment of breast milk with ß-glucuronidase/sulfatase, while the OTA concentration remained the same. This was an indication of the occurrence of OTalpha conjugates in milk. The lactational transfer of OTA was later reported by the same group by comparing 21 mother–child pairs with parallel collection of maternal blood, milk and of infant urine samples over a period of up to 6 months (EFSA, 2020). OTA was detected in almost all maternal blood plasma samples, at concentrations ranging between 72 and 639 ng/L. An average milk/plasma (M/P) ratio of 0.25 was reported. The authors observed that a higher fraction of circulating OTA was excreted in colostrum (M/P 0.4) than with mature milk (M/P ≤ 0.2). However, the M/P ratios varied substantially between individuals. OTA concentrations in infant urine correlated with OTA levels in concurrently collected milk samples (EFSA, 2020).

An experiment was conducted to investigate the influence of repeated ingestion of OTA at different levels (from 10 to 100 μg /kg of drymatter of feed) on milk production of lactating Holstein cows over 28 days, and the carry-over of OTA from the diets into the milk and tissues of the cows. Body weight, feed intake and daily milk yield in cows were not different among the three groups during the OTA-intake period. OTA residues were neither detected in the tissues, such as liver, kidney, muscles, fat and jejunum ileum, nor in the milk of any cows in the OTA intake groups (Hashimoto, Katsunuma, Nunokawa, Minato & Yonemochi. 2016).

Donkey milk is claimed to be similar to human breast milk in nutrient composition. Analysis of some cereal-containing donkey feed components (pellets, oats) by EIA revealed absence of OTA (< 4 μg/kg). A study reported the detection limit of the OTA EIA (9 ng/kg) clearly was below European Union regulation 1881/2006 for milk By using this enzymatic determination, no residues of OTA were found in different donkey milk samples (Gross, Ploetz & Gottschalk. 2019).

1.1.7.3 OTA exposure biomarker in urine

The known effects of OTA on renal tumors, renal failure testifies that kidney is the major organ that is damaged by OTA exposure and urine is one of the major biomarker for the analysis of OTA and OT α, although the their levels are lower than those in the blood. Usually the samples of urine are collected after 24 hours of the ingestion of contaminated diet as it could be considered as representative for the full day consumption of toxin although a daily fluctuation in excretion is known. In addition, there is variability in the volume of collected urine at different times and among individuals; this variability leads to changes in the concentration of excreted compounds in the samples. For this reason, OTA values should be normalized based on creatinine excretion (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman, 2016; Arce-López , Lizarraga,Vettorazzi & González-Peñas. 2020).

The highest OTA values found in urine samples have been recently detected in a study performed in Nigeria, where a total of 120 urine samples (19 of children, 20 of adolescents and 81 of adults) were analysed. It was found that 28.3% of the samples were OTA positive, with a mean value of 0.2 ng ml–1. Considering age group, adolescents and adults had higher levels (0.2 ng ml^{-1}) than children (0.1 ng ml^{-1}) (Soto, Ruiz, Manyes & Juan-García. 2016).

In southern Italy (Apulia region), urine samples from 52 volunteers (equally divided into males and females) were analysed. Mean OTA levels in urine were at 0.061 ng ml–1 and in almost all samples. This was not surprising because it is usually found in cereals and derived products, staple foods of Italian people (Soto, Ruiz, Manyes & Juan-García. 2016).

A comparison study was performed between two populations in Spain and Portugal. Ninety five urine samples were collected from individuals of six different regions from north to south in Portugal. The study was performed with a 1-year follow-up and levels in winter and summer seasons were compared. OTA was found to be present in 84% of the samples, and values were higher in winter (0.021 ng ml^{-1}) than in summer (0.016 ng ml^{-1}) (Soto, Ruiz, Manyes & Juan-García. 2016).The presence of OTA in urine for two different populations, Valencia (Spain) and Coimbra (Portugal), were compared. The percentage of positive samples ranged from 46% to 84%, the highest percentage belonging to the Spanish population. Urine samples from Spain had the highest OTA mean values (0.03 ng ml–1) compared with Portugal (0.019 ng ml–1), which was related to several factors: climatic conditions, different consumption habits and poor techniques of food preservation (Soto, Ruiz, Manyes & Juan-García. 2016).

The urinary levels of both OTA and OTα were analysed in a cohort of German adults (23 males, 27 females) fed with a regular diet. All samples were subjected to an enzymatic hydrolysis of biomarker conjugates prior to clean-up by liquid–liquid extraction and HPLC-FD analysis. The profile in the first individual showed small fluctuations over time: mean levels in urine were 0.06 ng/mL for both OTA and OTα. The other individual had mean levels of 0.24 and 2.22 ng/mL for OTA and OTα analytes in urine (Ali, Muñoz & Degen. 2017).

Total 152 urine samples collected from patients with BEN (Balkan endemic nephropathy) or urinary tract tumors and from the control families were analysed. OTA was detected in about 33% of the samples of urine (more often-in endemic villages than in non-endemic ones) in the range 5–604 ng/L and in healthy people in the range 5–43 ng/L (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016)

In the United States, OTA in urine samples from patients with chronic fatigue syndrome was analysed. Of 159 samples analysed (104 patients and 55 controls), 83% of them were OTA positive. OTA values from the control group ranged from 0.355 to 0.457 ng ml^{-1}, while in the patient group it went from 5.26 to 3.65 ng ml^{-1} (Soto, Ruiz, Manyes & Juan-García. 2016).

1.1.7.4 Multi-biomarker analysis

Multi-biomarker analysis is the most appreciated method for the analysis of concentration of OTA in different biological fluid samples such as milk/colostrum, blood and urine certify the presence of OTA concentration in body. They could be suggested as helpful parameter for the correlation study of diet exposure and cell/tissue damage in body. Plasma/serum analysis can confirm the long-term exposure to OTA, for high half-life, even though the exposure at low levels. Whereas the milk and urine sample confirm the exposure that is not very longer .The multi-biomarker analysis is rapid method to analyze the early and late OTA exposure (Malir, Ostry, Pfohl-Leszkowicz, Malir & Toman. 2016).

Concerning OTA exposure, a study performed on German and Turkish population assessed the infant exposure to OTA by determining the concentration of OTA in maternal plasma, breast milk and infants' urine samples. All maternal plasma samples analysed in the period of 2 weeks–4 months of breastfeeding period were positive for OTA with concentrations that ranged between 0.072 and 0.573 ng/ml. The average concentration that was found in breast milk and infants' urine was several times lower than OTA concentration in plasma samples with average values at 4

months of breastfeeding period 0.030 ng/mL and 0.036 ng/ml (Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019)

Pilot study conducted in Belgium analysed, as biomarkers of human OTA exposure, OTA and 4-OH OTA in 40 human urine samples; they found the presence of both compounds in only one sample, where the concentration of 4-OH OTA was lower than the LOQ concentration (< 0.24 ng/ml) and OTA concentration was 0.6 ng/mL. Furthermore, three samples contained OTα concentrations 5.1 ng/mL, 7.0 ng/mL and 15 ng/mL (Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019).

Human exposure to several mycotoxins takes place through the consumption of contaminated food such as cereals. It can also occur through the ingestion of products of animal origin, such as eggs and milk, if the animals have previously been fed with contaminated feed. For this reason the humans should be exposed to different class of mycotoxins. Structurally, mycotoxins can occur in three possible forms. “Unmodified” forms are biosynthesized by fungal metabolism (e.g., OTA, Aflatoxin B1, Zearalenone , Deoxynivalenol, fumonisin B1 , Patulin) and refer to the basic or free forms of mycotoxin structures. “Matrix-associated” mycotoxins form complexes with matrix compounds. Examples include Fumonisins bound to proteins and OTA bound to polysaccharides. Finally, “modified” mycotoxins have undergone chemical or biological modification to their structure (Arce-López, Lizarraga, Vettorazzi & González-Peñas. 2020).

Aflatoxins are produced by *Aspergillus* species. In the exposure study in rats, Aflatoxin B1 (AFB1), AFP1 and AFM1 were detected in urine. Urinary AFB1 metabolites, such as AFM1, AFP1, and AFQ1, Aflatoxin glucuronide, AFB1-N7-Gua and AFB1-mercapturic acid were used as biomarkers to assess the exposure of aflatoxins. As AFM1 can be detected in human breast milk it can also serve as a biomarker of maternal and infant exposure to AFB1. Based on human and animal studies, AFB1-N7-Gua adduct in urine is considered the most reliable short term biomarker, with a half-life of 7.5 h, for evaluating hazards and exposure to carcinogenic AFB1(Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019).

Fumonisins are produced by diverse *Fusarium* fungi species. Biomarkers of fumonisin B1 (FB1) in humans were detected in hair, nails, blood serum, urine and stool. Urinary biomarkers of fumonisins are FB1, FB2 and FB3 (Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019).

Zearalenone (ZEN), 6-(10-Hydroxy-6-oxo-trans-1-undecenyl)-betaresorcylic acid lactone, is produced by different species of *Fusarium*. Free ZEN and ZEN metabolites detected in humans are ZEN, α-Zearalenol (ZEL), β-ZEL, α-Zearalanolo (ZAL), β-ZAL, ZEN14GlcA. To date,

zearalanone (ZAN), a derivative of zearalenone that is produced by several species of *Fusarium*, has not been detected in the human body. The studies performed in pigs showed that ZEN metabolites can mainly be detected 24 and 48 h post administration in urine and faeces. Interestingly some biomonitoring studies in humans that investigated the presence of ZEN and its metabolites (Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019).

Deoxynivalenol (DON) that belongs to trichothecene group of mycotoxins. Human exposure to DON is monitored by analyzing the free or bioconjugated forms of DON such as DON-GlcA, DON-15-GlcA, DON-3- O-glucoside (DON3GlcA), DON-7-GlcA, DOM-1 and DON-3-GlcA. Biomonitoring studies in human reported higher incidence of glucuronidated forms of DON in urine, especially DON-15-GlcA, compared to free DON (Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019).

Studies in African countries assessed the occurrence of different mycotoxins, such as AFB1, FB1, OTA, ZEA and DON biomarkers in human biological fluids. Most of the biomonitoring studies originate from Tunisia and Nigeria reported the presence of mycotoxin biomarkers in urine, blood, blood components and maternal breast milk were the most commonly investigated matrix (Al-Jaal, Jaganjac, Barcaru, Horvatovich & Latiff. 2019). Table 1.3 showed different surveys performed in different European countries/years.

Table 1.3 Studies on Ochratoxin A during human biological monitoring (Arce-López, Lizarraga, Vettorazzi, & González-Peñas, 2020)

Country (year)	Analyte	Matrix	Total Samples	Positive Samples (%)	Detection Technique	Mean (µg/L) and/or [Range] (µg/L or pg/mg Albumin *)
China (2020)	OTA FB1 DON ZEA ZAN	Plasma	260	27.7 2.7 2.3 6.5 1.2	LC-MS/MS	1.21 [0.312–9.18] 0.69 [0.305–0.993] 2.60 [1.39–5.53] 0.16 [0.063–0.418] 0.26 [0.164–0.346]
Italy (2019)	GLIO OTA	Serum	110	31/55/33 33/65/74 74	LC-FLD	0.24//0.41/0.27 0.40/0.36/0.65
China (2018)	AFB1 AFB2 AFG1 AFG2 AFM1 STER CIT FB1 FB2 OTA	Plasma	60	13/33 17/23 3/3 3/3 3/0 13/40 0/3 3/7 3/3 0/3	LC-MS/MS	[0.95–1.78]/[1.23–4.56] [1.37–3.89]/[1.16–3.75] 0.61/0.55 0.43/0.46 0.57/n.d. [0.88–2.05]/[1.06–3.23] n.d./0.63 1.92/[1.35–2.78]2.03/1.57 n.d./0.83
Italy (2017)	AFB1 AFM1 DON DOM-1 FB1	Serum	213	22.9 50.2 19.5 13.1 13.7	LC-MS/MS	0.01 [0–0.73] 0.11 [0–1.91] 1.0 [0–27.9] 0.3 [0–12.7] 0.7 [0–5.6]

	GLIO OTA ZEA			21.2 82.9 5.4		2.3 [0–28.4] 0.36 [0–1.76] 0.1 [0–3.9]
Portugal (2018)	OTA 2'R-OTA EnB	Serum	42	100 81 100	LC-MS/MS	0.76 [0.36–4.99] 0.32 [0.08–0.51] 0.048 [0.01–0.15
Germany (2017)	OTA EnB	Blood	50	100 100	LC-MS/MS	0.204
Germany (2019)	OTA	Blood	16	100	LC-MS/MS	0.157 [0.079–0.262]
Czech Republic (2019)	OTA	Serum	50	48	LC-FLD	0.14 [LOD–0.83]
Bangladesh (2018)	OTA	Plasma	104	10098	LC-FLD	0.72 [LOD–6.63]0.38
Egypt (2016)	OTA	Serum	98	81.6	LC-FLD	0.33 [0.20–1.53]
Italy (2016)	OTA	Serum	50	54.8/44.2	LC-FLD	0.26/0.27
Germany (2016)	OTA	Blood	50	100	LC-MS/MS	0.211 [0.071–0.383]
Germany (2015)	OTA 2'R-OTA	Blood	34	100 100	LC-MS/MS	0.21 [0.071–0.383] 0.11 [0.021–0.414]

Key: *: pg/mg albumin, n.d: not detected, not indicated; 10-OH-OTA: 10-hydroxyochratoxin A; 2'R-OTA: 2'R-ochratoxin, LOQ: limit of quantification; OTα: ochratoxin α; OTA: ochratoxin A

Concerning the exposure of farm animals, in different regions of Sweden, determination of urinary biomarkers of DON, FB1, OTA, AFB1 and ZEN was performed, A multiple mycotoxin exposure was detected in all samples and the more frequent mycotoxin combinations were DON + ZEN + OTA followed by total DON + FB1 + ZEN + OTA. Urinary biomarker concentrations were used to estimate mycotoxin levels in feeds consumed by pigs. The overall mycotoxin levels in feeds were lower the European limits with the exception of 4% of the samples that were found to be contaminated with levels of either DON, OTA or AFB1 that were higher than the recommended/regulatory limits. Within the three regions monitored in this study (West, South and East), the pigs bred in the West region were more exposed to DON and ZEA compared to pigs in the South and East regions. Monitoring of urinary mycotoxin biomarkers was quite effective to assess pig exposure to mycotoxins, the mycotoxin levels in consumed grains and to identify the regions at higher risk for mycotoxin accumulation in the grains produced (Gambacorta, Olsen & Solfrizzo. 2019). Of course, the exposure to multiple mycotoxins of humans and farm animals, even if at low levels, could have serious repercussions on health due to a possible interaction of mycotoxins with each other, not yet carefully studied

1.2 THE SHEEP AS A TRANSLATIONAL IN VITRO ANIMAL MODEL

1.2.1 Reproductive physiology of the ewe

The ewe is a seasonal breeder, having polyestral seasonal reproductive activity linked to the negative photoperiod, in response to the shortening day length. Thus, in the northern hemisphere, estrous cycles in ewes occur from late summer (September) to early spring (March). Photoperiod also plays an important role in the onset of puberty (Downey, 1980; Noakes Parkinson, England, & Arthur. 2001).

The extent of the breeding season diminishes with increase of latitude. Thus, at the equator, ewes may breed at any time of the year, whereas in regions of high latitude – in both northern and southern hemispheres - the breeding season is restricted and distinct, with a prolonged phase of anoestrus after parturition. The breed of the ewe also influences the duration of the breeding season. Artificial manipulation of the photoperiod with the use of hormonal agents can help to advance the seasonal onset of reproductive activity.

The average length of the oestrous cycle in the sheep is 17 days. Estrous, the time during which mating behaviour is exhibited, lasts 24-36 hr in the ewe and is variable in length in different sheep breeds. The average duration of oestrus in mature ewes of British breeds is about 30 hours, and is at least 10 hours less in immature ewes. In Merinos, heat may last 48 hours (Noakes, Parkinson, England, & Arthur. 2001).

1.2.1.1 Estrous cycle

The estrous cycle is defined as the number of days between the start of two different periods of estrous, or heat. A complex regulatory system, involving a number of hormones, control the estrous cycle in domestic ruminants. The hypothalamic neurosecretion leads to the production of specific releasing (e.g., gonadotropin releasing hormone [GnRH]) and inhibiting factors. More recently, the role of kisspeptin in controlling GnRH secretion in sheep has been investigated (Smith, Geisert & Parrish. 2018).

GnRH is involved in the release of major gonadotrophin hormones from the anterior pituitary gland. The first occurring hormone, the follicle stimulating hormone (FSH), is involved in follicular growth and development, by exerting mitogenic activity on follicular cells and, in turn, it stimulates estrogens production within follicles. The second hormone, the luteinizing hormone (LH) plays the

primary role in the regulation meiosis resumption, follicular rupture and ovulation and initiates luteinization of follicular cells. It is the principal luteotropin responsible for the maintenance of corpus luteum. The third hormone, prolactin, apart its specific roles, is also involved in maintenance of the corpus luteum function (Downey, 1980; Noakes, Parkinson, England, & Arthur. 2001).

The ovarian hormones estrogens and progesterone have both inhibitory and stimulatory effects, at the anterior pituitary and the hypothalamic levels, and they significantly alter the magnitude of the GnRH effect. Estrogens are produced by granulosa cells in growing follicles whereas progesterone is produced in luteinic cells. To correlate with the estrous cycle, an increase in estrogen concentration in plasma preceeds the pre-ovulatory surge of LH and it appears to augment the pituitary responsiveness to GnRH. Conversely, the large amounts of progesterone, which is secreted after ovulation, may lower this response (Downey, 1980; Noakes, Parkinson, England, & Arthur. 2001). Figure 1.4 describes the hypothalamic–pituitary–gonadal axis (HPG-axis) loop in the female sheep, detailing feedback and interactions between the hormones and structures involved in its pathways.

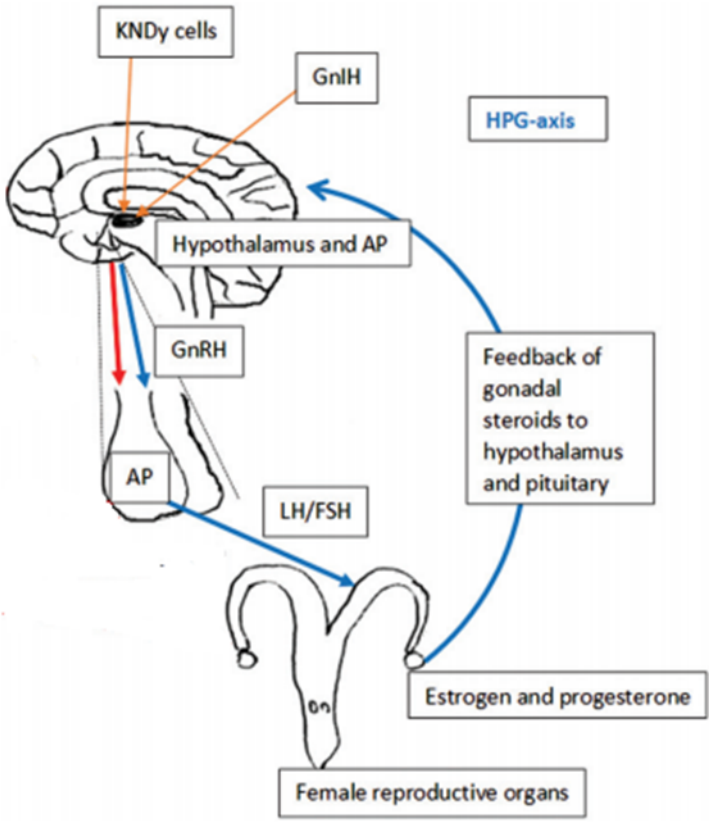

Figure 1.4. Hypothalamic–pituitary–gonadal axis (HPG-axis) loop in the female sheep, detailing feedback and interactions between the hormones and structures involved in its pathways. Kisspeptin/neurokinin B/dynorphin (KNDy) cells involved in generation of GnRH produced by hypothalamic neurons and it reaches the anterior pituitary gland (AP) where it stimulates FSH secretion. FSH targets the ovarian granulosa cells within the follicle. As response, in the follicle, estrogens are produced. These hormones act with a feedback mechanism on hypothalamus. In response to this feedback, there is a switch of AP activity from FSH to LH secretion. LH controls oocyte meiosis resumption, ovulation and corpus luteum formation. After ovulation, progesterone is produced in the corpus luteum and it acts on uterine endometrium and, with feedback mechanisms, on the hypothalamus (Narayan, Parisella. 2017).

