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Ochratoxin A induces craniofacial malformation in mice acting on *Dlx5* gene expression

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1. ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by fungal of *Aspergillus* species absorbed in human through contaminate food in gastrointestinal tract. OTA has been demonstrated to be teratogenic in a number of species including mice and potentially human. Mice exposed in uterus to OTA develop craniofacial abnormalities such as exencephaly, microencephaly, microphthalmia and facial clefts. An important role in differentiation of maxillofacial are exerted by the Hox related genes *Dlx* and *Msx*. In the present investigation we have confirmed that 2.75 mg/kg body weight OTA, given at gestational day 7.5, induces significant developmental craniofacial anomalies in mice and we have demonstrated the down expression of *Dlx5*, a member of *Dlx* gene family, that seems to be responsible of the observed deformities. These results support the hypothesis that *Dlx5* is a target for ochratoxin and the inhibition of its function, directly or indirectly, could be at origin of the observed differentiation defects.

2. INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, carcinogenic and teratogenic mycotoxin produced as a secondary metabolite by certain fungal of *Aspergillus* and *Penicillium* species (1). Mycotoxins as ochratoxin A, Citrinin and Sterigmatocystin, are isocumarinic derivatives of phenylalanine and are widespread contaminants of grains and agricultural products. OTA is commonly found in animal feeds and human foodstuffs including cereals products, dried fruit, dried fish, coffee (2;3). Considerable levels of OTA have also been found in red wines at concentrations up to 7 mg/l, meat and meat products, and in confectionary with contamination levels ranging from 0.1–3.8 mg/ kg (4) (3). In particular, the fungal production of this mycotoxins is optimized by humidity ranging 15-19% and temperature more than 15°C and pH 5.5 (5).

The OTA is absorbed in human through contaminate food in gastrointestinal tract, entheropatic

circulation and proximal tubule of nephron (6) accumulating in kidney, moreover is vehiculated and stabilized in serum by albumin, accumulating in lung, liver, kidney, hearth, adipose tissue and gut at different concentrations. Individual variation in plasma levels of ochratoxin A are founded in humans. (3).

Exposure to OTA has been demonstrated to be teratogenic in a number of species including rats (7), mice (8), hamsters (9), chick (10) and potentially humans.

Surprising despite the fact that OTA is a specific and potent animal teratogen, largely distributed in food and detected in all human plasma samples (11) with an half-life in humans approximately 6–13-fold longer than that reported in rats (12, 13) and that children and infants have been suggested to be more at risk from the toxic effects of OTA, very little or no research has been undertaken to identify possible risks for human fetal development.

Many effects of OTA administration *in vivo* in rats and mice are also observed in humans, but it is not known the mechanism.

The most common defects observed in mice exposed *in uterus* to OTA are craniofacial abnormalities such as exencephaly, microencephaly, microphthalmia, facial clefts, and hypoplastic jaws, all of which appear to be dependent on both the dose and the period of gestation during which the toxin is administered (13).

The period of gestation in which there is, in mice and in rat, the major sensibility to OTA, is between the day 6 and 8 post coitum (pc) (14, 15) with a minimal dose of 3mg/Kg body weight.

The timing of these effects coincides with the period of neurulation. Once the neuropores are closed, subsequent exposure has not been observed to be teratogenic. This may be due, probably, to reduced placental transfer later in development (16, 17) and for a specific action of the toxin on the developing nervous system (18, 19, 20).

For this reason it seems interesting to make an analysis of embryological malformations induced in mice by OTA exposure and evaluate the genetic events that are involved in these mechanisms. Our interest was to understand what genes are target for OTA, with particular interest for the genes involved in differentiation and, in particular, in the differentiation of maxillary and craniofacial. We focused our attention on homeotic genes and on some homeotic related genes.

For the correct morphogenesis of the different segments of mouse embryo are necessary the concerted action of Hox genes (21). These genes constitute a highly conserved family of homeobox genes that act as transcription factors. An important role in differentiation of maxillofacial are exerted by *Dlx, Msx* and *Otx* genes however, their mode of action as regulatory molecules, might be more complex as it has been shown that members of the *Dlx* family can form dimeric complexes with *Msx* homeoproteins mutually affecting their DNA-binding properties (22). With respect to their biochemical property *Msx* proteins act as transcriptional repressor, while *Dlx* protein are transcriptional activator (23).

In the mouse, there are at least six *Dlx* genes arranged as pairs and located near Hox clusters (*Dlx1* and *Dlx2* near HoxD; *Dlx3* and *Dlx7* near HoxB; *Dlx5* and *Dlx 6* near HoxC) (24, 25).