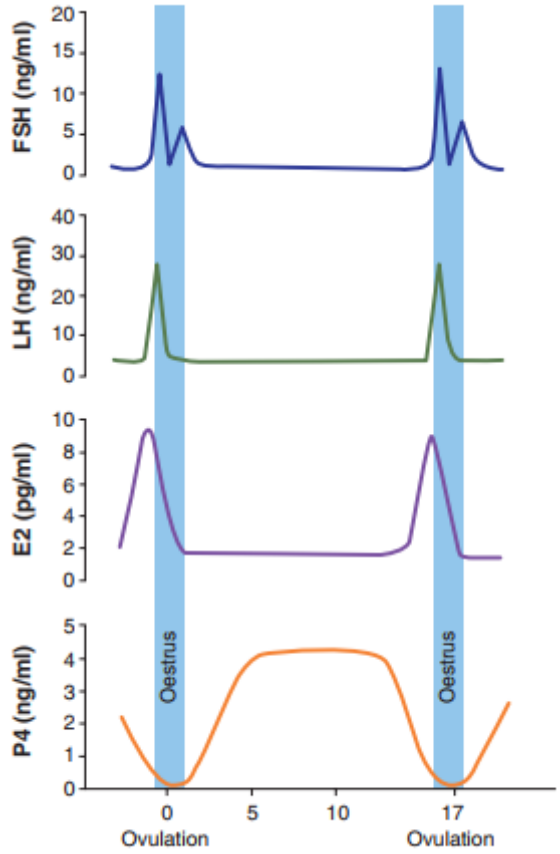

Figure 1.5. Schematic representation of the estrous cycle of the ewe (Downey, 1980; Noakes, Parkinson, England, & Arthur. 2001)

1.2.2 Background on assisted reproductive technologies in the sheep

In the sheep, two critical factors affecting the overall efficiency of in vivo embryo production (IVEP) are the large variation in the response to superovulation treatments and the traumatic surgical procedure of embryo recovery. In vitro embryo production can allow to overcome some of these limitations because superovulation is not always required and oocytes are recovered directly from the follicles in hormonally stimulated or non-stimulated females by laparoscopic ovum pick-up (LOPU). LOPU is a less traumatic intervention than laparotomy. Furthermore, IVEP allows the production of progeny from non-fertile females, prepubertal, pregnant, lactating, and even dead or slaughtered females (Paramio, & Izquierdo. 2016)

In vitro maturation

In vitro maturation (IVM) is the most critical part of the whole process of in vitro embryo production. For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. Due to the heterogeneous nature of immature oocytes, as they arise from a wide range of follicle stages, their response to the same IVM condition are different and unpredictable. In any way, the percentage of oocytes that resume nuclear maturation and reach the metaphase II stage is around 80% in the sheep (Paramio, & Izquierdo. 2014).

Conventionally sheep oocytes are in vitro matured in groups (25–50 oocytes with a ratio of 1 oocyte/2–5 µl medium) and incubated at 38–39°C in humidified atmosphere of 5% CO2 in air for 24–27 h. TCM199 medium, with bicarbonate buffer, mineral salts, carbon and energy sources (glucose, lactate and pyruvate) as well as vitamins and amino acids supplemented with L-glutamine, hormones (FSH, LH, 17b-E2,) plus complex fluids such as heat-treated serum and/or follicular fluid recovered from healthy non-atretic follicles is the most widely used medium for IVM. The atmospheric conditions of the IVM are 5% CO2 in air. This means that there are 3–4 times more O2 than in the oviduct, resulting in increased reactive oxygen species (ROS) production in the in vitro culture, which could be harmful for oocytes (Paramio, & Izquierdo. 2014; Zhu, Moawad, Wang, Li, Ren, Dai. 2018).

Gonadotrophin hormones and 17b-estradiol are used in IVM protocols to improve nuclear and cytoplasmic oocyte maturation as well as expansion of the surrounding cumulus cells in ovine. Gonadotrophin concentration commonly added to IVM medium are 5 µg/ml FSH, 5 µg/ml LH and 17beta – estradiol is added at 1 µg/ml in the sheep (Paramio, & Izquierdo. 2014; Zhu, Moawad, Wang, Li, Ren, Dai. 2018).

Conventionally, IVM media are supplemented with Serum and/or Follicular Fluid (FF), both of them with unknown composition. Their action is not fully understood, but it is believed that they provide proteins and/or some growth factors that contribute to the success of IVM and subsequent development. Despite the undefined and variable nature of serum composition, this supplementation is widely generalized in a conventional concentration of 10–20% (Paramio, & Izquierdo. 2014).

Growth factors are also added to IVM to improve embryo results. Epidermal growth factor (EGF) is the most widely used. Thus, EGF is routinely added to IVM medium plus serum in sheep IVM. The positive influence of growth hormone (GH) on oocyte maturation has been reported. In ovine IVM, addition of 300 ng/ml GH in serum-containing maturation medium during IVM has significantly

increased embryo development in terms of cleavage, blastocyst and hatching rates compared to the control (Paramio, & Izquierdo. 2014; Zhu, Moawad, Wang, Li, Ren, Dai. 2018).

Different laboratories use different protocols according to their specific oocyte quality. Thus, testing three different IVM protocols showed no differences in blastocyst production between (i) defined medium (TCM199 plus cysteamine plus EGF), (ii) TCM199 with FCS and (iii) complex medium TCM199 with serum, hormones, EGF, Insulin Like Growth Factor, vitamins, thiol compounds and ITS with 46% and 45% and 45% of blastocysts respectively. Nevertheless, the authors of these studies used 10% Oestrus Sheep Serum (OSS) in the fertilization medium and 10% FCS for embryo culture medium. IVM has significantly increased embryo development in terms of cleavage, blastocyst and hatching rates compared to the control (Paramio, & Izquierdo. 2014).

In vitro fertilization.

In vitro fertilization (IVF) is a complex procedure whose success requires appropriate oocyte maturation, sperm selection, sperm capacitation and IVF media. The first step is to select the motile and viable spermatozoa from fresh ejaculate or frozen-thawed sperm. The most common method used for separating the sample into motile and non-motile fractions from fresh ejaculate is the swim-up technique. An aliquot of sperm suspension (50–100 µl) is layered under 2 ml of capacitation medium in several tubes at 38.5°C and after 0.5–1 h the top medium (0.4–1 ml) is collected from each tube. The pooled medium containing highly motile spermatozoa is centrifuged (200 x g) for 10 min (Paramio, & Izquierdo. 2016).

For frozen-thawed semen, conventionally, motile spermatozoa are also obtained by centrifugation on a discontinuous Percoll gradient. Normally, two densities (45% and 90%) of Percoll solutions are used. The gradient is formed by pipetting 1.5 ml of 90% Percoll solution into a 15 ml conical tube and then overlaying it with 1.5 ml of 45% Percoll solution. Semen is placed over the top of the 45% layer and then centrifuged at 600–1000x g at room temperature for 10–15 min through the gradient. After removal of supernatant, the resulting pellet is washed twice by centrifugation and resuspension in 0.5–2 ml of medium is performed (Paramio, & Izquierdo. 2014).

Once the most viable and motile spermatozoa are selected, sperm capacitation is carried out in vitro. Several capacitating agents have been used to capacitate spermatozoa and to yield good fertilization and cleavage rates.

In mammals, freshly ejaculated spermatozoa cannot fertilize mature oocytes. Rather they become competent to sustain fertilization in the female genital tract, through a time-dependent process

called “sperm capacitation”. First described by Austin (1952) and Chang (1951), capacitation is a complex set of modifications that sperm undergo in the female reproductive tract whereby sperm acquire the ability to fertilize an oocyte. The duration of capacitation varies among species and ranges from 1–2 h in sheep and rat to 10–12 h in ferret. During capacitation, several changes have been described, particularly at sperm membrane level, such as increase in membrane fluidity, lateral movement of cholesterol to the apical region of the sperm head, and cholesterol displacement from the plasma membrane to the extracellular environment. During capacitation, sperm undergo a change in the motility pattern called hyperactivation and become competent to undergo a physiological secretory event known as acrosome reaction (Puga Molina, Luque, Balestrini, Marín-Briggiler, Romarowski, & Buffone. 2018). Capacitation occurs in vitro in defined media whose compositions are based on the electrolyte concentration of oviductal fluid. Typically, capacitation media contain energy substrates (e.g. pyruvate, lactate and glucose), a cholesterol acceptor (usually serum albumin), NaHCO3, Ca2+, low K+ and physiological Na+, some of which play an important role in the capacitation process (Grasa, Cebrián-Pérez & Muiño-Blanco. 2006).

The fertilization media mostly used in ovine IVF is the Synthetic Oviductal Fluid (SOF) medium. For IVF, groups of 15–30 oocytes are transferred to drops of 50–100 µl of IVF medium where are inseminated. The final sperm concentration used in the IVF drop is 1 x 106 spermatozoa/ml but it can vary from 0.5 x106 cells/ml to 12–15 x 106 cells/ ml depending on the male and IVF system used. Sperm and oocytes are incubated for 16–24 h at 38–39°C. Concerning the atmosphere used during IVF, most laboratories use a humidified atmosphere of 5% CO2 in air. Low oxygen atmosphere during the IVF period improves the number of high quality sheep blastocysts as evaluated through kinetic development to blastocyst stage. Semen can be sex sorted before IVF (Paramio, & Izquierdo. 2014).

In vitro embryo culture.

The last step of IVEP is the culture of the presumptive zygotes to reach the stage of blastocysts at 6–8 days after in vitro fertilization in ruminant. The post-fertilization culture is the period having the greatest impact on the blastocyst quality. The success of IVEP depends on various factors, such as osmolarity and ionic medium composition, temperature, pH and CO2, oxygen, carbohydrates, amino acids, lipids and fatty acids, proteins, growth factors and cytokines. Any deviation from the appropriate environment could lead to embryonic arrest at any stage of development (Paramio, & Izquierdo. 2014).

Embryonic genome activation (EGA), which occurs at the 8- to 16-cell stage in ruminants, coincides with an increase in metabolic activity, oxygen consumption and uptake of carbohydrates to the blastocyst stage. Thus, in vitro cultured early embryos may fail to develop across 8- to 16-cell stages in traditional culture media. For this reason, the co-culture with epithelial oviduct cells or with other cell monolayers could support sheep embryos.

Co-culture with somatic cells have several disadvantages such as contamination risks. Moreover, the results are not predictable because of the unknown physiological status of the cells. To avoid the inconveniences of co-culture in atmospheres with 20% O2, strategy are adopted for decreasing oxidative stress and oxidative damage. Thus IVEP can be performed under 5%O2, 5%CO2 and 90%N2 atmosphere. As well, natural or synthetic antioxidants can be added to the media (Martino et al., 2016; Paramio, & Izquierdo. 2014).

Different culture media have been successfully used for small ruminant embryo development such as TCM 199 and 'Sydney IVF Blastocyst' medium. However, the most widely used medium is the synthetic oviduct fluid (SOF) (Tervit, Whittingham, Rowson. 1972). Ruminant zygotes is successfully cultured in vitro leading to the blastocyst stage using SOF medium, based on the composition of ovine oviduct fluid.

The progress in understanding of the requirements of the developing embryo resulted in the development of sequential media where components change according to the needs of the embryo. These sequential media would mimic the change in environment experienced by the developing embryo in vivo, enabling the biochemical and morphological changes of embryos. Thus, physiological sequential media are formulated to reflect the carbohydrate levels of the reproductive tract and reduce cellular stress on the embryo (Paramio, & Izquierdo. 2014). Embryos are cultured in groups. In fact, most researchers report higher blastocyst rates and improvement embryo quality following group, compared to single culture, because oocytes and embryos stimulate reciprocally during their in vitro development (Paramio, & Izquierdo. 2014). In sheep, routinely presumptive zygotes are cultured for 8 days after insemination in an atmosphere of 5% O2 using SOF medium plus serum. Addition of serum increases blastocyst yield because its effect in the mitosis activation but also serum have a significant effect on the incidence of chromosomal abnormalities in the resulting blastocysts (Paramio & Izquierdo. 2014).

Efficiency of in vitro production of embryo in the sheep

In 1986, for the first time in vitro production of embryos by IVF and in vitro embryo culture and embryo transfer with offspring was successfully reported in Cambridge – UK (Cheng, Moor &

Polge. 1986). In vitro embryo production is considered as a trustworthy tool in the field of animal reproduction to reduce the problem of infertility and to upgrade the extent of reproduction in humans and animals. This technique has vast application as a treatment for reproduction failure and preservation of breeds with high genetic impacts. In the current era the negligence of In-vitro lab techniques is not possible. Reported in a study from the last five year 70-90% immature oocyte undergo maturation, 50-80% undergo fertilization, and only 20% to 50% of immature oocytes ever reach the blastocyst stage. Table 1.4 ~~6~~ illustrates the impact of IVEP in Italy and Europe during the last five years (data from Zhu, Moawad, Wang, Li, Ren, Dai. 2018).

Table 1.4 Summary of the ovine embryo in vitro production in Italy and European countries (Zhu, Moawad, Wang, Li, Ren, Dai, 2018)

Year	Countries	Season	Sheep Age	Sperm Type	Duration of IVF	Fertilization Rate (%)	Blastocyst rate (%)
2016	Italy	N/A	4-6	Frozen	22	74.5	59.2
2016	Italy	Breeding	30-40 days	Fresh	22	77.8	35.8
2013	Italy	Breeding	N/A	Fresh	20	80	42
2014	Uruguay	N/A	N/A	Frozen	22	79	41.3
2014	Spain	N/A	≥3m	Frozen	24	77.7	15.7
2014	Spain	Non Breeding/ Breeding	3-6m	Frozen	20	75.9	31.7
2013	Spain	Breeding	Adult	Fresh	20-24	87.8	37.7
2013	Spain	N/A	4 year	Frozen	24	52	N/A
2012	Spain	N/A	3-6m	Frozen	20	N/A	24.1
2012	Portugal	N/A	N/A	Frozen, Fresh	18	45	42.8

Juvenile In Vitro Embryo Production and Transfer (JIVET)

The incorporation of Juvenile in vitro Embryo Technology (JIVET) into breeding programs is advantageous because it can reduce the generation interval and increase the rate of genetic gain. Another advantage of oocyte collection from prepubertal ovaries is the early availability of large numbers of oocytes per female. Thus, this technology would allow a fast multiplication of unique and expensive animals such as endangered species. Nevertheless, the greatest limitation of JIVET is that the production of embryos and their development to term after transferring to recipient females is lower in comparison to their adult counterparts (Paramio & Izquierdo. 2014).

Low embryo development of oocytes obtained from prepubertal females might be caused because in the ovaries lower number of follicles larger than 3 mm are present. The direct relationship between follicle sizes, oocyte diameter and embryo development is well-known. Studies have

shown ultrastructural and functional deficiencies in prepubertal goat oocytes such as altered distribution of cortical granules and mitochondria, disorganization of microtubules and microfilaments and alteration in total RNA content, p34 (cdc2) and cyclin B1 expression and maturation promoting factor (MPF) activity (Paramio & Izquierdo. 2014).

Ovaries from prepubertal animals have a high percentage of antral follicles with a diameter smaller than 3 mm, making it difficult to release oocytes by follicular aspiration or select oocytes by their follicular diameter as is conventionally done in adult ovaries. Prepubertal female oocytes are routinely obtained by slicing the ovary surface recovering a pool of oocytes with a heterogeneous degree of growth and atresia and coming from unknown follicles. In this case, oocytes are selected by diameter and the morphological appearance of cumulus cells and oocyte cytoplasm.

Assessment of oocyte diameter is a time-consuming procedure which affects IVEP output because the long exposure of oocytes to light and harmful atmosphere. In order to select the larger and more competent oocytes avoiding these inconveniences, oocytes can be tested with Brilliant Cresyl Blue (BCB) staining to select fully-grown oocytes (Paramio & Izquierdo. 2014). Staining is based on BCB ability of the dye to be reduced by glucose-6-phosphate dehydrogenase (G6PDH) activity. Oocytes that have reached their growth phase and have low G6PDH activity cannot reduce BCB to a color less compound. Thus they exhibit a blue-colored cytoplasm (BCB+) whereas growing oocytes with high G6PDH activity are able to reduce the blue compound, which results in a colourless oocyte cytoplasm (BCB-). The absence of enzymatic activity of G6PD can be an indirect measure of fully-grown oocytes that have concluded their intraovarian growth phase (Paramio & Izquierdo. 2014).

Variability in IVEP output has also been observed according to season. IVEP programme concluding that the percentage of blastocysts was higher during the breeding season, but there were no differences in pregnancy and lambing rates among blastocysts produced throughout the year. In vitro blastocyst production in sheep is highly variable with many factors affecting these results. Assessment of the oocyte competence is one of the key points in IVEP programs (Paramio, & Izquierdo. 2014).

JIVET efficiency in the ovine species

Juvenile In vitro embryo transfer (JIVET) has a remarkable role in shortening generation interval, an increase in the rate of the genetic gain, and a reduction in the rate of interbreeding. JIVET gain commercial viability in the 1990s, donors selected for JIVET were evaluated on the bases of estimated breeding values (EBV). Besides the limitations to the JIVET technique the blastocyst

formation rate is stimulated to 40% in prepubertal lambs and ewes. Outcomes from JIVET can be enhanced by genomic selection. It enhances the multiplication of unique and expensive animals such as endangered species. Nevertheless, JIVET also has the greatest limitation in the shape of healthy embryos production and their development after transferring to recipient females (Granleese, Clark, Swan, & van der Werf. 2015).

Female reproductive technologies such as JIVET can boost rates of genetic gain but they can also increase rates of inbreeding. The competence of livestock breeding programs is improved by the use of JIVET as it increases the genetic gain, also it raises selection intensity placed on females also the decline in the optimum age limit of animal selection, thus leading to the reduction in the generation gap. However, JIVET has put forth several improvements in the animal breeding programs worldwide (Granleese, Clark, Swan, & van der Werf. 2015).

Cryopreservation of oocytes and embryos in the sheep

Cryopreservation is a storing technique used for biological materials, to preserve their viability for longer time duration with the help of cryopreservative agents and cooling temperature. Successful cryopreservation of sperm was first reported in 1949 (Polge, Smith, Parkes. 1949). Earlier cryopreservation of embryos and somatic was not successful. The physiology of these cells was a limitation as they were much bigger than the sperm cells. The water content of these cells was higher and proper dehydration was required to prevent ice crystal damage. In 1972 successful cryopreservation of mouse embryos was reported (Moore & Hasler. 2017). The major factor that made this technique successful for mammalians was to cool embryos with a first step from around 25°C to -7°C at a rate of -3/-5°C/min (rapid freezing) followed by seeding, and by a second step from around -7°C to -30 to -35°C at a rate of 0.5°C/min (slow freezing). The cooling method gives sufficient time to cells and enable them to get rid of surplus water osmotically. In result, the rate of intercellular ice crystal damage is decreased significantly. Later on further advancements were attained such as to drop the cooled (-30 to -35°C) embryos into liquid nitrogen before the cells get extremely dehydrated (Moore & Hasler. 2017)

Similarly to remove the cryoprotectant from cells after thawing was also a significant step to attain the maximum benefits from this technique. Essentially it is important the removal of cryoprotectant in a way that reduces the possibility of osmotic swelling. Today, cryopreservation of embryos is a very large component of Embryo transfer (ET) programs. Dimethyl sulfoxide was the cryoprotectant most often used for freezing embryos until it was replaced by glycerol in the early

1980s. The uptake of direct transfer with ethylene glycol as efficient cryoprotectant was rapid and had a positive effect on the ET industry (Moore & Hasler. 2017).

Conclusion

Assisted reproductive technologies in the sheep had greater impact on the percentage of the blastocyst formation rate. The blastocyst formation rate was higher in the breeding season but the rate of pregnancy and lamb production was remain constant throughout the year. Despite these techniques in past few decades decline the limitation to the breeding techniques in sheep but still number of factors need a room of improvement. As we know that a lot of factors are involved to attain the required results assessment of oocyte competence should be given importance to enhance the output.