Dlx genes are all expressed in spatially and temporally restricted patterns in craniofacial primordia, basal telencephalon and diencephalon. The pattern of expression of *Dlx5* differs from that of the other members of the family, in fact *Dlx5* is expressed much earlier than other *Dlx* genes (26) during development in territories that define the rostral and lateral border of the neural plate and the rostral prosencephalon, moreover *Dlx5* and *Dlx6* are expressed in all developing bones from the time of initial cartilage formation (24, 27).

A further indication of the possible importance of *Dlx5* in the control of bone differentiation comes from a study (28) in which it has been shown, that this gene is expressed at specific stages of osteoblast differentiation and could repress the *osteocalcin* gene expression by interacting with a single homeodomain-binding site in its promoter (29).

Studies of targeted inactivation of *Dlx 5* genes, as reported by Acampora *et al.* (29) , shown as homozygous mutant not survive longer than 24 hours after birth, and about the 12% of embryos presents exencephaly in addition to other severe phenotype modifications. *Dlx5* expression in brain begins around 10 days post coitum (dpc) and the first phenotypic difference, induced by the *Dlx5* homologous recombination inactivation, appears at 12.5 dpc, while craniofacial malformations were well visible at 14.5 dpc. Mutant embryos could be recognized by their shorter snout and open fontanelle, while in the few exencephalic embryos the craniofacial defects were more conspicuous. Moreover *Dlx5* mutant embryos at 14.5 dpc present severe malformations in maxillary region with a cleft secondary palate and the loss of horizontal laminae of the palatine bones, moreover the nasal and maxillary bones are shorter, resulting in a general reduction of the length of the snout. The palatine processes of the maxilla are reduced especially with respect to their posterior development and they fail to form proper connections with the palatine bones (29) .

At E16.5 *Dlx5* mutant have defects in olfactory placode and hypoplasia of frontonasal prominence derivatives more in particular they present pronounced asymmetry of nasal capsule with a nearly complete loss of the right nasal apparatus (30).

On the bases of these considerations we wanted to evaluate the possible regulatory effect, induced by OTA administration during a critical moment of gestation, on expression of some homeotic related genes in order to understand the possible mechanism of the mycotoxin in the

induction of differentiation defects. We have concentrated our attention principally on the two homeotic gene *Dlx5* and *Msx1*, that have opposite roles in the regulation of transcription, in fact, as mentioned above, *Msx* proteins act as transcriptional repressors, while *Dlx* protein are transcriptional activators (23, 31).

3. MATERIALS AND METHODS

3.1. Experimental animals

Sexually mature C57Bl6 female mice (Charles River Laboratory) were maintained on standard conditions, feed and water available *ad libitum,* in a temperature controlled and artificially illuminated room (12-h light:12-h dark cycle), free from any source of chemical contamination. After an acclimatization period of 1 week, females were mated with mature males of the same strain and the day on which were found vaginal plug was designated as day zero of pregnancy. After mating, the female mice were individually housed in polypropylene cages.

3.2 Ochratoxin Treatments

Pure ochratoxin A (Sigma Chemical Ltd), was dissolved in 0.1 M Sodium Bicarbonate solution. Pregnant female were injected intraperitoneally (i.p.) with a single dose of OTA at 3.0 mg/Kg body weight, in 100µl of 0.1M Bicarbonate vehicle, on day 7.5 of gestation and embryo were taken at day 16 (E16) post coitum (pc). Control mice was injected at same time with only 100µl of sodium bicarbonate vehicle.

3.3. Embryo analysis

Pregnant, OTA treated, females and control animals were killed by cervical dislocation on gestation day 16. Embryo E16 were removed from uterus and fixed in Bouin's fixative (1% saturated picric acid, 5% acetic acid, 24% formaldehyde, in distilled water).
The fixed embryo were photographed to study the morphology and the differentiation defects induced by OTA treatments. After no more than three days the embryo were washed in 70% ethanol, embedded in paraffin, according standard protocols, and sectioned at 5 µm slide for *In situ Hybridization* (ISH).

3.4. *Dlx5* and *Msx1* gene fragments purification

A 216 bp *Dlx5* and 350 bp *Msx1* gene fragments were prepared by reverse transcription polynucleotide chain reaction (RT-PCR) starting from total mRNA extracted from E15 mouse embryo**.**

Total mRNA was extracted from E15 embryo brain according Chomczynski and Sacchi method, using RNAzol (Invitrogen Co. Ltd) according the manufacturer's instructions and the integrity of purified RNA was verified by agarose gel electrophoresis. For reverse transcription 2 µg of total RNA in a final volume of 20 µl was reverse-transcribed by Avian myeloblastosis virus (AMV) reverse transcriptase (Gibco BRL- Invitrogen Ltd) according manufacturer's instruction in presence of random examer primers (Promega Ltd) at 37°C per 60 min.

PCR amplification of *Dlx5* genes fragment (216 bp), *Msx1* (350 bp) and *Actin* (500 bp) was performed by using a Gene Amp PCR system 9700 (Applied Biosystem Ltd) and hot start Taq Gold (Applera Ltd). *Actin* was used as housekeeping control gene. The *Dlx5*, *Msx1* and *Actin* PCR primer were: *Dlx5* Fw cca gcc aga gaa aga agt gg; Dlx5 Rw tca cc gtg ttt gcg tca gt; *Msx1* Fw agc tct gct gcc cta tac ca; *Msx1* Rw ggg ctc atc tct tga agc ac; *Actin* Fw gac tac ctc atg aag atc ct; *Actin* Rw gct tgc tga tcc aca tct gc. The PCR condition was for *Dlx5* and *Msx1*: initial denaturation at 95° for 10' followed by 36 cycles: 95°, 45''; 53° , 45'' and 72° 45'' with a final extention at 72° for 10', while for *Actin* initial denaturation at 95° for 10' followed by 32 cycles: 95°, 45''; 60°, 45'' and 72° 45'' and with a final extention at 72° for 10'. The amplification products were run on 1% agarose gel electrophoresis in 0.5 x TBE (Tris Borate EDTA) buffer for the control of the amplicons length.

3.5. *Dlx5* and *Msx1* riboprobe preparation for *ISH*

For the preparation of *Dlx5* and *Msx1* DIG-labelled riboprobes, the *Dlx5* 216 bp and the *Msx1* 350bp fragments, obtained by PCR, were purified from agarose gel by electroeluition and inserted in the PCR cloning vector pGEM T easy (Promega Co. Ltd) according the manufacturer's instructions, to obtain the pGEM-Dlx5 and pGEM-Msx1 recombinant plasmids.

The cloned *Dlx5* and *Msx1* exact sequences and the fragments orientation was controlled by sequencing using an automatic system (Primm Sequencing Core; Primm Italy) that shows how these fragments were both oriented in direction 3'-5'within the recombinant plasmids.

For the sense and anti-sense RNA-probes synthesis, 4µg of pGEM-Dlx5 and pGEM-Msx1 recombinant plasmids were linearized respectively with the restriction enzyme *Nco I* for the riboprobe sense (Sp6 transcription) and *Sal I* for the riboprobe anti-sense (T7 transcription), obviously, *Nco I* and *Sal I* restriction site are not present in the *Dlx5* and *Msx1* cloned sequences.

For the synthesis of sense and anti-sense DIG labelled RNA-probes, 1 µg of each linearized plasmid was transcribed using a DIG RNA labelling mix from Roche (Roche Applied Sciences Germany) according the manufacturer's instructions and the *Dlx5* and *Msx1* riboprobes, purified by ethanol precipitation in presence of 4 M Lithium chloride, were quantified by electrophoresis on agarose gel.

3.6. *Dlx5* and *Msx1 in situ* hybridization

Five-micrometer paraffin sections were dewaxed in xilol twice for 5' each time, rehydrated in graded concentrations of ethanol and rinsed in diethilpyrocarbonate-treated PBS. The sections were fixed in 4% paraformaldehyde in 0.5M NaCl, 0.1M MOPS, pH 7.5, for 30' at room temperature, then washed in 1x PBS. The slides were treated with 10µg/ml protease K in 100 mM Tris-HCl and 1mM EDTA at pH 7.2 for 7' at room temperature and rinsed in 1x PBS for 5' and then transferred in 5x SSC twice for 2' each time. After pre-hybridation performed at room temperature for 30' in Tris