1.3 THE DONKEY AS A TRANSLATIONAL IN VIVO ANIMAL MODEL

1.3.1 Reproductive physiology of the jenny

In the donkey, the reproductive activity seems to be less affected by the season. The month of conception was related to pregnancy length (PL), longer pregnancies were observed for conceptions that occurred at the beginning of the season, and decreasing subsequently. In the Northern Hemisphere, shortest gestation was reported when foaling occurs in January and the longest in April. The PL, defined as the time (days) between ovulation and the day of parturition, was recorded after foaling. the possible role of the environmental conditions (temperature and natural lighting) on estrous cycle length and reproductive endocrinology of the jenny and in seasonal anestrus onset, is still not completely investigated (Contri, Robbe, Gloria, De Amicis, Veronesi, & Carluccio 2014; Carluccio, Gloria, Veronesi, De Amicis, Noto, & Contri. 2015).

A study conducted in Teramo (Italy) put forth that no significant influence of age on PL was observed in Italian Martina Franca (MF) Jennies. The wide range in the MF donkey PL (333–395 days) suggested that other factors than gender could affect this parameter. Some of these factors could be related to the stallion or the jenny; however, these genetic parameters were not evaluated (Carluccio, Gloria, Veronesi, De Amicis, Noto, & Contri. 2015).

1.3.1.1 Estrous cycles

Estrous cycle is considered as the time elapsing between two consecutive ovulations, detected using ultrasonography. The day of the behavioral willingness to mating (heat) was considered the Day 1 of the estrus (Ed1). The day when the jenny refused the jackass was considered the Day 1 of the diestrus (Dd1). Thus, the estrus time was defined as the time, in days, between Ed1 to Dd1. All jennies (100%) showed estrous cycles along the whole year; heats were observed also in winter, and always coupled to ovulation. (Contri, Robbe, Gloria, De Amicis, Veronesi, & Carluccio. 2014).

Puberty, understood as the appearance of the first estrous cycle, is reached around 12-14 months. Nonetheless, it is preferable to wait 30 months to start the reproductive career, to guarantee a correct and complete body development of the future donkey.

The mean estrous cycle in the MF jennies consisted of 23.3± 0.8 days during the whole year, with similar values throughout different seasons. On average, the estrous length was 6.5 ± 0.6 days

and it was significantly longer in summer (7.1± 0.4 days) as compared to autumn (6.2± 0.6 days) and winter (6.1± 0.4 days). Estrous in spring was significantly longer than in winter. The mean duration of the diestruos during the whole year in MF Jennie was 16.8± 0.6 days. A significant reduction in diestrous length was reported in the summer compared with the other seasons. The diestrous was not significantly different among the other seasons (Contri, Robbe, Gloria, De Amicis, Veronesi, & Carluccio. 2014).

In estrus, the jenny displays signs, such as standing to be mounted by another female or male, mouth clapping, clitoral winking, urinating, and tail raising.

In donkeys progesterone concentration remains low up to the day after ovulation, it slowly increases until 4 to 6 days post-ovulation, plateaus by days 14 to 16 post-ovulation and then starts to decline to reach baseline concentrations (<1 ng/mL) in 2 days. 17b-estradiol increases from 10 pg/mL during early estrus to peak around 40 to 60 pg/mL at ovulation (Canisso, Panzani, Miró, & Ellerbrock. 2019).

Prostaglandin F2alpha (PGF2a) and its analogs have been used in the mare to manipulate ovarian function for several purposes, but mainly as a luteolysin to induce and/or synchronize estrus for breeding. Minimal effective dose in mares when administered 5 days after ovulation or later is 9 mg/kg PGF2a (Carluccio, Panzani, Contri, Tosi, De Amicis, & Veronesi. 2008).

Follicle-stimulating hormone concentrations remain low throughout the estrous cycle, and reach the peak 3 - 9 days post-ovulation. Luteinizing hormone (LH) concentrations increase pre-ovulation, peak 2 days after ovulation, and then decrease to baseline throughout the remainder of the estrous cycle. Follicular deviation occurs 8 to 9 days before ovulation at approximately 19 to 20 mm follicular diameter. In most donkeys the dominant follicle grows 2 to 3 mm/d from deviation to ovulation; however, in the large breed Catalonian donkeys, follicles grow up to 4 mm/d. Jennies have 1 follicular wave during the estrous cycle, ovulatory diameter varies from 30 to 48 mm with a positive association between body frame (Canisso, Panzani, Miró & Ellerbrock. 2019).

Several hormones can be used to induce ovulation, including human Chorionic Gonadotrophin (hCG), Crude Equine Gonadotrophin (CEG), and Gonadotrophin Releasing Hormone (GnRH). The most commonly used hormone is hCG, and several studies have assessed its efficacy for this purpose in cycling donkeys. More than 90% of ovulations occurred within 48h of treatment with 2000 IU hCG when a follicle of at least 35 mm diameter had been detected. It is recommend that donkeys be treated with hCG no more than twice during a breeding season. Because this would be unsuitable in embryo transfer programs and for some other reproductive

management goals, alternative ovulation induction (Carluccio, Panzani, Tosi, Faustini, De Amicis, & Veronesi. 2007).

1.3.1.2 Pregnancy and parturition

Gestation length is approximately 12 months (331–421 days). As in all equids, donkey placentation is diffuse, epitheliochorial, and non-invasive. The donkey chorioallantois has a higher concentration of microcotyledons per area when compared with the mare owing to extensive branching of the villi. This feature makes the donkey placenta more efficient and may explain why it is relatively more common for jennies to deliver live twins than mares. Pregnancy diagnosis may be possible by transrectal ultrasonography starting 9 days after ovulation, although the chance of detecting a pregnancy so early is just 9% to 33% (Canisso, Panzani, Miró, & Ellerbrock. 2019).

The presence of the conceptus blocks and the synthesis of PGF2α by the uterus. In this way, CL continues to produce progesterone, necessary for the maintenance of gestation. The mechanisms of maternal-fetal recognition are not yet well known in equid: an important role seems to be attributed to the movements of the zygote in utero in the days preceding nesting. However, some studies suggest that trophoblastic invasion may be implicated in this process (Klein, & Troedsson. 2011).

In clinical practice, the first pregnancy diagnosis should be performed 12 to 15 days after ovulation. Maternal recognition of pregnancy in donkeys seems to be similar to horses, with the embryonic vesicle mobile in the uterine lumen until day 16 post-ovulation, at which point the embryonic vesicle fixes at the base of a uterine horn. The embryonic vesicle starts losing its spherical shape around days 16 to 18, and the embryo proper appears at the ventral pole of the vesicle around days 19 to 21(Canisso, Panzani, Miró, & Ellerbrock. 2019).

While there have been no studies assessing the fetal heart rates in donkeys or hybrid fetuses under physiological and pathological conditions, current study reported a tendency for donkey fetal heart rate to increase the week before parturition. Assessment of the caudal placental pole (e.g., edema, intracervical fluid accumulation, and combined thickness of uterus and placenta (CTUP) has been used to assess equine pregnancy for signs of ascending placentitis. In the healthy pregnancy, the asinine CTUP grows linearly from the sixth month of pregnancy until foaling, with a substantial increase from the ninth to the twelfth month of pregnancy (Canisso, Panzani, Miró, & Ellerbrock. 2019).

The study reported the first stage of foaling in jennies lasted on average 65 minutes (20–135 minutes). Although the exact timing of the first stage of parturition could not be, clear because jennies, similar to mares, may not show obvious signs of the first-stage labour. The stage 2 of the parturition was also consistent with the timing observed in the horse, in which the complete expulsion can range between 5 and 60 minutes and the foals were expelled on average at 17 minutes or 20 minute. In normal eutocic jennies, the stage 3 of parturition was completed in 58 minutes (from 10–175 minutes) (Carluccio, Gloria, Veronesi, De Amicis, Noto, & Contri. 2015).

Following expulsion, the placenta must be carefully inspected to assess its integrity and main characteristics (Carluccio, Panzani, Contri, Tosi, De Amicis, & Veronesi. 2008). The healthy placenta has a reddish colour and a velvety appearance, given by the presence of the chorionic villi (Figure 1.6).

Figure 1.6 Placental morphology of donkey after birth

Immediate postpartum care must also be given to the newborn. In particular, it must be ensured that the umbilical cord breaks about 5 cm from the foal's abdomen, in correspondence with a slight depression of the cord itself. This normally occurs within minutes of expulsion. The stump of the cord must be thoroughly disinfected with a diluted iodine solution. It is also good to ascertain the foal's viability and verify that he takes the first colostrum intake within 2 hours of birth. The expulsion of meconium must also be recorded: in fact, in healthy infants, the first feces are expelled within about 90 minutes of delivery (Carluccio, Gloria, Veronesi, De Amicis, Noto, & Contri. 2015).

1.3.1.3 Lactation

Milk has been a staple part of the human diet since the agricultural revolution, as it supplies the body with important nutrients, hence playing a critical role both in nutrition and in health. Milk has

all nutritional requirements for every mammalian newborn of different species. Cow's milk represents the most common feeding during the infant weaning and widely used as a substitute for human milk, but the cow's milk is one of the most common food allergies in children, it can lead to an abnormal immunological response. In the last few years, milk from non-bovine mammals has been studied to identify the best natural substitute for human milk. Donkey milk has been successfully through clinical trials on children with cow milk allergy and found to provide nutritional adequacy and good palatability (Madhusudan, Ramachandra, Udaykumar, Sharnagouda, Nagraj. 2017).

Donkey milk production differs greatly from that of other dairy species, especially in terms of milk supply. As with all mammals, lactation is triggered by birth. Interestingly, an investigation in Sicily showed that the local latitude determined small photoperiod oscillations between different seasons, and under these conditions the donkeys have a continuous reproductive cycle (Giosuè, Alabiso, Russo, Alicata, & Torrisi. 2008).

The highest milk yield can be obtained with three milking per day compared to two per day, with an increase in milk fat content. The average milk yield remains constant for up to 8–10 months of lactation. Milk production can be affected by both breed and the breeding season. For instance, the donkeys that gave birth in an autumn–winter period yielded more milk than donkeys foaling in spring–summer period since seasonal thermal stress can have detrimental effect on the quantity and quality of milk (Aspri, Economou, & Papademas. 2017).

Chemical composition of donkey milk

Donkey's milk composition resembles that of human milk rather than dairy animals (e.g., Cow, buffalo, sheep, goat, and camel). More precisely, donkey and human milk have similar lactose, protein content. Donkey milk shows a homogeneous nutritional profile that is particularly important since it can be utilized by sensitive populations (infants and elderly). The reason why donkey milk is so similar in macro-composition to that of human milk is still unclear, especially as donkey and humans are not phylogenetically related. Donkey milk is unique containing various protective proteins like α-lactalbumin, lysozyme, lactoferrin, lactoperoxidase, immunoglobulins which exert antioxidant, antibacterial, antiviral, antifungal, hypoglycaemic, antiparasitic propertiess, growth promotion, aging prevention, autoimmune diseases and anti-tumour activity (Aspri, Economou, & Papademas. 2017).

1.3.2 Mycotoxins and potential health risk for donkey's milk intake

Research interest in donkey milk has increased in Europe, especially in Italy. Main hazards possibly present in raw donkey milk, including bacteria, fungal toxins, parasites, and chemical pollutants.

Microbiological hazards

Bacterial microflora isolated from donkey milk, mainly in Europe, can be briefly summarized as follows: *Bacillus (B.) cereus, Campylobacter* spp., coliforms, *Cronobacter (Cr.) sakazakii* (formerly *Enterobacter sakazakii*), *Enterobacter (En.) cloacae, En. agglomerans, Escherichia coli*, *Escherichia* (*E.*) *hermannii*, *Listeria* spp., *Pseudomonas (Ps) aeruginosa*, *Staphylococcus (S.) aureus S. chromogenes, S. intermedius*, *S. sciuri, S. warneri*, *S. xylosus*, *Streptococcus (Str.) hycus*, *Str. epidermidis, Str. equi*, *Str. equisimilis*, *Str. intermedius*, *Str. zooepidemicus*, *Str. dysgalactiae*. On the basis of the above, donkey milk contamination by microsporidia and consequent human infection cannot be ruled out (Conte, & Panebianco. 2019)

Mastitis is a well-known problem for dairy farms. Inflammation can be caused by a large variety of bacteria including *S. aureus*, some coliforms, and Brucella (for some EU countries); they are frequently found in infected animals. The development of bacterial resistance to antimicrobial agents poses a serious threat to human health. Raw milk may be a source of bacteria (primary or opportunistic pathogens) that are resistant to antimicrobials. In raw milk from donkey, antibiotic resistance (AR) was described in two *En. sakazakii* (at present *Cr. sakazakii*) strains (Conte, & Panebianco. 2019).

Chemical hazard

Donkey milk is claimed could be a convenient tool to analyze aflatoxin M1 (AFM1), Ochratoxin A (OTA) and Zearalenone (ZEN) in donkey milk. For AFM1 and OTA analysis, milk could be analysed by EIA without sample pre-treatment. For ZEN, heat treatment at 78 °C for 30 min prior EIA analysis was required to avoid false positives. All donkey milk samples were negative for all three toxins. Satisfactory quantitation was achieved for spiked samples (Gross, Ploetz, & Gottschalk, 2019).

For OTA, no specific regulation according to European Union regulation 1881/2006 for milk exists, but the lowest maximum level set for 'dietary foods for special medical purposes intended specifically for infants' is at 500 ng/kg (Gross, Ploetz, & Gottschalk. 2019).

A study reported the excretion of aflatoxin M1 (AFM1) and M2 (AFM2) in milk after feeding trials with contaminated feed. Samples were analyzed for AFM1 and AFM2 for 15 days after the contaminated feed administration. No AFM1 or AFM2 were detected in milk after 28 h from the last contaminated feed administration. The carryover from AFB1 to AFM1 and from AFB2 to AFM2 was found to be 0.02 and 0.31%, respectively (Tozzi, Liponi, Meucci, Casini, Dall'Asta, Intorre, & Gatta. 2016).

Donkey milk did not pose any toxicological risk of persistent organic pollutants for consumers. Residues of organic pollutants may accumulate in donkeys through contaminated food and inhaled air, stored in fat-rich tissues of the animal, and then excreted into the milk. Donkey milk is rich in selenium, zinc, copper, and iron, while it has low amounts of manganese and chromium. These results did not postulate any dangerous residues for human health. Indeed, mercury (Hg) and Arsenic (As) are within the permitted range, while cadmium (Cd) and Lead (Pb) were infrequently reported (and only for children) (Aspri, Economou, & Papademas. 2017).

Milk-producing animals accumulate pesticides residues through contaminated feed, grass/hay and by air; donkey milk could be contaminated in the same way. Current literature on pesticides in donkey milk is very scanty (Conte, & Panebianco. 2019).

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AIM OF THE STUDY

Food and feed contaminants have different routes of mother-to-child passage and their direct effects on reproductive cells/tissues, due to pre or postnatal exposure, must be analysed. To this aim, livestock models of accepted translational relevance are needed, also because many contaminants have widespread occurrence in food and feed. Ochratoxin A (OTA) is a mycotoxin produced by some toxigenic fungal species of the genera *Aspergillus* and *Penicillium* and it induces nephrotoxic, immunotoxic, reprotoxic, embryotoxic and teratogenic effects.

The sheep is a relevant model for human reproductive medicine, particularly for mechanisms controlling ovarian follicle development. As a ruminant, it is known to be relatively resistant to toxic effects of OTA, due to its degradation to the less toxic metabolite Ochratoxin alpha by rumen microbiota. However, in ewes fed with OTA-contaminated feed, OTA was detected in serum where it accumulates with exposure time and administered dose.

The donkey is a monogastric species, thus with high sensitivity to OTA, and is a suitable model to follow, like human, the route of natural exposure from feed to jennies during pregnancy and in foals after delivery. Moreover, donkey milk is of high interest for its nutritional value and its industry uses.

Given the premises explained in the Introduction, the aims of the present PhD Thesis were to determine:

1) as an *in vitro* pre-natal exposure model, the effects of ovine oocyte exposure to OTA on oocyte and embryo development;

2) as an *in vivo* post-natal exposure model, OTA levels in the spinneret feed, blood and milk samples of jennies and related foals.

CHAPTER 2 EXPERIMENTAL SECTION

Subchapter 2.1 Ochratoxin A affects oocyte maturation and embryo development in the juvenile sheep model

Abstract

Ochratoxin A (OTA) is a mycotoxin ubiquitously present in human foodstuffs and animal feed, reported as toxic for oocyte maturation and embryo development. Studies on the effects of OTA on female fertility have dealt with micromolar concentrations and no information is available to date on effects of naturally occurring nanomolar concentrations. The aims of the present study were to evaluate, in the juvenile sheep model, the effects of oocyte exposure to OTA, in a micromolar to nanomolar concentration range, during in vitro maturation (IVM) on maturation, fertilization and embryo development and to determine whether different mechanisms of action could underly OTA toxic effects at different exposure levels. Cumulus-oocyte-complexes (COCs) were exposed to 10μmol/L-0.1nmol/L OTA during *in vitro* maturation and evaluated for cumulus viability, oocyte maturation and bioenergetic/oxidative status or subjected to *in vitro* fertilization, in vitro culture for embryo development and embryo quality assessment via morphology, viability and bioenergetic/oxidative status. At micromolar concentrations, OTA induced cytotoxic effects, by reducing cumulus expansion, increasing cumulus cell apoptosis and inhibiting oocyte nuclear and cytoplasmic maturation, expressed as altered mitochondria distribution pattern and reduced activity. Exposure to OTA during IVM did not apparently alter embryo development. However, blastocysts, even morphologically normal, showed increased (boosted) mitochondrial activity and altered mitochondria/ROS colocalization. At nanomolar concentrations, OTA did not affect COC morpho-functional parameters but it increased blastocyst apoptosis. In conclusion, in the female germ cell, cytotoxic nonspecific effects characterize OTA-induced toxicity at micromolar exposure levels, whereas long-term carryover effects, not associated with altered COC viability and integrity, characterize OTA toxic action at nanomolar levels.

Keywords: Ochratoxin A, juvenile sheep oocyte, in vitro maturation, in vitro fertilization, in vitro embryo development, bioenergetic/oxidative status.

2.1.1 Introduction

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by fungi of *Aspergillus* and *Penicillium* genera (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016). Because of its ubiquitous presence in a variety of human foodstuffs and animal feed (Bhatnagar, Yu, Ehrlich 2002), it exerts various hazardous effects on human and animal health (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016; Kőszegi & Poór. 2016). OTA chemical structure consists of a para-chlorophenolic moiety containing a dihydroiso-coumarin group that is amide-linked to L-phenylalanine (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016). The molecule is characterized by considerable stability (El Khoury & Atoui. 2010) and long blood half-life, particularly in humans, but also in different animal species and for this reasons it is of concerns (Shen , Zhang , Xu , Liang , Zheng , Luo , Wang & Huang. 2013). OTA shows several toxicological effects, including nephrotoxicity, teratogenicity, mutagenicity, genotoxicity, carcinogenicity, immunotoxicity, neurotoxicity and hepatotoxicity and it has been classified it into group 2B as possibly carcinogenic to human (Pfohl-Leszkowicz & Manderville. 2007; Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016). OTA mechanisms of action are highly complex and have been described in detail in the Introduction part (Rached, Pfeiffer, Dekant, Mally. 2006; El Khoury & Atoui. 2010; Kőszegi & Poór. 2016; Gupta, Lasher Miller Mukherjee, Srivastavam & Lall 2017; Tao, Xie, Xu, Liu, Wang, Chen, Pan, Huang, Peng, Wang & Yuan. 2018).