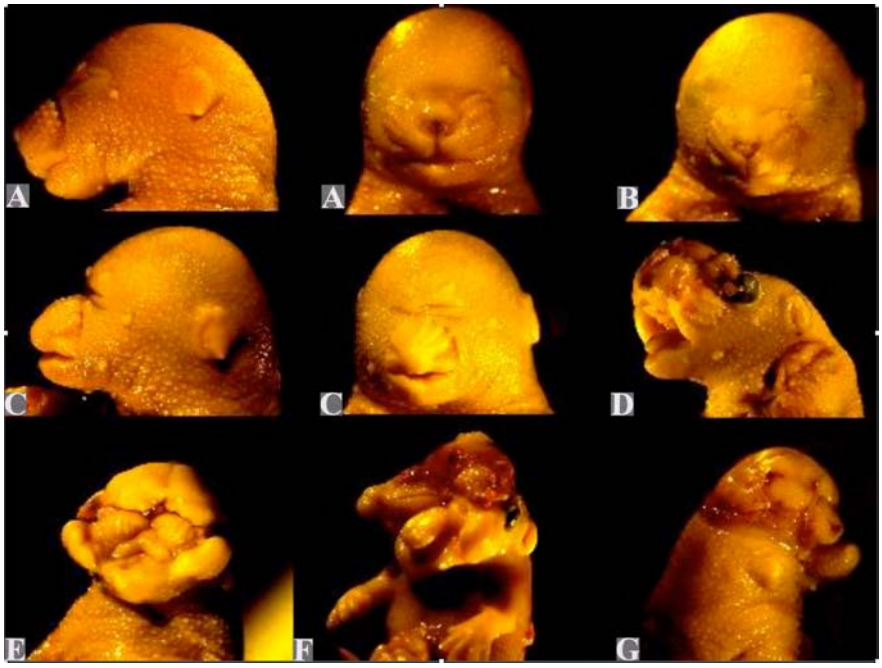

Figure 1. E16 embryo following OTA treatments on gestation day 7.5. A: lateral and frontal vision of E16 normal control embryo without OTA treatments. B: OTA treated embryo showing maxillary asymmetry. C: frontal and lateral vision of embryo presenting synophthalmia with absence of normal eye replaced by a central pseudoocular formation. D: absence of normal head formation with evident encephalocele. E, F, G: loss of head formation with severe exencephaly.

glycine buffer at pH 7.2, the sections were hybridized, in a humidified chamber, overnight at 60°C in a buffer containing 40% deionized formamide, 5x SSC, 1x Denhardt's solution, 100 µg/ml sonicated salmon testes DNA, 100 µg/ml transfer RNA and 80 ng digoxigenin-labeled *Dlx5* or *Msx1* complementary RNA probe. After incubation, the slides were washed 3 times in 5x SSC for 20' each and then in posthybridization buffer (0.5x SSC, 20% deionized formamide) at 60°C for 40 ' and incubated in NTE (0.5M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.0) containing 10 µg/ml ribonuclease A for 30' a 37°C.

The slides were rinsed in NTE for 15' at 37°C, then washed in post-hybridization buffer for 30 min at 60°C and rinsed in 2x SSC for 30' at room temperature. The sections were incubated in 1% blocking solution (1% blocking reagent Roche Diagnostics, Basel, Switzerland) in MBT buffer, (0.1M maleic acid, 0.15M NaCl, pH 7.5) for 10' before the overnight incubation at 4°C with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics), diluted 1:2000 in MBT buffer. The slides were rinsed 4 times in TBS (25 mM Tris-HCl pH 7.5, 0.15M NaCl, 2.5mM KCl) pH 7.4 for 10' each and then in solution B (0.1% Tween 20, 0.5 mg/ml levamisol) for 10'. The colour detection substrate: 1ml BM purple, 10 µl of 100x solution B (Roche Diagnostics), was applied and the incubation was carried out overnight in the dark at room temperature. The reaction was terminated by rinsing the sections in PBS 1x, 1mM EDTA for 10' at room temperature. The slides were dehydrated and mounted.

4. RESULTS

4.1. Differentiation defects induced by ochratoxin A treatment

The OTA treatment at a very early gestation time consists in a very dramatic differentiation defect, specially concentrated in maxillary craniofacial body segment.

When we compare the normal head development, in untreated mice (Figure 1A), with the pups derived from OTA treated dam we may observe, in the same progeny, different malformation degree not apparently correlated to an experimental difference in the procedure or in quantity and timing of drug administration. On the basis of these observation we can confirm a generalized high toxicity with a different sensibility of different embryos ranging from little to monstrous malformation. In fact, in the same progeny, we observe embryos with not apparently severe differentiative defect, consisting essentially in loss of symmetry in maxillo-facial formation (Figure 1 B) or a more severe deformity with a loss of an ocular formation with replacement of a large central eye (Figure 1 C). On the other hand we can observe progeny with a very high malformation degree probably correlated to a major dam sensitivity to the drug. In fact, embryo derived from an other mouse present in our experiment very dramatic generalized malformation with macro encephalocele in frontal region (Figure 1 D) or