In human, despite the interest for this mycotoxin due to its relatively long half-life (Studer-Rohr, Schlatter, Dietrich. 2000) and demonstrated placental passage in the fetus and passage through breast milk (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri & Rossi. 2011), no studies are available on its effects on female fertility and most of the studies have been performed in animal models. OTA has been reported to induce reproductive and developmental toxicity in animals (Malir, Ostry, Pfohl-Leszkowicz & Novotna, 2013; Gupta, Lasher Miller Mukherjee, Srivastavam & Lall. 2017). However, few studies have been published to date on direct effects of OTA exposure on female gametes. Huang & Chan (2014) firstly investigated the effects of OTA on mouse oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and subsequent development. In that study, OTA was shown to significantly impair oocyte maturation, and decrease IVF rates and embryonic development *in vitro*. A subsequent comparative study between mouse and porcine oocytes confirmed a significant reduction of the maturation rate *in vitro* in both species and revealed that porcine oocytes are more sensitive to OTA than mouse

oocytes (Lu, Zhang, Liu, Zou, Jia, Su, Sun & Sun. 2018). A recent study using high-throughput technologies showed that OTA exposure alters the expression of multiple genes in oocytes, indicating its molecular effects on oocyte maturation. As cellular changes following OTA treatment, it was shown that OTA adversely affects porcine oocyte polar body (PB) extrusion, by delaying CDC2-mediated cell cycle progression and that it disrupts meiotic spindle formation, by altering phosphorylated MAPK expression. RNA-seq screening showed that OTA induces aberrant oocyte mitochondria distribution and oxidative phosphorylation defects, which then causes oxidative stress, followed by early apoptosis and autophagy (Lan, Zhang, Wan, Pan, Xu & Sun. 2019). Another multitechnological study in the mouse (Jia, An, Cheng, Jiang, Xu, Zhao, Peng, Zhang & Su. 2019) showed that OTA decreases oocyte maturation and fertility by inducing oxidative stress and epigenetic changes. In detail, OTA intraperitoneal injection induced ovarian dysfunction with decreased offspring number. OTA exposure disrupted spindle formation and chromosome alignment, preventing first PB extrusion. In addition, OTA caused oocyte apoptosis as a result of enhanced oxidative stress during meiosis. Oocytes exposed to OTA also exhibited damaged mitochondria and insufficient energy supply, leading to meiotic failure. Epigenetic modifications were also affected in mouse oocytes (Jia, An, Cheng, Jiang, Xu, Zhao, Peng, Zhang & Su. 2019). Oocyte preincubation with OTA during IVM was shown to have negative long-term effects on embryo quality and viability. Huang and Chan (2016) reported that preincubation of oocytes with OTA during IVM increased post-implantation embryonic resorption and reduced the blastocyst total cell number. Particularly, the number of cells of the inner cell mass was reduced more than that of the trophoblast. The same authors reported that apoptosis, via p53- p21 and caspase-3-dependent regulatory mechanisms, increased in blastocysts derived from the OTA-preincubated oocytes compared with the untreated group (Huang & Chan 2016).

Above mentioned studies analyzed the effects of OTA on oocyte maturation and developmental competence at micromolar concentrations and no studies have been reported to date on OTA effects at nanomolar concentrations on cumulus-oocyte complex (COC) viability and developmental competence. An assessment of OTA effects in micro to nanomolar concentration range could give more complete information, as micromolar concentrations can be attained under experimental conditions whereas the nanomolar concentrations represent relevant, naturally occurring, conditions (Gekle, Sauvant & Schwerdt. 2005), being those detected in human (Schulz, Gekle & Schwerdt, 2019) and animal (Weidenbörner 2011) blood. Moreover, in other cell systems, OTA has been reported as inducing nonspecific cytotoxic effects at high doses (>1 μmol/L) and specific cell signalling-mediated effects at low doses (<1 μmol/L; Gekle, Sauvant & Schwerdt. 2005). Thus, the

aims of the present study were to determine the effects of oocyte exposure to OTA at a nanomolar to micromolar concentration range on nuclear and cytoplasmic maturation, fertilization and embryo development and quality and to assess whether OTA toxic effects could be mediated by different concentration-related mechanisms at the COC level

2.1.2 Materials and methods

2.1.2.1 Chemicals

All chemicals for in vitro cultures and analyses were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise indicated.

2.1.2.2 Preparation of OTA stock and working solutions

OTA was purchased by Sigma Aldrich, Milan, Italy (cod O1877-5 mg). OTA stock solution was obtained by dissolving 5mg OTA in 5 ml Methanol for HPLC (MeOH; ≥99.9%), as vehicle, to obtain the concentration of 1 mg/mL. Then, two mL of this solution were diluted in 3 mL of MeOH to obtain a final concentration of 1 mmol/L. Aliquots were stored at −20 °C until use.

2.1.2.3 Processing of ovaries and oocyte collection

Slaughterhouse ovaries were (Fin. Sud Import s.r.l.; Conversano, Bari) obtained from juvenile ewes (under 6 months) subjected to routine veterinary inspection in accordance with the specific health requirements stated in Council Directive 89/556/ECC. After transport, within 2-4 hours from slaughter, they were processed at the laboratory, by the slicing procedure (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli, & Dell'Aquila. 2012) to obtain COCs collected in phosphate buffered saline (PBS) solution. For further in vitro culture only those COCs with intact cumulus cell layers and homogeneous cytoplasm were used.

2.1.2.4 In vitro maturation (IVM) medium

Following was the composition of IVM medium: TCM-199 medium with Earle's salts buffered with 5.87 mM HEPES and 33.09 mM sodium bicarbonate and supplemented with 0.1 g/L L-glutamine, 2.27 mM sodium pyruvate, calcium lactate pentahydrate (1.62 mM Ca2+, 3.9 mM Lactate), 50 μg/mL gentamicin, 20% (v/v) fetal calf serum (FCS), 10 μg/mL ovine follicle

stimulating hormone (FSH), 20 μg/mL ovine luteinizing hormone (LH) and 1 μg/mL 17β estradiol (Martino et al., 2012). IVM medium and paraffin oil were pre-equilibrated for 1 hour under 5% CO2 in air at 38.5°C. Then, IVM medium was placed in 4-well plates (Nunc Intermed, Roskilde, Denmark). Each culture well contained 400 μL IVM medium and was covered with 400 μL of lightweight paraffin oil to prevent evaporation of culture and bacterial contamination.

2.1.2.5 IVM culture

In vitro maturation (IVM) was performed as previously reported (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli, & Dell'Aquila. 2012). COCs were placed in four-well dishes (Nunc Intermed, Roskilde, Denmark) containing 400 μL of IVM culture medium perwell of a four-well dish, covered with paraffin oil and cultured in vitro for 24 hours at 38.5°C under 5% CO2 in air. On the day of experiments, OTA stock solution was diluted in a 1/10 micromolar to nanomolar concentration range from 10 μmol/L to 0.0001 μmol/L in IVM medium, according to the experimental design. The highest concentration was selected on the basis of published mouse data (Huang & Chan 2016) while the nanomolar concentration were chosen as corresponding to OTA blood levels in sheep, after oral ingestion of OTA contaminated feed (Weidenbörner. 2011). Medium with 1% MeOH was used as vehicle control. For each experiment and condition, at least two runs/replicates were performed, where a run/replicate was a group of 20-25 COCs cultured for IVM in one well of a 4-well Nunc plate.

2.1.2.6 Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End labeling (TUNEL) assay

Cumulus cells collected in MPM with 20% FCS were spun down at 300g for 5 min and analzyed with the Click-iT® Plus TUNEL Assay, Molecular probe Life Technology, code: C10617, according the manufacturer's instructions. Briefly, cumulus cells were fixed in 4% paraformaldehyde in PBS for 15min at room temperature, washed with PBS and subsequently permeabilized for 20 minutes with 0.5% Triton X-100. Cumulus cells were washed twice with deionized water, placed in 50 μl drops of TUNEL reagent and incubated in the dark for 1 hr at 37°C in a humidified chamber. The cells were washed with 3% Bovine Serum Albumin (BSA) in PBS and stained with 2.5 μg/ml Hoechst 33258 in 3:1 (v/v) glycerol/PBS, mounted and kept at 4°C in the dark until observation. The samples were examined using an E-600 Nikon fluorescent microscope equipped with a 365 nm excitation filter.

2.1.2.7 In vitro fertilization (IVF) and in vitro embryo culture

In vitro fertilization was performed in Synthetic Oviductal Fluid Medium (SOFM, Tervit et al. 1972) supplemented with 2% oestrous sheep serum and 1 μg/mL heparin (Martino, Ariu, Bebbere, Filioli Uranio, Chirico, Marzano, Sardanelli, Cardinali, Minervini,Bogliolo & Dell'Aquila. 2016). Oocytes were cultured for 22 hours with frozen-thawed ram semen (1.5×10^6 spermatozoa /mL) at 38.5°C and under a 5% CO_2, 90% N_2 atmosphere in four-well dishes. Presumptive zygotes were partially denuded by gently pipetting using finely-drawn glass pipettes and cultured for 7 days in four- well dishes in SOFM (Walker, Hill, Kleemann & Nancarrow. 1996)) and 0.4% BSA under mineral oil, in maximum humidified atmosphere with 5% CO_2, 90% N_2 at 38.5°C. Embryos were examined at day 7 classified according to expansion and hatching status (Martino, Ariu, Bebbere, Filioli Uranio, Chirico, Marzano, Sardanelli, Cardinali, Minervini, Bogliolo & Dell'Aquila. 2016).

2.1.2.8 Oocyte and blastocyst staining for mitochondria and ROS

Oocytes and blastocysts were washed with PBS with 3% BSA and incubated with MitoTracker Orange CMTM Ros (280 nmol/L; Molecular Probes) for 30 minutes at 38.5°C under 5% CO2 (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli,& Dell'Aquila 2012;Somoskoi, Martino, Cardone, Lacalandra, Dell'Aquila, Cseh. 2015). Structures were subsequently washed in PBS with 0.3% BSA and incubated for 15 minutes with PBS 3% BSA containing 10 μmol/L 2',7'- dichlorodihydrofluorescein diacetate (H2DCF-DA), at 38.5°C under 5% CO2, to detect the dichlorofluorescein (DCF) and localize intracellular sources of ROS (Yang, Hwang, Kwon, Kim, Choi, 1998). After washing in PBS, samples were fixed overnight at 4°C in 2% paraformaldehyde in PBS (Ambruosi, Filioli Uranio, Sardanelli, Pocar, Martino, Paternoster, Amati & Dell'Aquila. 2011). Exposure to light was avoided during all procedures.

2.1.2.9 Nuclear chromatin evaluation of oocytes and embryos

Oocytes and embryos were fixed 2% paraformaldehyde solution in PBS and stained with Hoechst 33258 (2.5 μg/mL) in 3:1 (v/v) glycerol/PBS and mounted on microscope slides with coverslips, sealed with nail polish and kept at 4°C in the dark until observation using an epifluorescence microscope (Nikon Eclipse 600; 400x magnification) equipped with a B-2A (346 nm excitation/460 nm emission) filter. Oocytes were classified as germinal vesicle (GV), metaphase to telophase I (MI

to TI), MII with the 1st PB extruded, or as activated or degenerated (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli & Dell'Aquila 2012). Fertilization assessment was performed as described (Tessaro Modina, Crotti, Franciosi, Colleoni, Lodde, Galli, Lazzari & Luciano 2015). Normally fertilized oocytes contained two pronuclei (2PN) and two polar bodies (2PB). The occurrence of only one PN (1PN), a sperm head (SH) that failed chromatin decondensation and 2PB were considered as uncompleted pronuclear formation. The occurrence of a second meiotic spindle (MII) with a PB indicated unfertilized matured oocytes. Cells with more than two pronuclei (>2PN), possibly resulted from either polyspermic fertilization or parthenogenetic activation, were classified as abnormal.

2.1.2.10 Assessment of mitochondrial distribution pattern

Oocytes at blastocysts were examined at 600x magnification in oil immersion with a Nikon C1/TE2000-U laser scanning confocal microscope; 25 optical series were made per oocyte and embryo with a step size of 0.45 μm. MitoTracker Orange CMTM Ros was imaged using a helium/neon laser at 543 nm and the G-2A filter (551 nm excitation and 576 nm emission), while DCF was imaged with an argon ion laser at 488 nm and the B-2A filter (495 nm excitation and 519 nm emission) The mitochondrial distribution pattern was evaluated on the basis of previous studies. Homogeneously distributed mitochondria indicated low energy cytoplasmic condition; perinuclear and subplasmalemmal mitochondria were considered indicative of healthy cytoplasmic condition (P/S); irregularly distributed of mitochondria were classified as abnormal (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli,& Dell'Aquila 2012,2013; Somoskoi, Martino, Cardone, Lacalandra, Dell'Aquila, Cseh 2015). Oocytes and embryos where mitochondria and ROS were overlapping were considered healthy (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli,& Dell'Aquila 2012, 2013; Somoskoi, Martino, Cardone, Lacalandra, Dell'Aquila, Cseh. 2015).

2.1.2.11 Quantification of MitoTracker Orange CMTM Ros and H2DCF- DA fluorescence intensity

For the oocytes, MitoTracker and DCF fluorescence intensities were measured at the equatorial plane using the EZ-C1 Gold Version 3.70 image analysis software on the whole cytoplasmic areas. For the blastocysts, all the 25 acquired focal planes were used. Sample signals were expressed as arbitrary densitometric units (ADU). Parameters related to fluorescence intensity were maintained at constant values for all measurements (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira,

Silvestre, Pizzi, Desantis, Accogli,& Dell'Aquila 2012, 2013,2016; Somoskoi, Martino, Cardone, Lacalandra, Dell'Aquila, Cseh. 2015).

2.1.2.12 Mitochondria-ROS colocalization analysis

Colocalization analysis of mitochondria and ROS was performed with the EZ-C1 Gold Version 3.70 software. Degree of mitochondria/ROS colocalization was reported as the overlap coefficient. Mitochondria/ROS colocalization was considered as indicating healthy oocytes and embryos (Martino, Ariu, Bebbere, Filioli Uranio, Chirico, Marzano, Sardanelli, Cardinali, Minervini,Bogliolo & Dell'Aquila, 2016,2013; Dell'Aquila, Bogliolo, Russo, Martino, Filioli Uranio, Ariu, Amati, Sardanelli, Linsalata, Ferruzzi, Cardinali, Minervini 2014; Somoskoi, Martino, Cardone, Lacalandra, Dell'Aquila, Cseh. 2015).

2.1.2.13 Statistical analysis

The proportions of oocytes showing the different chromatin configurations and mitochondria distribution patterns, the proportions of cleaved embryos and blastocysts and the percentages of cumulus cells showing DNA fragmentation were compared among groups by Chi-square test. The Yates' correction was adopted according to the sample size. The fluorescence values of mitocondrial function, ROS and colocalization were plotted by box and whisker plot that showed the median, 1 st and 3 rd quantile, the minimum and maximum by using SigmaPlot software. The fluorescence values were compared by one-way ANOVA Kruskal-Wallis non-parametric test. Differences with $p<0.05$ were considered to be statistically significant.

2.1.3 Results

2.1.3.1 OTA at micromolar concentrations affects cumulus viability and oocyte maturation

First, the effects of OTA on cumulus expansion and apoptosis and oocyte maturation were analyzed. For each experimental condition, 6 to 16 replicates were performed. At 10 μmol/L, OTA reduced cumulus expansion and oocyte nuclear maturation rates, and increased percentages of apoptotic cumulus cells ($p<0.0001$; Table 2.1). At 1 μmol/L, it significantly reduced the cumulus expansion rate ($p<0.0001$) even if it did not affect the cumulus cell apoptotic index. At this concentration, OTA reduced the maturation rate and increased the percentage of oocytes found at the GV stage ($p<0.01$). At 0.1 nmol/L, OTA did not affect cumulus expansion and apoptosis nor

nuclear maturation. In Fig. 2.1, representative photomicrographs of OTA-induced inhibition of cumulus expansion (panel a) and apoptosis observed at analyzed concentration (panel b) are shown. Fig. 2.2 shows oocyte chromatin configurations observed at analyzed concentrations. It can be seen that, at any analyzed OTA concentration, chromatin configuration of specific meiotic stages had normal appearance. Abnormal configurations consisted in chromatin dispersion into multiple groups.

Table 2.1 Effects of in vitro exposure to OTA during IVM on oocyte meiotic progression and maturation

OTA concentration (µM)	**N° of cultured COCs**	**Cumulus expansion rate N (%)**	**N. (%) of apoptotic/ examined cells**	**N° of evaluated oocytes**	**Nuclear chromatin configurations N (%)**			
					GV	**MI to TI**	**MII**	**Abnormal**
0 (1%MeOH)	342	181 (53) a	5/160 (3) a	307	97 (32) a	32 (10)	131 (43) a	47 (15)
10	333	57 (17) e	56/160 (35) e	281	106 (38)	41 (15)	75 (27) e	59 (21)
1	269	81 (30) e	9/160 (5)	232	103 (44) c	32 (14)	60 (26) e	37 (16)
0.0001	151	80 (53)	5/160 (3)	127	39 (31)	19 (15)	44 (35)	25 (20)

Chi Square test: Comparisons OTA-exposed *versus* vehicle control (1%MeOH) a,c=$p<0.01$; a,e=$p<0.0001$

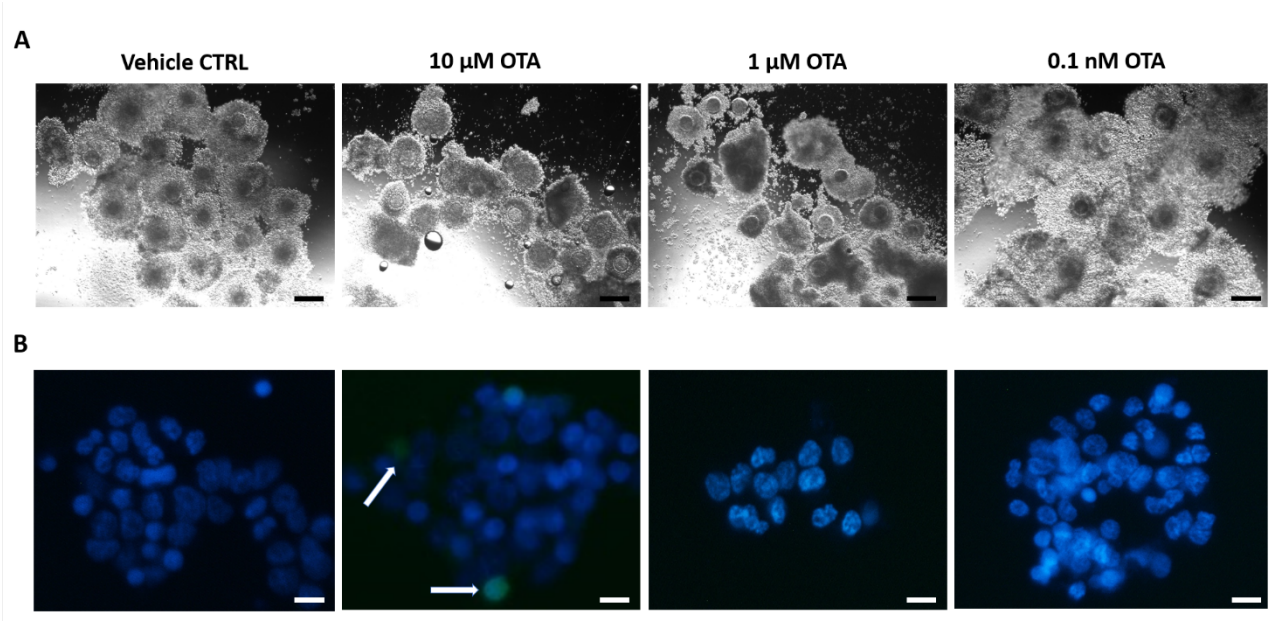

Figure 2.1 Effects of OTA on cumulus expansion and apoptosis. (Panel a) Representative phase contrast photomicrographs of COCs exposed to OTA during 24 hours IVM and observed under stereomicroscopy. COC expansion was inhibited in presence of OTA as assessed by cumulus morphology. Indeed, cumuli with continuous edges, consisting of cells in close contact each other, were seen in samples exposed to 10 and 1 µmol/L OTA, whereas in cumuli cultured in control conditions or in presence of 0.1 nmol/L OTA, the edges were discontinuous following cell detachment and production of a viscous extracellular matrix. Black scale bars represent 200 µm. (Panel b) Representative images of cumulus cells observed after IVM in presence of OTA and TUNEL assay. Merge of green and blue fluorescence are shown and were related to Alexa Fluor 488 and Hoechst 33258 staining, respectively. White arrows indicate TUNEL positive cumulus cells (green fluorescence). White scale bars represent 10 µm. Numbers of analyzed cumulus cells per experimental condition are indicated in Table 2.1.