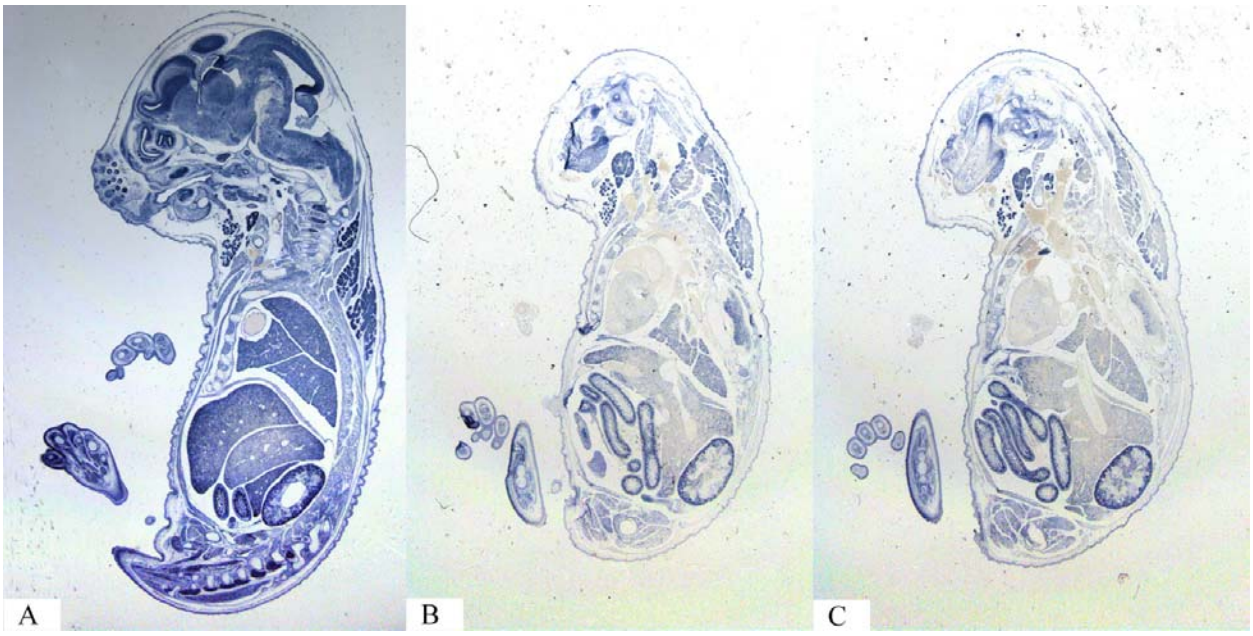

Figure 2. *Dlx5 in situ Hybridization* following OTA treatments on gestation day 7.5. A: E16 normal control embryo without OTA treatments showing high expression of *Dlx5* gene in almost all brain and maxillo-facial structures. B: *Dlx5 ISH* of OTA treated embryo showing pseudo-ocular formation (see Figure 1 C) present a confused or absent *Dlx5* expression overlapping between retina and the hypothalamus inferior part (see text). C: *Dlx5* ISH of OTA treated embryo with severe craniofacial malformation showing low and confuse *Dlx5* expression, that do not identifies the anatomic structures in cranial formation.

monstrous loss in head formation as in (Figure 1 E) or a severe exencephaly with absence of cranial formation (Figure 1 F)**.**

4.2. *Dlx5 in situ* hybridization

In E16 embryos obtained from pregnant mice that did not received OTA we observed high *Dlx5* gene expression in almost all brain and maxillo-facial structures (Figure 2 A), which were morphologically highlighted by the expression of *Dlx5* so that we could detect easily the differences in malformations induced by OTA treatment.

Interestingly *Dlx5* expression was very reduced in almost all embryonic structures when we administered OTA at pregnancy day 7.5. More in detail, in the different craniofacial malformed structures we found an altered *Dlx5* expression pattern. In particular, in OTA treated deformed mice, we observed an absence of correct development of the facial prominence together with absence of the face bone, olfactory epithelium and palatal-sheet development, of course in these structures there was a confused or absent expression of *Dlx5*, that was instead clearly expressed in the same parts in control mice (Figure 2 B, C).

The wisker-follicles, that clearly identify rostral prominence and olfactory part and show a strong *Dlx5* expression in control mice (Figure 2 A), are not evident in OTA treated embryos, in agreement with a strong anatomical alteration in the development of this body district.

Any *Dlx5* expression was found in tongue and in the face bone, because these structures was strongly reduced in size and deformed (Figure 2 B,C).

Some embryos, obtained from OTA treated mothers, as results from morphological analysis (Figure 1 C), seem to be *monocular* showing just one big pseudo eye in the middle of the face and they presented also a *Dlx5* expression overlapping between retina and the hypothalamus inferior part, that seems to correspond to an anatomical overlapping of these structures, quite confused in treated embryos (Figure 2 B). At contrary in the normal mice the retina and hypothalamus *Dlx5* expression was so clear to identify the anatomical boundary of the structures (Figure 2 A).

Despite a quite normal developed cranial box, analyzing the brain tissues, we found an evident down expression of *Dlx5* in all the brain structures, while in normal mice there was a clear and strong expression of the gene in neocortex, striatum, thalamus and hypothalamus, superior and inferior colliculus, tegmentum, inner and middle ear (Figure 2A). Much more difference of *Dlx5* hybridization, between treated and untreated mice, was observed in pons, medulla and cerebellum (Figure 2 C) that were considerably damaged by OTA administration.

It is important to underlie that, in OTA treated mice, the absence of Dlx5 expression in some anatomical parts is due to the absence of the correct structures formation.

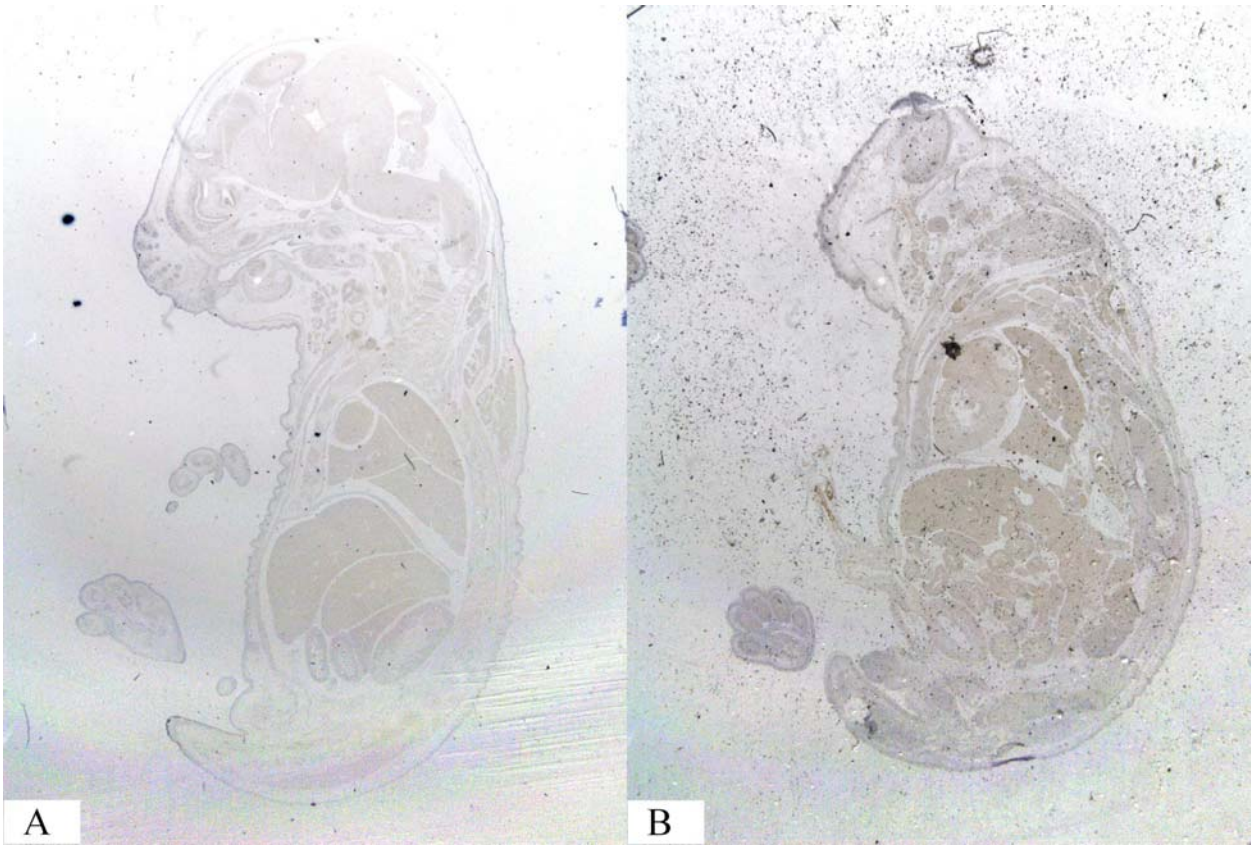

Figure 3. *Msx1* is very little expressed in embryo mouse at stage E16, and the expression of this gene does not differ in embryo derived from OTA treated mice. A. untreated control E16 embryo; B: OTA treated E16 embryo.