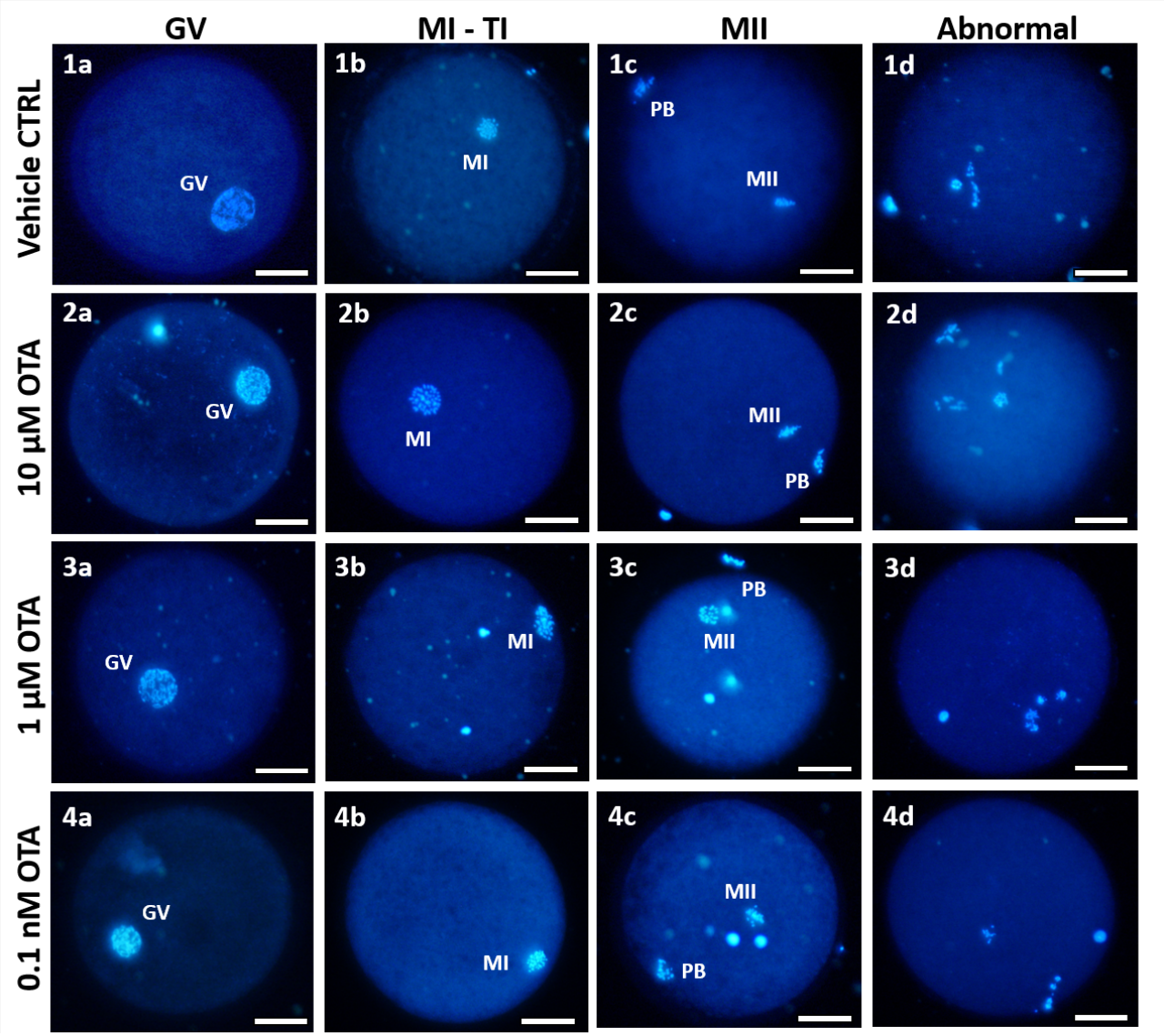

Figure 2.2 Representative photomicrographs showing nuclear chromatin configuration of oocytes in different meiotic stages obtained after IVM in presence or absence of OTA. Scale bars represent 40 µm. GV = Germinal Vesicle; MI = Metaphase I; TI = Telophase; MII = Metaphase II.

2.1.3.2 OTA at micromolar concentrations affects oocyte bioenergetic/oxidative status

For each experimental condition, the metaphase II oocytes issuing from at least 5 previously described IVM runs were assessed for mitochondria distribution pattern by LSCM analysis. The percentage of oocytes showing heterogeneous perinuclear and subcortical (P/S) pattern, indicating cytoplasm maturity and competence, was significantly reduced after exposure to 10 μmol/L OTA ($p<0.0001$; Table 2.2). This parameter was not altered at the other tested concentrations. Part of the oocytes underwent quantification and colocalization analysis. Mitochondrial membrane potential and intracellular ROS levels were significantly reduced after culture in presence of 10 μmol/L and 1μmol/L OTA compared with controls (Fig. 2.3, panels a, b; $p<0.05$). As well, mitochondria/ROS colocalization was significantly reduced at these OTA concentrations (Fig. 2.3, panel c; $p < 0.05$). Instead, at 0.1 nmol/L, OTA did not affect any biomarker of oocyte bioenergetic/oxidative status. In Fig. 2.4, representative photomicrographs of a control oocyte (lane 1) and oocytes exposed to examined OTA concentrations (lanes 2-4) are shown. Oocytes exposed to 10 and 1μmol/L OTA showed lower MitoTracker and DCF fluorescence intensities (Fig. 4, 2c, 2d and 3c, 3d) compared with controls and oocyte exposed to 0.1nmol/L OTA.

Table 2.2 Effects of in vitro exposure to OTA during IVM on oocyte mitochondrial distribution pattern

OTA concentration (μM)	**N° of evaluated MII oocytes**	**Mitochondria distribution pattern N (%)**	
		perinuclear/subcortical	**small aggregates**
0 (1%MeOH)	85	48 (56) a	37 (44) a
10	68	15 (22) e	53 (78) e
1	36	14 (39)	22 (61)
0.0001	35	14 (40)	21 (60)

Chi Square test: Comparisons OTA-exposed *versus* 1%MeOH control: a, e=P<0.0001

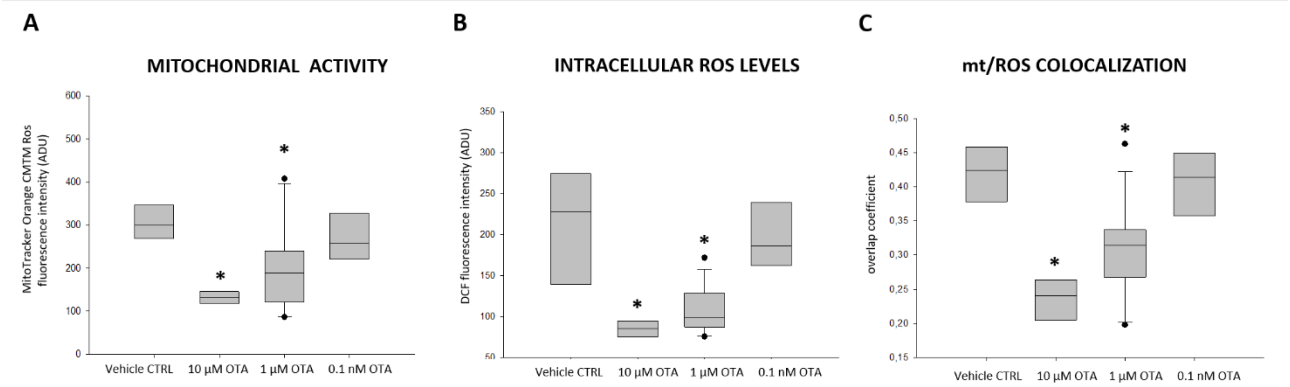

Figure 2.3 Boxplot representing mitochondrial activity, intracellular reactive oxygen species (ROS) levels and mitochondria/ROS colocalization in MII oocytes cultured in absence of OTA and in presence of 10 µmol/L, 1 µmol/L and 0.1 nmol/L OTA. Mitochondrial activity and ROS levels are expressed as MitoTracker Orange CMTM Ros (panel a) and DCF (panel b) fluorescence intensity in arbitrary densitometric units (ADU); mitochondria/ROS colocalization is expressed as overlap coefficient (panel c). Oocytes matured in presence of 10 µmol/L and 1 µmol/L OTA showed significantly reduced mitochondrial activity, intracellular ROS levels and mitochondria/ROS colocalization. No effects were noticed on oocytes matured in presence of 0.1nmol/L OTA. One Way ANOVA Kruskal-Wallis non-parametric test, comparisons OTA-exposed vs control: * $p < 0.05$.

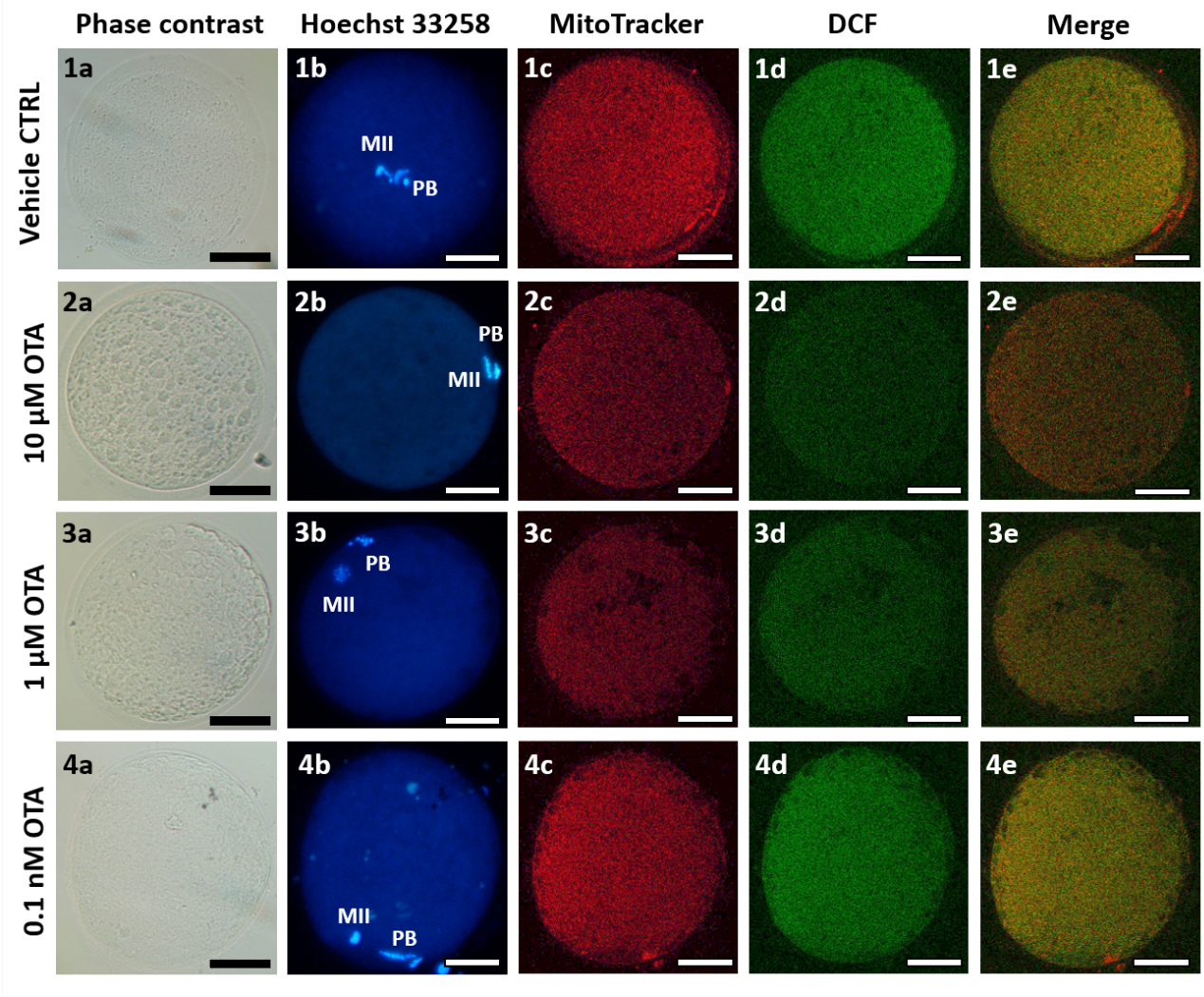

Figure 2.4 Photomicrographs showing representative images of a control oocyte (1) and of oocytes exposed to 10 µmol/L (2), 1 µmol/L (3) and 0.1 nmol/L OTA (4), respectively. Corresponding phase-contrast images showing cell morphology (column a), epifluorescence images showing nuclear chromatin configuration (column b: Hoechst 33258) and confocal images showing mitochondrial distribution pattern and activity (column c: MitoTracker Orange), intracellular ROS localization and levels (column d: DCF) and mitochondria/ROS colocalization (column e: Merge). Confocal images were taken at the oocyte equatorial plane. Decreased mitochondrial activity and intracellular ROS levels, expressed as decreased MitoTracker (2c and 3c vs. 1c) and DCF fluorescent intensity (2d and 3d vs. 1d) are visible in oocytes exposed to 10 µmol/L and 1 µmol/L OTA. Scale bars represent 40 µm. OTA = Ochratoxin A; DCF = dichlorodihydrofluorescein; ROS = reactive oxygen species

2.1.3.3 OTA affects oocyte fertilization by delaying pronuclear growth and migration

Next, we evaluated whether OTA exposure during IVM, affected oocyte fertilization and pronuclear formation. At any tested concentration, OTA exposure during maturation significantly affected oocyte fertilizability (Tables 2.3). In detail, it significantly reduced the normal fertilization rate at 10 μmol/L ($p<0.001$), 1 μmol/L, 1nmol/L and 0.1 nmol/L ($p<0.05$, Table 2.3) expressed as the percentage of oocytes showing two pronuclei (PN). In oocytes exposed to 0.1 nmol/, OTA significantly increased the percentage of abnormally fertilized oocytes showing 3 or 4 or more PNs (Table 2.3; $P<0.05$). This trend was also observed at 10 μmol/L, even if it was not quite statistically significant ($p=0.0915$). In Fig. 2.5, representative micrographs of 2PN (1a) and more than 2PN (1b, 1c) obtained after IVM culture in presence of OTA and IVF are displayed.

Table 2.3 Effects of in vitro exposure to OTA during IVM on oocyte fertilization

OTA concentration (μM)	**N° of cultured COCs**	**N° of evaluated oocytes**	**Nuclear chromatin configurations N (%)**	
			Normally fertilized oocytes with 2PN	**Abnormally fertilized oocytes (>2PNs)**
0 (1%MeOH)	265	173	53 (30.6) a	23 (13.3) a
10	140	116	12 (10.3) d	25 (21.5) (*)
1	178	136	24 (17.6) b	24 (17.6)
0.1	131	101	22 (21.7)	15 (14.8)
0.01	139	106	23 (21.6)	12 (11.3)
0.001	153	121	23 (19.0) b	17 (14.0)
0.0001	232	168	34 (20.2) b	39 (23.2) b

Chi Square test: Comparisons OTA-exposed *versus* vehicle control (1%MeOH) a, b =$p<0.05$; a,d=$P<0.001$.

(*) $p=0.0915$.

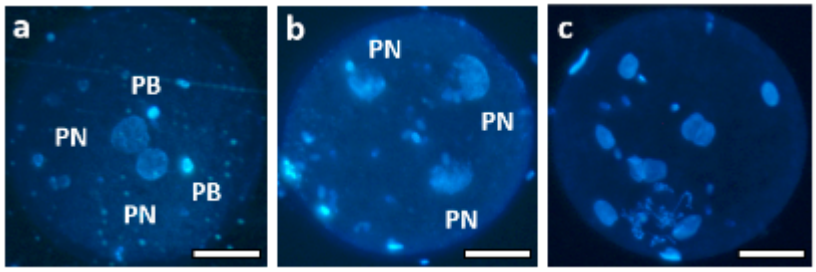

Figure 2.5 Representative photomicrographs of nuclear chromatin configuration of normally and abnormally fertilized oocytes after exposure to OTA during IVM. (a) normal zygote with 2 PN and 2 PB, obtained after IVM in control condition and IVF; abnormally fertilized oocytes with 3 (b) or more than 2 PN (c), obtained from oocytes cultured in presence of OTA and IVF. Scale bars represent 40 µm.

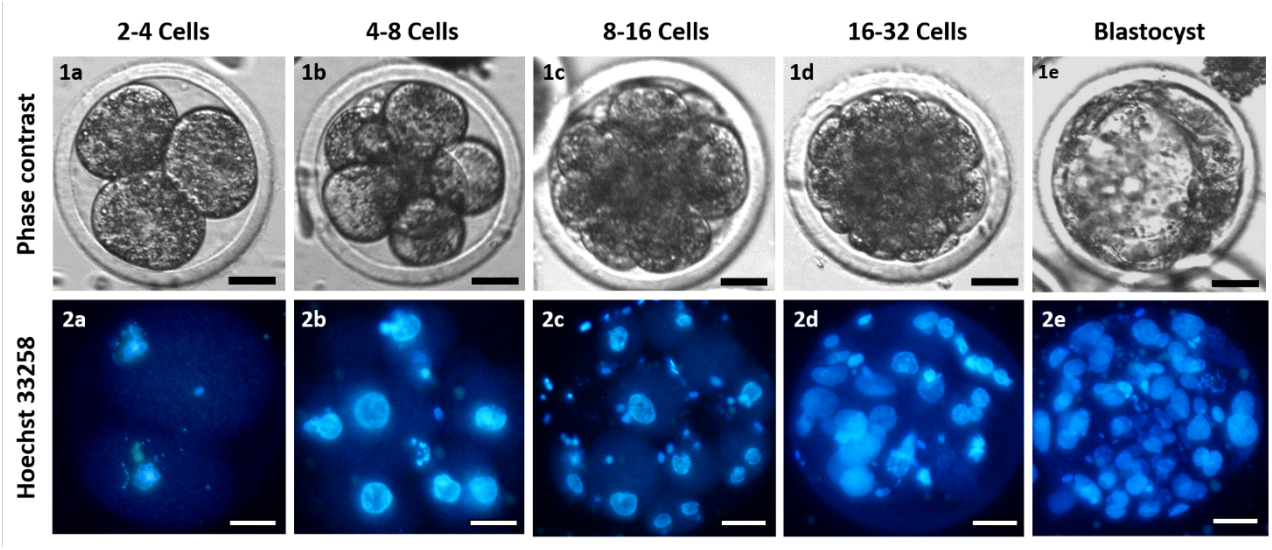

Figure 2.6 Photomicrographs representative of different embryo cleavage stages observed under phase contrast microscopy (lane 1; a-e) and epifluorescence microscopy after fixation (lane 2; a-e). Scale bars represent 40 µm.

2.1.3.4 OTA alters embryo development and blastocyst quality

To evaluate whether in vitro oocyte exposure to OTA could affect embryo development, per each experimental condition, five to ten independent IVM runs with subsequent IVF and embryo culture were performed. Embryo blastomere nuclear chromatin configuration was assessed under epifluorescence microscopy after fixation at Day 7. At 10 μmol/L, exposure to OTA during IVM increased the rate of embryos arrested at the 2-4 cell stage ($p<0.01$). At 0.1 μmol/L, OTA increased the rates of embryos arrested at the 8-16 cell stage ($p<0.05$) and, correspondingly, it reduced the rates of embryos at the 4-8 cell stage ($p<0.05$). At 0.1 nmol/L, it reduced the rates of embryos developed up to the 4-8 cell stage ($p<0.05$) and to the 8-16 cell stage ($p=0.05$). Despite these differences observed at specific stages of embryo development, the total cleavage and blastocyst formation rates did not change compared with controls, at any examined OTA concentration (Table 2.4). In Fig. 2.6, photomicrographs representative of different embryo cleavage stages observed under phase contrast microscopy (1a-1e) and epifluorescence microscopy after fixation (2a-2e) are shown.