4.3. Msx1 *in situ* hybridization

The results obtained from *Dlx5 In situ Hybridization* induce us to hypothesize the existence of a negative regulation of genes involved in the craniofacial differentiation after treatment with ochratoxin A.

On this base also the *Msx1* gene, that acts as transcriptional inhibitor, contrary to *Dlx5*, could be hyper expressed or remain constant in a system were we hypothesize the inhibition of a genetic pattern.

The *Msx1 In situ Hybridization* results have not demonstrated differences in the expression rate of this gene and considering that *Msx1* is very little expressed in embryo mouse at stage E14 (23) and E16, and that the expression of this gene does not increase in embryo derived from OTA treated mice (Figure 3 A, B) we can arrive to the conclusion that OTA do not interact with *Msx1* promoter and that there is not inhibition induced by a major presence of *Msx1* gene product.

More in deep we could hypothesize that genes controlled by *Msx1* are not down regulated from a major presence or *msx1* protein, after OTA treatment.

The teratogenic effects observed, after OTA administration, are probably correlated to the loss of transcriptional activation of genes controlled from the *Dlx5* gene product.

5. DISCUSSION

The present study, was undertaken to determine as a single intra peritoneal dose of ochratoxin A (2.75 mg OTA/kg body weight), was teratogenic for the offsprings of pregnant C57Bl6 mice, when given at the 7.5 gestation day, during the major organogenesis period, and if there is a subset of gene, target of OTA, that are responsible for the correct development of a particular body segment, in the aim to explain what is the possible mechanism for the induction of differentiation defects observed after administration of ochratoxin A or others mycotoxins.

OTA has been suggested by various researchers to mediate its toxic effects via induction of apoptosis, disruption of mitochondrial respiration and/or the cytoskeleton or via generation of DNA adducts (32) .

Teratogenic effects of OTA have been well documented in mice (33), with craniofacial abnormalities being the most commonly observed malformations (14).

High lipophilic nature, efficient absorption of OTA from the gastrointestinal tract, an extremely high affinity to the serum albumin and other macromolecules,

with the consequent extremely long serum half-life (34), and a very low extent of biotransformation led to persistence of OTA in the body of consumers (35) .

In the present investigation we have confirmed that 2.75 mg/kg body weight OTA proved to be an effective dose to exert significant developmental toxicity in the foetuses of the pregnant mice. The early stage of gestation as GD 7 and 8 was found to be the most critical for the induction of various types of anomalies in the embryos. After treatment on these days, the highest percentages of gross and skeletal malformations were observed.

The types of malformations and their sites predominantly were the regions of head and face and were, in general, similar to those reported by earlier workers (36, 37, 38, 39).

It was evident that the specific action of OTA, during the early neurulation stage in embryonic development, was critical to induce anomalies, mostly in the craniofacial region.

The embryo defects observed in our experiments are in accord with the results obtained by Wei and Sulik in their magnificent work (14).

Wei and Sulik demonstrated, as after OTA treatment of pregnant mice at gestation day 7 - 8, the cell death, resulting from vital staining with Nile blue sulphate, is localized in selected cell population, and interestingly the area of major cell death was localized in the somatopleuric portion of the lateral plate mesoderm, that is the precursor of the body wall. As demonstrate by these authors staining was heavy in the frontonasal prominence, the region rostral to the developing eyes, when compared with staged untreated control. Some E16 embryos, furthermore, shown exencephaly while the craniofacial malformation, well evident at this stage, consist in a remarkable deformation or absence of nasal prominence derivative, frequently associated with midline cleft, and anterior neural tube closure defects, that proper derive from excessive cell death in the neuroepithelium and premigratory neural crest cells. The ocular structures frequently were reduced in size and closed positioned with hypotelorism or synoftalmia (14).

Being not clear the basis for the vulnerability of selected cell population to OTA, and the correlated teratogenic mechanism, we have presupposed the involvement of some Hox related gene that, if down or up expressed, can deregulate the correct gene cascade activation necessary for the differentiation program.

Dlx5 and *Msx1* seem to play an important role in palatal formation and more in general in craniofacial differentiation, and for this motif we have studied the expression of these two genes in malformed embryos derived from mice treated with ochratoxin.