Table 2.4 Effects of in vitro exposure to OTA during IVM on embryo development

OTA concentration (μM)	N° of cultured oocytes	N° of evaluated oocytes	Embryo developmental stages Fluorescence microscopy-based nuclear chromatin evaluation N (%)						
			2-4 cells	4-8 cells	8-16 cells	16-32 cells	Blastocyst	Total cleaved	Cleaved degenerated
0 (1% MeOH)	225	171	13 (7.6) a	30 (17.5) a	24 (14) a	11 (6.4)	5 (2.9)	82 (48)	8 (4.6)
10	199	182	32 (17.6) c	21 (11.5)	18 (9.9)	5 (2.7)	9 (4.9)	86 (47.2)	2 (1.1)
1	125	103	8 (7.7)	16 (15.5)	21 (20.4)	9 (8.7)	3 (2.9)	57 (55)	9 (8.7)
0.1	127	93	4 (4.3)	7 (7.5) b	23 (24.7) b	2 (2.1)	4 (4.3)	40 (43)	9 (9.6)
0.01	125	95	12 (12.6)	14 (14.7)	8 (8.4)	5 (5.2)	4 (4.2)	43 (45)	8 (8.4)
0.001	100	73	7 (9.5)	18 (24.6)	10 (13.7)	5 (6.8)	1 (1.3)	41 (56)	5 (6.8)
0.0001	180	137	19 (14)	10 (7) b	9 (6.5) (*)	6 (4)	7 (5.1)	51 (37)	10 (7)

Chi square test: Comparisons OTA-exposed *versus* vehicle controls (1%MeOH): a, b = $p<0.05$; a,c = $p<0.01$; (*)= p=00549

Blastocyst morphology (whether early, expanded or hatched) was not affected by oocyte maturation in the presence of OTA. Actually, OTA tended to reduce the blastocyst expansion rate and, particularly at 10 μmol/L and 0.1 nmol/L, some blastocyst developed a barely visible blastocoelic cavity faster, but the differences did not attain statistical significance (Table 2.5). Total numbers of nuclei were not affected. However, as shown in Fig. 2.7, the percentage of apoptotic nuclei was significantly higher after oocyte exposure to OTA low concentrations (starting from 0.1 μmol/L to 0.1 nmol/L) compared with controls (Table 2.5). Blastocysts obtained in at least three runs/condition, under a micromolar (10 μmol/L) and a nanomolar concentration (0.1 nmol/L) were analyzed for bioenergetic and oxidative status and compared with controls. As a long-term effect of oocyte exposure during IVM, OTA significantly increased blastocyst mitochondrial membrane potential at both tested concentrations ($p<0.05$; Fig. 2.8, panel a, Fig. 2.9 column c). Intracellular ROS levels were not affected (Fig. 2.8, panel b and Fig. 2.9, column d) and mitochondria/ROS colocalization was significantly reduced at both tested concentrations ($p<0.05$; Fig. 2.8, panel c and Fig. 2.9, column e).

Table 2.5. Effects of in vitro exposure to OTA during IVM on blastocyst quality

OTA concentration (μM)	N° of evaluated Blastocysts	Blastocyst quality parameters N (%)			
		Expanded morphology N (%)	Number of nuclei (mean±sd)	Apoptotic index (N. of apoptotic cells/blastocyst)	Range of apoptosis
0 (1%MeOH)	5	4 (80)	53±7.5	39/265 (15) a	(6.3-24)
10	9	5 (56)	58±26	101/521 (19)	(9.6-46.9)
1	3	3 (100)	66±6.5	27/199 (14)	(7.5-23)
0.1	4	3 (75)	57±24.7	50/229 (22) b	(4.6-82)
0.01	4	4 (100)	56±44.2	59/224 (26) c	(10.5-83)
0.001	1 (*)	0 (0)	28	24/28 (85)	(85)
0.0001	7	3 (43)	51±21.5	116/357 (33) e	(6.8-71.9)

Chi Square test: Comparisons OTA-exposed *versus* vehicle controls (1%MeOH): a, b $p<0.05$; a, c=$P<0.01$; a,e $p<0.0001$. (*) data of this blastocyst were not statistically analyzed.

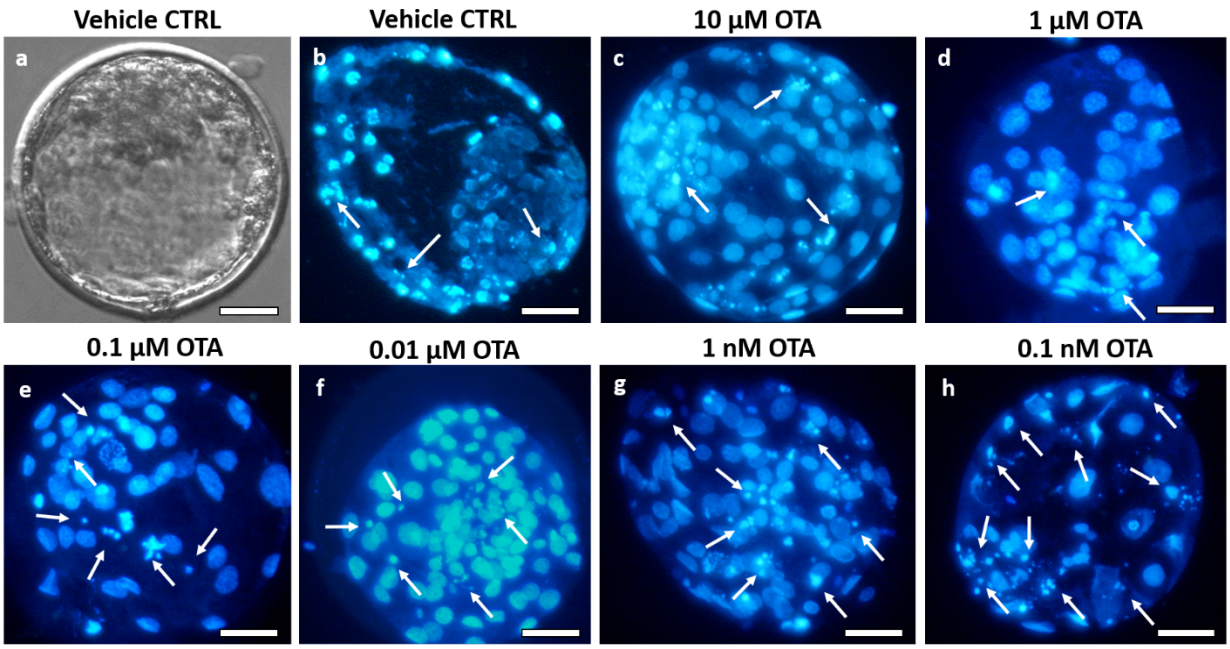

Figure 2.7 Photomicrographs representative of blastocysts obtained after IVM in presence of OTA and observed as fresh (a) or after fixing and Hoechst 33258 staining (b-h). Blastocysts derived from oocytes exposed during IVM to lower OTA concentrations (from 0.1 µmol/L to 0.1 nmol/L) showed increased apoptotic index (see Table 7). Arrows indicate apoptotic nuclei. Scale bars represent 40 µm. OTA = Ochratoxin A; IVM = *in vitro* maturation. *p<0.05.

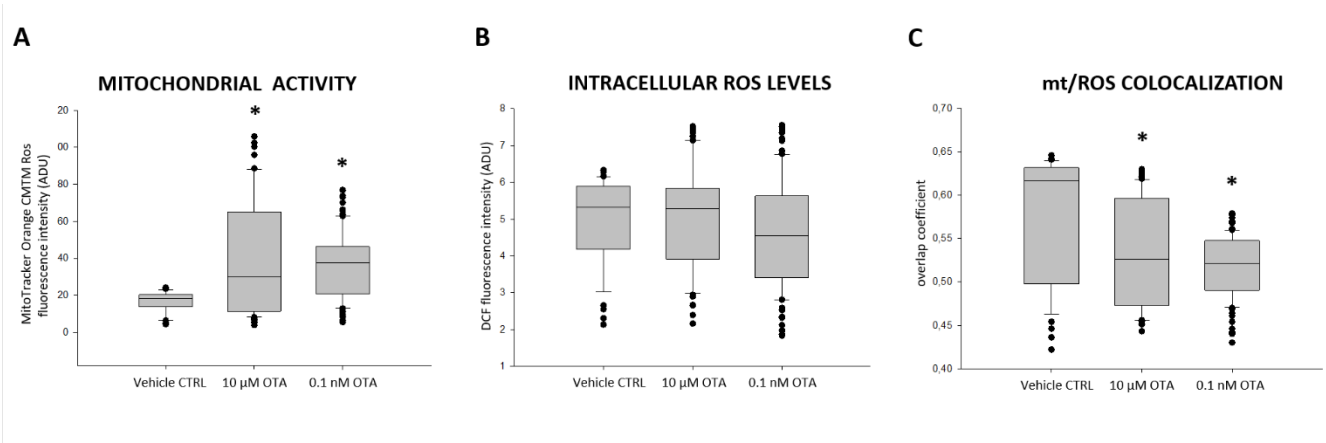

Figure 2.8 Boxplots representing mitochondrial membrane potential (panel a) and intracellular ROS levels (panel b) in control blastocysts and blastocyst derived from oocytes exposed to 10 µmol/L and 0.1 nmol/L OTA. In panels A and B, values are presented as fluorescence intensities of MitoTracker Orange and DCF, respectively. In panel c, mitochondria/ROS colocalization is presented as overlap coefficient. One-way ANOVA Kruskal-Wallis non-parametric test, comparisons OTA-exposed vs control. * p<0.05.

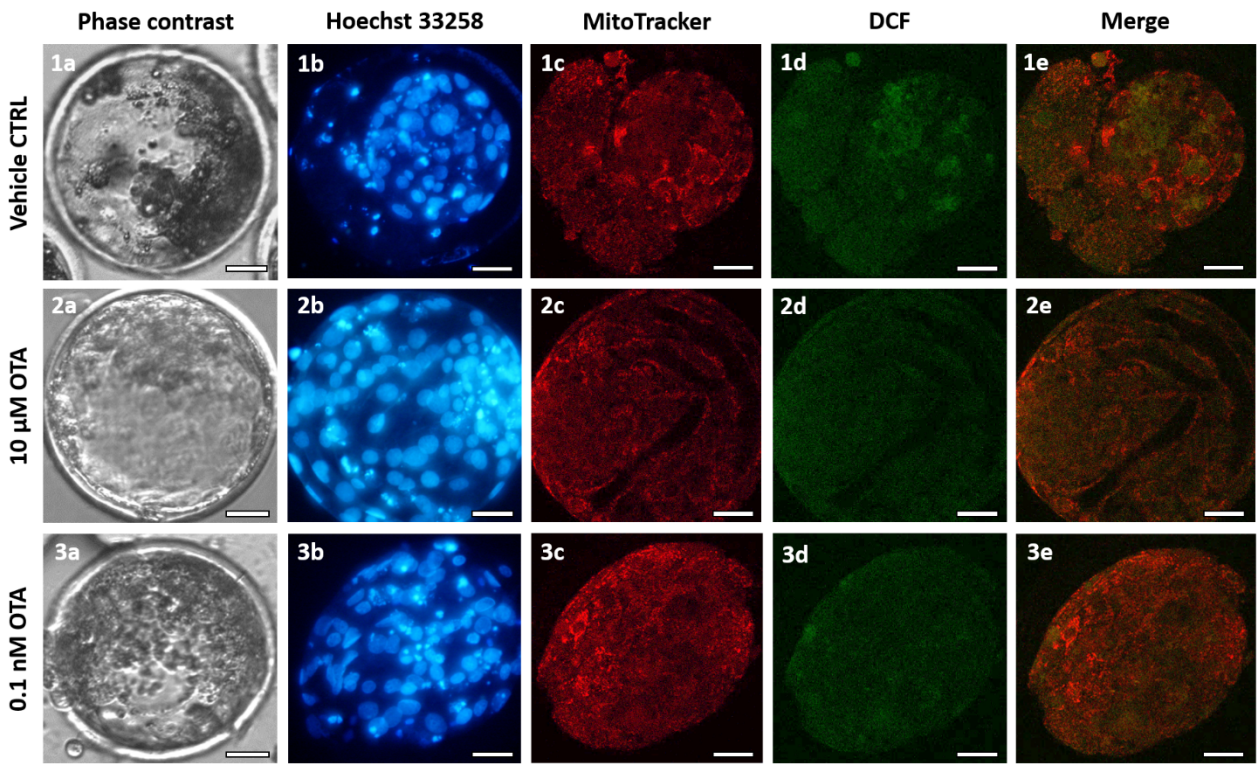

Figure 2.9 Photomicrographs showing representative images of a control blastocyst (lane 1) and of blastocysts obtained from oocytes exposed to 10 µmol/L (lane 2) and 0.1 nmol/L (lane 3) OTA, respectively. MitoTracker Orange and DCF were used to label mitochondria and ROS, respectively. Nuclear chromatin was stained with Hoechst 33258. For each blastocyst, the corresponding phase-contrast images showing cell morphology (column a), epifluorescence images showing nuclear chromatin (column b) and confocal images showing the mitochondrial distribution pattern (column c), ROS localization (column d), mitochondria/ROS merge (column e) are shown. Scale bars represent 40 µm. DCF = dichlorodihydrofluorescein; ROS = reactive oxygen species

2.1.4 Discussion

In the present thesis, OTA concentrations were selected in a micromolar to nanomolar range, in order to compare data obtained under experimentally-induced conditions (micromolar) with those of naturally occurring conditions, as nanomolar concentrations are those detected in human and animal blood (Schulz, Gekle, Schwerdt 2019; Weidenbörner. 2011). To the best of our knowledge, this is the first study examining the effects of OTA on oocytes at nanomolar concentrations and the first study performed in the sheep, a large animal model of recognized translational relevance for human reproductive medicine (Baird 1983; Ledda, Bogliolo, Calvia, Leoni & Naitana 1997; Campbell, Souza, Gong, Webb, Kendall, Marsters, Robinson, Mitchell, Telfer & Baird, 2003; Cotterill, Harris, Collado Fernandez, Lu, Huntriss, Campbell, Picton 2013; Leoni, Palmerini, Satta, Succu, Pasciu, Zinellu, Carru, Macchiarelli, Nottola, Naitana, Berlinguer. 2015). Due to current dietary regimens including cereal-based feed, small ruminants are increasingly exposed to mycotoxins and, for this reason, play a role as models for human. Moreover, the juvenile sheep model is of particular interest as it allows to study the effects of juvenile exposure to contaminants on female fertility, thus mirroring exposure during pediatric and adolescent age.

In our experiments, OTA induced a dose-dependent inhibitory effect on cumulus expansion and oocyte maturation. The expansion rate was reduced ($p<0.0001$) at both examined micromolar concentrations, indicating a cytotoxic effect. Correspondingly, significantly increased cumulus cell apoptosis was observed, but only at 10 µmol/L OTA and not at 1µmol/L OTA, indicating that a dose dependent effect on DNA fragmentation. These two parameters are fundamental biomarkers of COC viability and maturation as they indicate cumulus cell viability and metabolic activity, such as the ability to synthesize extracellular hyaluronic acid-based matrix and to interrupt gap junction-mediated intercellular communications, physiological events of oocyte maturation and ovulation (Sanchez & Smitz 2012). Our data are in agreement with a previous study in porcine oocytes reporting that OTA disturbed cumulus and granulosa cell diffusion and proliferation (Lan, Zhang, Wan, Pan, Xu & Sun. 2019). Interestingly, exposure at 0.1 nmol/L OTA did not affect cumulus expansion and viability, indicating that nanomolar doses do not induce cyctotoxic effects.

OTA affected oocyte maturation only at micromolar concentrations. In fact, exposure to 10 and 1 µmol/L OTA reduced the MII rate ($p<0.0001$). At 1µmol/L OTA the reduction of the MII rate was associated with significant increase of the percentage of oocytes remaining at the GV stage ($p<0.01$). No effects on nuclear maturation were noticed at 0.1 nmol/L OTA. Findings at micromolar concentrations are in agreement with previously reported studies in mice (Huang &

Chan 2016; Jia, An, Cheng, Jiang, Xu, Zhao, Peng, Zhang & Su. 2019) and pigs (Lu, Zhang, Liu, Zou, Jia, Su, Sun & Sun. 2018) in which, similarly to our results, significant inhibition of oocyte maturation was reported at 10 μmol/L. However, data on meiosis progression with effects on earlier meiotic stages were not reported in those studies. In another study in porcine oocytes, OTA was found to significantly reduce progression to the MII stage with a higher percentage of oocytes at the germinal vescicle (GV)/germinal vesicle breakdown (GVBD) stages (Lan, Zhang, Wan, Pan, Xu & Sun. 2019). None of these previous studies analyzed the effects of nanomolar OTA concentrations on oocyte maturation.

In order to identify mechanisms underlying OTA-induced oocyte damage with possible implications on its developmental competence, the effects of OTA on ooplasmic quality of matured oocytes were assessed in terms of bioenergetic/oxidative status. At high concentration (10 μmol/L), OTA changed the cytoplasmic distribution of mitochondria, leading to structural change in the bioenergetic status. Moreover, at 10 and 1μmol/L, OTA reduced mitochondrial activity and ROS generation, inducing functional damage. These results highlight multiple aspects of oocyte bioenergetic damage caused by OTA. Indeed, the LSCM-based multiparametric method for assessment of oocyte mitochondrial energy/redox status allows the combined analysis of qualitative and quantitative parameters able to distinguish severe damage (loss of cell viability, expressed as reduced mitochondrial activity and ROS generation) from mild damage (oxidative stress expressed as an adverse boosting of mitochondrial membrane potential leading to increased intracellular ROS levels). The role of mitochondria as indicators of oocyte developmental competence is well known and indeed standard bioenergetic activities of mitochondria include the generation of ATP, managing calcium homeostasis, regulation of cytoplasmic redox state and signal transduction (Mastrorocco, Martino, Marzano, Lacalandra, Ciani, Roelen, Dell'Aquila & Minervini. 2019). Abnormally functioning mitochondria can compromise the quality of the oocyte, including an inability to undergo meiosis, reduced fertilization potential and apoptosis (Brookes, Yoon, Robotham, Anders & Sheu. 2004). In healthy oocytes of most mammalian species, including sheep, mitochondria are distributed around the nucleus and below the plasma membrane. Scattered mitochondrial clusters and empty vacuolated areas, on the other hand, indicate a damage. The majority of oocytes exposed to OTA demonstrated a uniform mitochondrial distribution pattern in small aggregates. Thus, besides causing effects on oocyte nuclear maturation, OTA may have acted by preventing redistribution of mitochondria that is considered as a biomarker of cytoplasmic health conditions. These data are in agreement with those reported in a recent study in porcine oocytes by

(Lan, Zhang, Wan, Pan, Xu & Sun. 2019) who described that OTA altered mitochondria distribution pattern.

OTA-related reduction of ROS was most likely due to functional mitochondrial damage since mitochondria are the major source of ROS (Cadenas & Davies. 2000). In this case, our data are not in agreement with those of previous studies which reported increased ROS levels indicating oxidative stress (Lan, Zhang, Wan, Pan, Xu & Sun. 2019; Jia, An, Cheng, Jiang, Xu, Zhao, Peng, Zhang & Su. 2019). These discrepancies could be due to age-related differences. Indeed, adult oocytes are possibly more resistant to OTA-induced oxidative stress whereas in our study juvenile oocytes were used, possibly having higher sensitivity to OTA due to less developed antioxidant defences (Jiao, Cao, Cui, Lian, Miao, Wu, Han, Tan. 2013; Piras, Menéndez-Blanco, Soto-Heras, Catalá, Izquierdo, Bogliolo, Paramio. 2019).

Moreover, colocalization of active mitochondria and intracellular ROS, a putative marker of the oocyte healthy conditions (Martino, Lacalandra Filioli Uranio, Ambruosi, Caira, Silvestre, Pizzi, Desantis, Accogli,& Dell'Aquila. 2012), decreased following OTA exposure, suggesting that ROS could be shifted in non-physiological districts with consequent cellular damage. These toxic effects on mitochondrial function and ROS generation ability may have affected oocyte maturation, subsequent fertilization and embryo development and quality.

The effects of OTA on fertilization were tested as pronuclear formation rate. Data obtained on micromolar concentrations are in agreement with those of two previous studies. Mouse oocytes exhibit a reduced ability to be fertilized by fresh sperm upon exposure to10 μmol/L OTA during IVM but not at 1μmol/L (Huang & Chan 2016; Jia, An, Cheng, Jiang, Xu, Zhao, Peng, Zhang & Su. 2019). Here we demonstrate that, both at the highest and at the lowest tested concentration, OTA inhibited the normal fertilization rate. Indeed, previous studies, in other cell systems, reported that OTA affects Ca2+ signalling, pH- and energy-homeostasis and mitogen-activated protein kinases (MAPKs) pathways (Gekle, Sauvant & Schwerdt. 2005). All these pathways are involved in the regulation of oocyte maturation (Sanchez & Smitz. 2012), thus it is plausible to speculate that such modifications could have affected oocyte fertilizability. Further studies are necessary to explore these effects.

Surprisingly, after having observed impairment of oocyte maturation and fertilization, total embryo cleavage and blastocyst formation rates were apparently not affected by oocyte exposure to OTA. This result was contrary to our expectations because previous studies in the mouse reported that oocyte exposure to OTA during IVM inhibits subsequent embryonic development (Huang and Chan

2014; Jia, An, Cheng, Jiang, Xu, Zhao, Peng, Zhang & Su. 2019). A possible explanation for these discrepancies may lie in species-specific effects. Indeed, OTA-induced species-specific effects have been reported (Lu, Zhang, Liu, Zou, Jia, Su, Sun & Sun. 2018). At any tested concentration, OTA did not affect blastocyst morphology and growth but, at nanomolar concentrations, it increased the apoptotic index. This result is not in line with those by Huang and Chan 2014 who observeded significantly increased apoptosis in mouse blastocysts derived from 10μM OTA-pretreated oocytes. Possible explanations could be related either to species-specific differences or to different methods used in our study and in the study by Huang and Chan 2014, evidencing two not necessarily related aspects of apoptosis, chromatin damage (as in our study) and DNA fragmentation (as in the study by Huang and Chan. 2014), respectively.

At both micro and nanomolar concentrations, OTA pushed blastocyst mitochondrial activity and reduced mitochondria/ROS colocalization. To the best of our knowledge, this is the first study investigating mitochondria activity in blastocysts obtained in vitro by OTA-exposed oocytes. Previous studies were performed on in vivo produced mouse blastocysts, exposed in vitro for 24 hours to OTA during the morula/blastocyst transition. These studies reported altered mitochondrial function and oxidative stress, expressed as loss of mitochondria membrane potential and increased ROS generation (Hsuuw, Chan & Yu. 2013; Huang, Wang & Chan. 2019). A possible explanation of discrepancies with our study could be the exposure phase. In fact, in the studies in the mouse, embryos were exposed to OTA during in vitro embryo culture, which may have acted with immediate stronger effects, whereas in our study, OTA exposure occurred during IVM with long-term effects on blastocysts.

Going in more details on effects of OTA exposure during embryo development, in the study by Hsuuw et al., 2013, the effects exerted by OTA on the blastocyst stage of mouse embryos was investigated. Mouse blastocysts were incubated at micromolar (1, 5, or 10 μM) OTA concentrations for 24 h. An interesting mix of in vitro (cell proliferation and growth, apoptosis by TUNEL assay and in vitro embryo implantation by fibronectin-coated culture dishes) and in vivo studies (post-implantation development after embryo transfer, ET) was performed. Blastocysts treated with 10 μM OTA displayed significantly increased apoptosis and reduced total cell number. No differences were observed in implantation success rate between OTA-pretreated and control blastocysts either during in vitro embryonic development or after in vivo ET. However, in vitro treatment with 10 μM OTA was associated with increased resorption of post-implantation embryos by the mouse uterus, and decreased fetal weight upon ET. These results collectively indicate that, in line with our results, in vitro exposure to OTA induces apoptosis-mediated injury of blastocysts, by promoting

mitochondrion-dependent apoptotic signaling processes that impair subsequent embryonic development, and affects post-implantation development after ET. The same research group later reported that liquiritigenin (a flavonoid isolated from Glycyrrhiza radix) protects mouse blastocysts against OTA-mediated apoptosis, opening the possibility of detoxifying strategies (Huang, Wang & Chan. 2019).

In conclusion, this study demonstrated that, in the juvenile sheep model, OTA at micromolar concentrations induced cytotoxic effects on the COC, by affecting cumulus cell viability and expansion, oocyte nuclear maturation and mitochondrial activity. Blastocyst development occurred with apparently normal morphology and viability but it was associated with pushed mitochondrial activity. At nanomolar concentration, OTA did not affect viewable morpho-functional COC parameters but it increased blastocyst apoptotic index. These different effects noticed between the two concentration levels demonstrate that, in the COC, as previously observed in other cell systems, nonspecific effects characterize OTA-toxicity due to high levels of exposure, having minor toxicological relevance, whereas relevant nanomolar concentrations do not excert "classical" toxic action. Rather, they induce specific, possibly cell signaling-mediated effects, without alterations of cell viability or integrity. Further studies are necessary to analyze the pregnancy rate of embryos derived from OTA exposed oocytes. Overall, the data are of interest for the animal production industry. Moreover, given the translational relevance of the juvenile sheep model, they are important for the assessment of OTA exposure to human female fertility.

2.1.5 References

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Subchapter 2.2 Natural occurrence of Ochratoxin A in blood and milk samples from jennies and their foals during pregnancy and after delivery

Abstract

An assessment of the natural Ochratoxin A (OTA) exposure of seven Martina Franca jennies was carried out by analysing feed, blood and milk samples collected during late pregnancy and after delivery. Fifty-three feed samples were collected from January to September and analysed by a reference method (AOAC Official Method No. 2000.03) for OTA content. A total of 67 and 34 blood samples were collected from jennies and foals, respectively, and analysed by ELISA. A total of 33 milk samples were collected from jennies and analysed by HPLC/FLD method based on IAC clean-up. Although feed samples showed low OTA levels, up to 2.7 ng/g with an incidence of 32%, in jennies the OTA incidence rate of blood samples was 73%, with median value of 81 ng/L and concentrations ranging from < LOD to 6,000 ng/L. A season-effect on OTA levels in positive blood samples was observed with increases of 45% of the positive ones from April to June. Concerning foals, the incidence rate of blood samples was 50% with median value of 52 ng/L and concentrations ranged from < LOD to 4034 ng/L. The incidence of milk samples was 36% with levels ranging from < LOD to 82 ng/L. In conclusion, the results showed a natural exposure of jennies and foals to OTA and its presence in jenny milk could pose a risk for human new-borns considering its well-known nutritional and health properties.

Keywords: Jennies, Ochratoxin A, pregnancy, milk

2.2.1 Introduction

Ochratoxin A (OTA) is a major mycotoxin produced by several species of fungi, including *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicillium verrucosum* (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016). Contamination generally occurs because of suboptimal drying practices and poor storage conditions of commodities. OTA has been reported as a ubiquitous natural contaminant of food and feed (Bui-Klimke, & Wu. 2015; Duarte, Pena, & Lino. 2010; Gallo, Giuberti, Frisvad, Bertuzzi, & Nielsen. 2015). Cereals, such as oat, wheat, barley and their by-products, such as bran, being widely used as feed raw materials, are the major cause of OTA exposure to animals. The intake of feed contaminated by OTA represents a potential risk for animal health and a food safety issue mainly due to the transfer of the toxin through the food chain to humans (Fink-Gremmels. 2008). Interest in OTA increased when OTA was classified by the International Agency for Research on Cancer as a possible human carcinogen (Group 2B), based on

evidence of carcinogenicity in experimental animal studies (Malir, Ostry, Pfohl-Leszkowicz, Malir, & Toman. 2016). Ochratoxin A has a potent toxicity, and its nephrotoxic, hepatotoxic, teratogenic, carcinogenic, and immunosuppressive effects have been demonstrated in several mammalian species (Malir, Ostry, Pfohl-Leszkowicz, & Novotna. 2013). The use of OTA-contaminated feed during a long rearing period involves serious risk as a result of reduced feed efficiency, with decreased growth and weakening of the immune system (Battacone, Nudda, & Pulina. 2010). Monogastric (without rumen) species, for example humans, swine, and ruminants (with immature/partially functioning rumen) are at higher risk, lacking appropriate ruminal microbiota and microbes for the degradation of OTA to less toxic compounds (Mobashar, Hummel, Blank, & Sudekum. 2010) consequently, regular exposure to OTA can be a threat to monogastric species. Chronic human exposure to low levels of OTA occurring frequently in foods is more alarming instead of acute exposure to higher doses (Bui-Klimke, & Wu. 2015). In addition, human fetus and new-born are exposed to OTA through transplacental transfer (especially in early gestation), inducing possible reprotoxicity, embryotoxicity and teratogenicity, or through OTA-contaminated human breast milk or infant formula (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri, & Rossi. 2011; Galvano, Pietri, Bertuzzi, Gagliardi, Ciotti, Luisi, Bognanno, La Fauci, Iacopino, Nigro, Li Volti, Vanella, Giammanco, Tina, & Gazzolo. 2008). Several studies have demonstrated transplacental transfer of OTA in swine although contrasting reports have been published showing no residues in piglets of sows fed diets containing OTA through gestation or no placental transfer after ingestion of OTA by a pregnant sow (Mortensen, Hald, Larsen, Madsen, 1983; Patterson, Roberts & Small. 1976). In contrast, some authors found OTA transmission to piglets in uterus with blood concentrations ranged from 0.075 to 0.12 ng/mL (Gallo, Giuberti, Frisvad, Bertuzzi, & Nielsen. 2015). Other authors (Minervini, Giannoccaro, Nicassio, Panzarini, & Lacalandra. 2013) reported OTA exposure of mare and transplacental transfer with consequent foals exposure. The mean ratio of OTA maternal blood/fetal blood was 1.96 ± 0.94, probably due to its active transport across the placenta, as a consequence of OTA chemical characteristics (such as the similarity between the chemical structures of OTA and phenylalanine, low molar mass, lipophilic character, serum binding property (Minervini, Giannoccaro, Nicassio, Panzarini, & Lacalandra. 2013). Concerning jennies, their feed typically consists simply of grass, hay and minerals, excluding the possibility of mycotoxins exposure. In case of professional jenny breeding farms and for lactating animals, cereals and cereal-based pellets are added to the diet with consequent carry-over of mycotoxins into milk that is considered to be similar to human breast milk in nutrient composition and it represents a valid alternative in the infant's diet in terms of nutritional adequacy for subjects affected by allergy to cow milk proteins

(Carminati & Tidona. 2017). It is rich in lactose, whey proteins, contributing to the intestinal absorption of calcium, essential for bone mineralization. The presence of endogenous bioactive compounds increases some other alleged health benefits, as the antibacterial activity, the stimulation of immune system, the prevention of inflammatory diseases, and antiaging properties (Carminati & Tidona. 2017). To our best knowledge, only one study has been reported in the literature showing no OTA occurrence in jenny's milk analyzed by ELISA (Gross, Ploetz, & Gottschalk. 2019).

At present, no data are available on the jenny's OTA exposure and the relevant transplacental transfer. The purpose of this study was to assess the natural exposure to OTA of jennies and their foals, reared extensively in open stabling, both by performing analysis of feed, blood and milk samples from jennies and their foals during the late time of pregnancy and three months after delivery

2.2.2 Materials and Methods

2.2.2.1 Chemicals

Acetonitrile and methanol (both HPLC grade) were purchased from Carlo Erba Reagents (Milan, Italy). Dichloromethane and hydrochloric acid (both HPLC grade) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA). Ochratoxin A (OTA), sodium chloride (NaCl, ACS grade), sodium hydrogen carbonate ($NaHCO_3$, ACS grade), Tween 20 and acetic acid were purchased from Sigma-Aldrich (Milan, Italy). OTA immunoaffinity columns (OchraTest™) were obtained from VICAM, A Waters Business (Milford, MA). Glass microfiber filters (GF/A) and paper filters (No. 4) were purchased from Whatman (Maidstone, UK). OTA immunoaffinity columns (OchraPrep®) and ELISA test kits (RIDASCREEN® Ochratoxin A 30/15) were purchased from R-Biopharm (R-Biopharm AG, Darmstadt, Germany).

2.2.2.2 Animals and clinical data

The study was conducted from January to September. Seven pregnant Martina Franca jennies, with ranges of age 5-11 years and weight 370-510 kg, were enrolled at animal farm, University of Teramo, Italy. All pregnant jennies were in good health and body conditions. The 7 jennies were grouped in small herds and were kept in outdoor paddocks. Daily jennies were fed ad libitum with

standard hay and supplemented with concentrate feed at different amounts in relation to physiological conditions two months before and three months after the delivery.

All animal procedures performed in this study met the requirements of Italian law on the use of animals for experimental and other scientific purposes (Legislative Decree 26/2014, implementing Directive 2010/63/EU on the protection of animals used for scientific purposes) and the research protocol was approved by Ministry of Health (authorization n° 370/2020-PR).

2.2.2.3 Feed, blood and milk sampling

Fantini S.r.l. Italy (a feed mill Company) prepared a cereal mixture (concentrated feed) composed by several cereals at different percentages. The complete feed for the jennies comprised: oats (35%), flacked barley (35%), flacked corn (10%), bran (15%), soy bean (4.5%) and mineral vitamin components (0.5%). Concentrate feeds were packed in 25 kg bags and stored at room temperature.

Sampling of feed, blood and milk samples was performed during the interval from January to September at animal farm which belongs to the Faculty of Veterinary Medicine, University of Teramo, Italy.

The sampling of concentrated feed samples was carried out according to the sampling procedures of the EU Regulation (CE) N. 152/2009 at the opening of each bag. A total of 53 feed samples (about 500 g each) were collected and stored at room temperature. Before the preparation of concentrated feed, each component of the mixture was collected following the sampling protocol as previously described (European Commission (EC). Commission Regulation (EC) No 152/2009).

Concerning blood sampling, samples (n=67) were collected from jennies every fifteen days. After delivery, at the same time interval, blood samples were collected from foals (n=34). Blood samples (2-5 mL per jenny and the respective foal) were collected in individual sampling tubes and stored at -20°C until analysis. At time of delivery and after delivery, every 15 days for consecutive three months, 2-3 mL milk samples (n=33) were collected from each jenny. Milk samples were stored at -20°C until analysis.

2.2.2.4 OTA extraction and purification

Feed samples

Feed samples were comminuted/homogenized by mixing with the Ultra Turrax IKA T50 mixer (IKA Werke GmbH & Co. KG., Staufen, Germany) after adding three times its weight of water (water/matrix ratio of 3:1). After 10 min mixing, slurry samples were stored at -20°C until HPLC analysis. For the determination of OTA, homogenized feed samples were extracted following the AOAC Official Method No. 2000.03, with minor modifications (Entwisle, Williams, Mann, Slack, Gilbert, 2000). The extraction procedure for 100 g slurry-mixing portions (equivalent to 25 g feed) was carried out by shaking (60 min) using 112.5 mL acetonitrile in order to obtain the same ratio among sample, acetonitrile and water, as in the official method procedure. The extraction mixture was filtered through filter paper (Whatman No. 4) to remove particulate matter. A 10 mL volume of filtered extract was diluted with 40 mL distilled water, mixed, and filtered through a glass microfiber filter (Whatman GF/A); 20 mL of the diluted extract was loaded onto a Ochra Test™ immunoaffinity column and passed through the column at a flow rate of about 1 drop/s. The immunoaffinity column was washed with 10 mL wash buffer (2.5% NaCl, 0.5% $NaHCO_3$, 0.01% Tween 20) and 10 mL distilled water at a flow rate of 1–2 drops/s. OTA was eluted with 1.5 mL methanol and collected in a silanized vial. The eluted extract was dried under a nitrogen stream at about 50°C, and reconstituted with 500 µL HPLC mobile phase [acetonitrile–water–acetic acid (99+99+2, v/v/v)].

Serum samples

OTA determination in blood samples was performed by ELISA test kits (RIDASCREEN® Ochratoxin A 30/15) according to the protocol provided by the manufacturer (R-Biopharm AG, Darmstadt, Germany), with minor modifications. The extraction procedure for 2 mL of thawed and vortexed serum samples was carried out by shaking (5 min) using 2.5 mL of 1 N HCl and 4 mL of dichloromethane. Shaking was followed by centrifugation for 15 min at 3500 g and 15°C. The upper aqueous layer was removed. Dichloromethane layer was filtered by filter paper (Whatman No. 4) and 2 mL of filtrate was extracted with 2 mL of sodium hydrogen carbonate buffer (0.13 M, pH 8.1). After shaking (5 min) the sample was centrifuged for 5 min at 3500 g and 15°C. The step of extraction with sodium hydrogen carbonate buffer was repeated twice. The double layers of sodium hydrogen carbonate buffer were combined and extracted with 2 mL of dichloromethane and 0.75 mL of 1 N HCl. After shaking (10 min) and centrifugation (5 min at 3500 g and 15°C) the buffer layer was discarded. The extract was dried under a nitrogen stream at about 50°C, and reconstituted with 1 mL sodium hydrogen carbonate buffer. Samples were kept at 2-8°C until ELISA analysis. A subset of randomly selected samples (n=23) was also purified using immunoaffinity column, according to Curtui et al. (Curtui, & Gareis. 2001), with minor

modifications, and analyzed by HPLC to confirm ELISA results. In particular 840 µL of reconstituted sample was diluted with 4.2 mL sodium hydrogen carbonate buffer, mixed, and 4 mL of diluted sample was loaded onto an OchraTest™ immunoaffinity column and passed through the column at a flow rate of about 1 drop/s. The immunoaffinity column was washed with 10 mL wash buffer (2.5% NaCl, 0.5% NaHCO3, 0.01% Tween 20) and 10 mL distilled water at a flow rate of 1–2 drops/s. OTA was eluted with 1.5 mL methanol and collected in a silanized vial. The eluted extract was dried under a nitrogen stream at about 50°C, and reconstituted with 250 µL HPLC mobile phase [acetonitrile–water–acetic acid (99+99+2, v/v/v)].

Milk samples

OTA determination in milk samples was performed by using the HPLC method based IAC purification reported by Bascaràn et al (Bascarán, de Rojas, Chouciño, & Delgado. 2007), with some modifications. Whole milk samples (2 mL) were loaded onto an OchraPrep® immunoaffinity column and passed through the column at a flow rate of about 1 drop/s. The immunoaffinity column was washed with 10 mL distilled water at a flow rate of 1–2 drops/s, twice. OTA was eluted with 3 mL methanol and collected in a silanized vial. The eluted extract was dried under a nitrogen stream at about 50°C, and reconstituted with 250 µL HPLC mobile phase [acetonitrile–water–acetic acid (99+99 +2, v/v/v)].

2.2.2.5 HPLC Analysis: OTA standard solutions and recovery experiments

An aliquot (100 µL) of reconstituted feed, serum or milk extract (corresponding to 0.107 g, 0.32 mL and 0.8 mL of feed, serum and milk, respectively) were injected into the chromatographic system. HPLC analyses were carried out using an Agilent 1260 Series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) equipped with a fluorometric detector (model G1321B, $\lambda_{ex} = 333$ nm, $\lambda_{em} = 460$ nm). The analytical column was a Zorbax SB-C18 (5 µm, 4.6 × 150 mm; Agilent Technologies), preceded by a 0.5 µm Rheodyne guard filter (IDEX Health & Science, Wertheim-Mondfeld. Germany); mobile phase was acetonitrile–water–acetic acid (99+99+2, v/v/v) and a flow rate of 1 mL/min was used. OTA concentrations are reported as ng/g, ng/L, ng/L for feed, serum and milk samples, respectively. The detection limits (S/N 3:1) of methods were 0.1 µg/kg, 50 ng/L and 15 ng/L for feed, serum and milk, respectively.

OTA stock solution was prepared by dissolving solid commercial toxin in toluene/acetic acid 99:1 (v/v) at a concentration of 1 mg/mL. An OTA standard solution in methanol, at the concentration of 10 µg/mL, was prepared and spectrophotometrically tested (ε=6,330 cm^2/mmol, at λ=332 nm in

methanol). For spiking purposes in recovery experiments and for the preparation of standard solutions for HPLC, a solution of OTA was prepared in methanol at a concentration of 250 ng/mL.

Concerning serum, recovery experiments were performed by spiking three serum samples with OTA at level of 500 ng/L. Spiked samples were extracted and analyzed by ELISA, showed an average recovery of 104%, with relative standard deviations lower than 25%. Concerning milk, recovery experiments were performed in triplicate by spiking milk samples with OTA at level of 100 ng/L. Spiked samples were extracted and analyzed by HPLC method, showed an average recovery of 79%, with relative standard deviations lower than 10 %.