Also if *Msx1* and *Dlx5* act independently in the development of craniofacial skeleton (31) their expression in the developing head appears to be complementary. *Msx* proteins are mainly transcriptional repressors (40), while *Dlx* proteins are usually activators (41). For this reason, the possibility that the Msx1 protein may normally repress *Dlx5* expression in the palate appeared likely. Since, the *Dlx* and *Msx* homeoproteins are known to form heterodimers *in vitro* and the interaction leads to abrogation of their DNA-binding and transcriptional activities (22), some of the phenotypes observed in *Msx1* or in *Dlx5* mutant animals could be due to altered activity of the cognate protein partner. On this basis the ochratoxin could interfere in the heterodimer formation.

Several mechanisms have been proposed for *Dlx* function. *Dlx* genes may instruct cell precursors of the palatal early in development (i.e. neural crest or arch ectomesenchyme, where they are expressed), or may control expression of secreted diffusable molecules, or the cleft is the consequence of a generalized deformation of the cranium. (31) .

Moreover a variety of molecules have been implicated in signalling during morphogenesis of facial primordia, including secreted molecules (Shh, Bmp, Wnt, Fgf) and transcription factors (*Dlx*, *Otx*, *Msx*, *Gli* and *Tbx*) (42, 43, 44) . *Dlx5* could act directly as an inhibitor of bone morphogenetic protein expression as Bmp4, or could modulate Bmp function by regulating the expression of Bmp antagonists, such as Noggin, Chordin, or Follistatin. Bmp4 is expressed at sites of fusion between prominences of the head primordium, including the palate (45) .

In the mouse, palatal cleft is often associated with a down regulation of Bmp4 in the anterior palate. More precisely Bmp4 do not function alone but in concert with the others bone morphogenetic protein as Bmp2 and Bmp7 (46) .

As demonstrated by Acampora *et al.*, disruption of *Dlx* genes causes palatal cleft (29, 30, 47), although disruption of *Dlx5* leads to a less severe cleft, as compared to *Msx1*.

The widely described role of *Dlx5* in the maxillofacial structures formation supports our results, in fact the widespread shutdown of *Dlx5* gene in embryos, derived from pregnant mice treated with OTA, it seems closely related to the observed deformities.

These results support the hypothesis that *Dlx5* gene is target for this toxin and the inhibition of its function, directly or indirectly, could be at origin of deformities caused by this mycotoxin. It remains to be demonstrated whether the OTA inhibits the transcription of *Dlx5* binding to the promoter of this gene or acting on other genes, which in turn regulate the expression of *Dlx5*.

Our *in situ* hybridization experiments, concerning *Msx1*, did not reveal any change in the expression of this gene between control embryos and those derived from OTA treatment. This does not exclude the involvement of *Msx1* gene in more earlier stage, even considering that at E16 stage, as well as E14 one, this gene is expressed at very low level. Moreover, since the proteins

derived from *Msx1* and *Dlx5* form eterodimers, the reduced expression of *Dlx5* could reduce the possibility of formation of these eterodimers, the molecules that are proper functionally active in regulating the development of maxillo-facial segment. Another hypothesis is that the phenotypic changes observed do not depend by *Dlx5* expression inhibition, but by the resulting deregulation of the downstream genes, like those coding for bone morphogenetic proteins, specifically involved in the formation of the palate. A reduced expression of *Dlx5* then could act indirectly by altering the expression of these effector proteins, that results in an alteration of this differentiation pattern.

Because of widespread contamination and increased incidence of ochratoxin in human food (48, 49) and the presence of OTA in human blood, cord blood samples from pregnant women, colostrum and milk, fetal exposure of OTA might pose a potential risk in pre-natal and post-natal life for the human infants (50, 51). On the basis of these considerations we think it is important to improve the studies on mycotoxins food contaminations and on the molecular mechanism involved in the determination of differentiation defects.

6. ACKNOWLEDGEMENTS

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Abbrevations: OTA: Ochratoxin A; Dlx: distal-les 5; Otx: orthodenticle ; Msx: muscle segment homeobox; Hox: homeotic gene; DIG: dioxigenin; Tbx: T- box gene; Bmp: bone morphogenetic protein; Shh: sonic hedgehog gene; Wnt: wongless gene; Fgf: Fibroblast Growth Factor.

Key Words: Ochratoxin A; mycotoxin; mouse teratogenicity; craniofacial abnormalities; maxillary differentiation; Homeotic gene; Dlx5 gene; Msx1 gene

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