2.2.2.6 Statistical Analysis

Statistical analysis was performed using the SigmaPlot™ software v.12 (Systat Software, Inc., SigmaPlot for Windows). Concerning blood and milk samples, a value corresponding to half the detection limit was assigned to all values below the detection limit (WHO 2009). Before testing for group differences, normality of the data distribution was assessed in OTA contamination of pooled serum samples collected for each jenny using the Shapiro–Wilk test. Since the data were not normally distributed, the median, 25th percentile and 75th percentile of the OTA concentration in serum samples are reported as descriptive statistical measures.

The comparison among pooled serum data of each jenny or foal was performed by one-way ANOVA Kruskal-Wallis non-parametric test. The relation between OTA levels in serum samples from jennies and serum/milk OTA ratio was performed by using linear regression analysis.

2.2.3 Results

2.2.3.1 Animals

During *in vivo* experiments, jennies recruited for this study showed normal pregnancy and parturition. All jennies had a normal course of pregnancy and complied with the criteria for normal parturition. All foals (three females and four males) were born at term and by spontaneous eutocic parturition. During all period time, any toxic effects were observed in the jennies and in the foals.

2.2.3.2 OTA contamination in feed

Table 2.6 shows the OTA levels found in feed samples and grouped into three major groups according to the level of OTA contamination. The incidence of positive feed samples was very low (32%); OTA concentrations found in feed samples ranged from 0.3 to 2.7 ng/g and were far below the guidance OTA values in feed materials (cereals and cereal based products) and composed feeds for pigs, poultry, cats and dogs reported by the Commission Recommendation 2006/576/EC.

The analysis of each cereal component present in the feed showed similar low OTA levels (0.15-0.18 ng/g) in bran, corn flake and soy, whereas barley and oat resulted uncontaminated (detection limit of 0.1 ng/g). Toxin concentrations were far below the guidance values of OTA in feed materials reported by the Commission Recommendation 2006/576/EC .

Table 2.6 Incidence (%) and concentrations (ng/g) of Ochratoxin A (OTA) contaminations in feed samples for jennies assessed by HPLC analysis

Feed samples	**Number of feed samples with OTA concentration**		
Contamination ranges (ng/g)	<0.3[a]	From 0.3 to 1	From 1 to 3
Number of samples	36	14	3
Incidence of contamination	68%	26%	6%
Range of OTA level (ng/g)	-	0.3-0.7	1.4-2.7

[a] Limit of quantification (LOQ)= 0.3 ng/g

2.2.3.3 OTA occurrence in blood samples collected from the jennies

In jennies, the OTA incidence rate of blood samples (with OTA levels higher than the detection limit) was 73%, with OTA median value of 81 ng/L and range from < LOD to 6000 ng/L. As observed in Table 2.7, for each jenny different incidence of OTA occurrence in serum samples was observed, ranging from 50 to 100%, with OTA median ranging from 44 to 114 ng/L.

Table 2.7 Occurrence of OTA in blood samples collected from jennies and assessed by ELISA

Jenny name	Period time of sampling	Positive/total samples (incidence)	Median (ng/L)	25th-75th percentile (ng/L)	Range (ng/L)
Adelaide	January-July	7/11 (64%)	55	< LOD[a]-176	< LOD[a]-1796
Francisca	February-May	5/9 (55%)	97	< LOD[a]-1489	< LOD[a]-6000
Gaia	May-August	4/8 (50%)	44	< LOD[a]-101	< LOD[a]-624
Etiopia	February-June	10/11 (91%)	125	78-181	< LOD[a]-1620
Antica	January-May	8/9 (89%)	82	51-157	< LOD[a]-1467
Filaria	March-July	5/9 (55%)	52	< LOD[a]-110	< LOD[a]-2215
Eritrea	May-September	10/10 (100%)	114	79-166	57-1255

[a] Limit of detection (LOD)= 50 ng/L

Although, no significant differences among OTA levels in jennies were observed a season-effect on the levels of OTA in the blood was found with significant increase from 2 to 120 times in 45% of the positive ones collected from April to June, as observed in Figure 2.10.

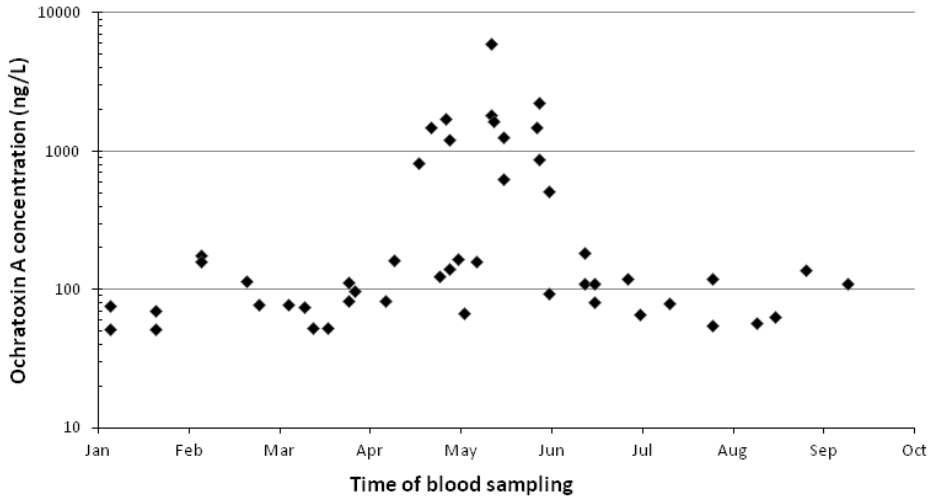

Figure 2.10 Seasonal distribution of OTA levels in positive blood samples collected from jennies.

After delivery, the mean incidence of positive blood samples was 73% with median value of 109 ng/L and similar OTA range. No significant differences among OTA levels in blood samples collected from jennies were found.

2.2.3.4 OTA contamination in blood samples from foals

At the time of delivery, no OTA was recorded in blood samples collected from all foals, attesting no placental transfer. As observed in Table 2.8, the total number of positive blood samples was 17 whereas the incidence rate was 50% in foals. Median value was 52 ng/L with a range from <LOD to 4034 ng/L. No statistical differences on OTA levels in blood samples were found among foals. No relation between OTA levels in blood samples of jennies and related foals was recorded because animals were reared extensively in open stabling, typical condition for donkeys, that favour the sucking of milk even by different jennies.

2.2.3.5 OTA contamination in milk samples

As observed in Table 2.8, the total number of positive milk samples was 12, whereas the incidence rate was 36%. The median value was of 7.5 ng/L and ranged from < LOD to 82 ng/L. In six jennies incidence of OTA contamination ranged from 25 to 60% with levels up to 82 ng/L. Only one jenny produced one milk sample with 41 ng/L of OTA and another jenny did not excrete OTA by milk. Francisca and Antica were the only jennies with higher value of incidence and consequent quantified OTA amount in milk and this condition could be related to the period of delivery.

Table 2.8 Ochratoxin (OTA) levels in blood samples collected from jennies and foals and milk samples after delivery

	Jenny's blood samples		Milk samples		Foal's blood samples	
Jennies(date of delivery)	Positive/total samples (%)	Median (Range) ng/L	Positive/total samples (%)	Median (Range) ng/L	Positive/total samples (%)	Median (Range) ng/L
Adelaide (17th March)	4/6 (67 %)	53.5 (<LOD[a]-1796)	2/5 (40 %)	<LOD[b] (<LOD[b]-43)	3/5 (60 %)	109 (<LOD[a]-4034)
Francisca (31st March)	4/5 (80 %)	1489 (<LOD[a]-6000)	3/5 (60 %)	21 (<LOD[b]-82)	4/5 (80 %)	144 (<LOD[a]-594)
Gaia (18th June)	2/5 (40 %)	<LOD[a] (<LOD[a]-109)	0/5 (0 %)	<LOD[b] (-)	0/5 (0 %)	<LOD[a] (<LOD[a])
Etiopia (1st May)	5/5 (100 %)	181 (118-1620)	2/5 (40 %)	<LOD[b] (<LOD[b]-30)	4/5 (80 %)	84 (<LOD[a]-108)
Antica (11th March)	3/4 (75 %)	119.5 (<LOD[a]-1467)	2/4 (50 %)	12.25 (<LOD[b]-55)	3/5 (60 %)	87 (<LOD[a]-306)
Filaria (31st May)	2/4 (50 %)	67.5 (<LOD[a]-2215)	1/4 (25 %)	<LOD[b] (<LOD[b]-41)	0/4 (0 %)	<LOD[a] (<LOD[a])
Eritrea (13th July)	5/5 (100 %)	109 (57-138)	2/5 (40 %)	<LOD[b] (<LOD[b]-30)	3/5 (60 %)	123 (<LOD[a]-367)

[a] Limit of detection (LOD) in blood samples = 50 ng/L

[b] Limit of detection (LOD) in milk samples = 15 ng/L

As observed in Figure 2.11, when considering only the positive milk and blood samples, a significant linear correlation was observed between the OTA level in serum and the OTA serum/OTA milk concentration ratio ($r^2 = 0.442$; $p<0.001$).

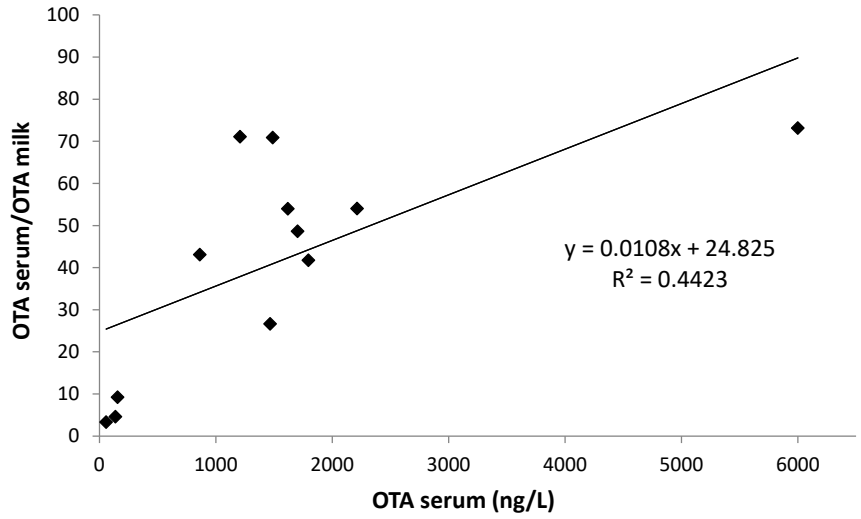

Figure 2.11 Relationship between serum OTA and serum/milk OTA ratio in samples collected from jennies.

2.2.4. Discussion

In contrast to *in vivo* toxicological studies, in which defined amounts of mycotoxins are administered and it is possible to determine their blood levels, this study was planned to monitor and to evaluate exposure of jennies to OTA under natural conditions, using non-artificially contaminated feed and keeping animals reared extensively in open stabling, as donkeys are normally bred. The natural exposure of jennies and their foals to OTA was assessed by analyzing feed samples and the relevant biomarker, i.e. OTA in blood and milk samples, in order to test the individual exposure of jennies and their foals and to check the safety of milk for minimizing hazards to human health.

OTA contamination in bulk of cereals is often randomly distributed in localized "hot spots" with high variability and this characteristic property of the contamination may reflect the fact that OTA is typically produced during storage. Nonetheless, it is very difficult to determine the extent of mycotoxin contamination in feedstuffs because mycotoxins are unevenly distributed in feed, introducing a significant amount of sampling error into sample analyses.

According to the European Commission (EC) regulation, official control of the levels of OTA in lots of cereals and cereal products has to be performed by collecting incremental samples (up to 100 samples) to obtain a laboratory sample that should be representative of the entire lot. In order to overcame an underestimation of OTA contamination and to provide an accurate and precise OTA determinations values the slurry-mixing procedure is strictly necessary as reported (Lippolis, Pascale, Valenzano, & Visconti, 2012). This procedure was used in this study to reduce the variability related to the sampling of concentrated feed. The OTA levels found in single component of mixture and in the concentrate mixture were lower than values laid down by the Commission Recommendation 2006/576/EC (European Commission Recommendation (EC) No 2006/576 of 17 August 2006) equal to 250 mg/kg in the case of cereals and cereal-derived products and for complementary and complete feedstuffs are 50 and 100 mg/kg for pigs and poultry, respectively. This paper confirmed that, although feed sampling was conducted according to European regulations and despite the use of the slurry, feed analysis is not an accurate approach to assess the exposure of animals to OTA. In fact, the incidence of OTA contaminated feed samples was lower than the incidence of OTA in blood samples, validating the usefulness of biomarkers as a more accurate approach for assessing OTA exposure. Concerning blood samples from jennies, OTA levels resulted not toxic for the health of jennies and their pregnancy. In addition the median value and the incidence of OTA level in blood samples collected from jennies were lower than those

found in horses (121.4 ng/L and 83% respectively), probably due to diet composition (Minervini, Giannoccaro, Nicassio, Panzarini, & Lacalandra. 2013). In fact the majority of horses (from 64% to 80%) fed with commercial feed, hay and oats had less OTA in serum (mean value = 150 pg/mL) than horses fed with bran (mean value = 339 pg/mL) (Minervini, Giannoccaro, Nicassio, Panzarini, & Lacalandra. 2013). Instead the jennies, notoriously more rustic animals than horses, have a diet mainly represented by hay with a supplement of feed during pregnancy and lactation. An increased OTA level in blood samples seasonal-related was recorded probably due to possible OTA contamination of the hay. As reported (Fink-Gremmels. 2008), the ensiled grass or hay may contain a complex mixture of mycotoxins, originating from a pre-harvest contamination by *Fusarium* spp. toxins, as well as from post-harvest contamination with toxins produced by fungal species that are common in silage during the storage, such as *Penicillium*, *Aspergillus*, *Monascus*, and *Trichoderma*, some of them OTA producers (Fink-Gremmels. 2008). Concerning foals, no placental transfer was observed because the blood samples collected from umbilical cords after delivery did not contain OTA. These results were in disagreement with data reported for horses (Minervini, Giannoccaro, Nicassio, Panzarini, & Lacalandra. 2013) on the exposure of foals, after delivery of mares, consequent to the placental transfer with higher blood OTA concentrations (from 69.5 to 252.6 ng/L). The ratio between mare's and foal's OTA serum levels was variable and no correlation ($r = -0.07$) was found. The absence of a correlation between OTA levels in umbilical and mare serum samples was in agreement with the results reported in pigs after *in vivo* exposure (EFSA 2004), and could be explained by several factors, such as exposure time (gestation period and related placental vascularization) and/or placental structure. Concerning the gestation period, during the early stage of pregnancy, OTA or its metabolites could pass through the placenta into the foetal circulation and accumulate in the foetal tissues, exerting developmental toxicity. Concerning placental structure, swine and equine placenta are characterized by a chorionic epithelium which could prevent or interfere with the access of toxin to the umbilical blood *in vivo* and consequent OTA foetal uptake, strictly related also to the developmental stage of the placenta. The disagreement of placental transfer between mare and jennies could be related to different anatomical structure of placental on both animal species; in fact, as reported by Veronesi et al. (Veronesi, Villani, Wilsher, Contri, & Carluccio. 2010) although strong morphological similarities exist between the allantochorion of the horse and jenny, the jenny develops more complex microcotyledons, as judged stereologically, and exhibits a lower placental efficiency. The OTA exposure of foals after delivery can be realized through milk as confirmed by Francisca and relevant foal, showing the highest levels of OTA both in the blood of jenny and foal and in milk samples. For the other jennies was not possible to find any relationship probably due to the animals were reared extensively in open stabling. A positive

relationship between serum OTA level and serum/milk OTA concentration ratio was found in this study and was in agreement to data found in humans (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri, & Rossi. 2011). The same pattern of OTA excretion found in women and jennies indicated that, at increasing OTA blood level, the carry-over to milk rises less than proportionally, suggesting a decrease in the efficiency of carry-over and probably a saturation of the transport system (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri, & Rossi. 2011). The levels of OTA found in jenny's milk were 10 times lower than those reported in woman's milk samples at the same incidence (Biasucci, Calabrese, Di Giuseppe, Carrara, Colombo, Mandelli, Maj, Bertuzzi, Pietri, & Rossi. 2011). On the contrary, similar levels, but with higher incidence (almost 60%) of human milk samples, was reported (Galvano, Pietri, Bertuzzi, Gagliardi, Ciotti, Luisi, Bognanno, La Fauci, Iacopino, Nigro, Li Volti, Vanella, Giammanco, Tina, & Gazzolo. 2008). The major OTA contamination/incidence found in human milk samples should be due to both different sources of OTA exposure (different foods suspected of contamination) and to the high half-life (35 days) in humans. Up to now, no data are available concerning the OTA half-life in equine species. The occurrence of OTA in milk samples collected from jennies in this study was in disagreement with results reported by (Gross, Ploetz, & Gottschalk. 2019) probably due to the different analytical determination used (ELISA respect to HPLC) and to the low number of milk samples (n=6). In our study, continuous monitoring of donkey milk has allowed to shown the presence of OTA in the blood and milk probably due to a batch of hay of poor quality and containing OTA. The seasonal effect observed on OTA presence in milk samples should indicate the need of continuous monitoring of OTA levels in monkey milk in order to guarantee the safety of this food intended for infant consumption. Further studies should be carried out in order to confirm the contamination of donkey's milk with OTA, which could pose a real health risk to new-borns.

2.2.5. References

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CONCLUSIONS

People are constantly exposed to toxic compounds present in the environment, food, drugs, and for instance cosmetics or due to occupational exposure. Together with environmental toxic compounds, exposure to food contaminants, such as mycotoxins, can also affect animal and human reproductive function. These effects were found when exposure occured at different times throughout the lifetime from the prenatal to the adult period, possibly due to different mechanisms.

The *in vitro* tests are projected with a clear knowledge on the main targets of reproductive toxic compounds and their involved mechanisms, avoiding the use of a large number of laboratory animals. The possibility to use slaughterhouse material for in vitro tests related to gametes and early embryo development appears as an important option to diminish the number of in vivo tests. Mycotoxins like deoxynivalenol (DON), zearalenone (ZEA) and its metabolites (alpha- and beta-zearalenol) can cause reproductive failure. These mycotoxins were tested on bovine and porcine models in order to test the influence on oocyte maturation, the fertilization and the embryo development (Santos et al, 2014).

Our studies integrated the list of mycotoxins by assessing the effects of OTA on ovine oocyte maturation and embryo development as translational model for reprotoxicity studies at toxicological and environmental concentrations. In the sheep model, we found that it affects oocyte maturation with carryover effects on embryo quality. The findings suggest that OTA may be a major threat to female fertility and could be a risk factor for oocyte reduced maturation and reduced embryonic development in the infertile couple. The long term exposure to different doses and their possible side effects need to be further investigated at clinical level. The safe dosage limit needs to be acknowledged in order to prevent overexposure to OTA.

The exposure to mycotoxins during pregnancy may have detrimental effects on pregnancy outcomes. Kyei et al (2020) identified ten studies that evaluated the effects of maternal or fetal exposure to Aflatoxins (AFLs) on the occurrence of intrauterine growth restriction. Only three studies evaluated the effect of a blood AFL biomarker on gestational age or preterm birth. Concerning fumonisins, only two studies were reported on maternal fumonisin B (FB) exposure and adverse pregnancy using the blood sphinganine/ sphingosine (sa:so) ratio that was associated with an increased risk of neural tube defects. More prospective studies, using pregnancy and birth surveillance systems or using linked databases, are still needed to firmly establish the effects of mycotoxin exposure on adverse pregnancy outcomes in humans. In particular, studies should take advantage of recent development in biomarker analysis.

In this study, Martina Franca jennies were used as in vivo translational model to assess the fetal exposure ot OTA. Analysis of blood samples collected from jennies at late pregnancy indicated the

exposure to OTA at low levels, but the absence of OTA in umbellical blood collected from delivery, attested the lack of OTA placental tranfer. These results indicated that jennies cannot be used as a translational model to assess fetal exposure to mycotoxins in woman. On the contrary, jennies results a good translational model to assess the exposure to newborns through the milk. In fact a good correlation, similar to that found in women by Biasucci et al (2011) was found between the transfers of OTA from blood to milk. The occurrence of OTA in jenny's milk should be further monitored, due to its known high nutritional value. These data integrate previous reports on the occurrence of different mycotoxins, such as AFLs, fumonisins, zearalenone, deoxinivalenol in human breast milk (Memis and Yalcin, 2019).