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Zinc, thymic endocrine activity and mitogen responsiveness (PHA) in piglets exposed to maternal aflatoxicosis B_1 and G_1

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Abstract

Growth retardation, thymic involution and impaired peripheral immune efficiency are constant events in piglets exposed to maternal aflatoxicosis. Zinc may play a key role because of its requirement for good immune responses, including thymic endocrine activity. Zinc is required to activate a thymic hormone, i.e. thymulin (ZnFTS), which is responsible for cell-mediated immunity. Zinc deficiency and decreased thymic endocrine activity are present in piglets fed from sows exposed to aflatoxins (AF) B_1 and G_1 as compared with healthy control piglets. In particular, active ZnFTS is decreased while concentrations of inactive thymulin (FTS) are high. The in vitro addition of zinc up to the plasma samples induces a reduction of inactive thymulin. The lymphocytes mitogen responsiveness (PHA) is decreased and a thymic cortical lymphocyte depletion is also present. These data suggest that the thymic defect, followed by impaired peripheral immune efficiency, may largely depend by the low peripheral zinc bioavailability to saturate all thymulin molecules produced. © 1998 Elsevier Science B.V.

Keywords: Zinc; Thymulin; Mitogen responsiveness; Aflatoxins; Piglets

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1. Introduction

Aflatoxins (AF) B_1 and G_1, are secondary mould metabolites by *Aspergillus flavus parasiticus*, chemically related to polycyclic furan compounds (for review, see the work of McLean and Dutton, 1995). Sows are very susceptible to AF and the toxic effects of AF on productive parameters are dose and time dependent (fertility, perinatal mortality, number of weaned pigs and food intake) (Miller et al., 1981; Harvey et al., 1988). Piglets from AF-exposed sows show growth retardation associated with various abnormalities. Namely immune functions are markedly compromised. Thymic involution associated with impairment of peripheral immune efficiency are common and constant events leading to early death (Panangala et al., 1986; Harvey et al., 1988; Scott et al., 1991; Neldon-Ortiz and Qureshi, 1992; Silvotti et al., 1995; Griffiths et al., 1996; Marin et al., 1996; Dimitri and Gabal, 1996). Thymulin (ZnFTS), one of the best known thymic hormones, requires zinc in order to be biologically active (Dardenne et al., 1982) and, in turn, zinc is relevant for body growth (Hill and Miller, 1983) and functioning of the entire immune system (Chandra, 1983). Low peripheral zinc bioavailability is the main cause of reduced active thymulin plasma levels (Fabris et al., 1984). In marginal zinc deficiencies, including infectious diseases, largely due to zinc intestinal malabsorption, the reduced thymulin activity is not due to intrinsic thymic failure but to low peripheral bioavailability of zinc to saturate all thymulin molecules produced (Fabris and Mocchegiani, 1995). Zinc supplementation increases thymic endocrine impairment and restores the crippled peripheral immune functions with increased survival in infectious diseases (Fabris and Mocchegiani, 1995). An intestinal malabsorption occurs in piglets from AF-exposed sows with defects related to a reduced zinc intestinal absorption (Miller et al., 1981). This work aims: (i) to measure both active ZnFTS and zinc plasma levels in piglets farrowed and fed from AF B_1 and G_1-exposed sows; (ii) to verify whether the thymic defect is intrinsic or due to a low zinc peripheral bioavailability. We also tested both zinc-unbound inactive thymulin (FTS) and total thymulin (Active ZnFTS + inactive FTS) plasma levels (Fabris et al., 1984). Morphological analysis of the thymus and lymphocytes mitogen responsiveness (PHA) have also been carefully performed.

2. Material and methods

2.1. Experimental design and animals

Twenty-four large white healthy sows (2–3 years old; mean body weight = 200 kg) with previous normal pregnancy history, were used. After natural insemination, with boars of the same breed, the pregnancies were echographically verified at day 23. Ten days before aflatoxins exposure (50 day of pregnancy), the echographic evaluation was repeated and four groups of six sows each with a minimum of 10 foetuses were randomly chosen. Group 1: aflatoxins free diet (Control group). Group 2: diet containing 800 ppb aflatoxin G_1. Group 3: diet containing 800 ppb aflatoxin B1. Group 4: diet containing 800 ppb aflatoxin B_1 and G_1, anaparte (400 ppb aflatoxin B_1 plus 400 ppb aflatoxin G_1).

Before adding purified aflatoxins (Carl Roth, Karlsruhe, Germany), the sows were fed during pregnancy and lactation periods with the same aflatoxin free HPLC diet (A. Bonomi, personal communication).

Groups 2, 3 and 4 received the diets containing aflatoxins from day 60 of pregnancy up to day 28 of lactation.

Piglets received maternal milk for 28 days. They also received, ad libitum, a weaning aflatoxins free diet from day 5 post-partum.

Piglets and sows were housed at a constantly controlled temperature (+20°C) and humidity (50%).

To determine zinc, on day 90 of pregnancy and day 15 of lactation, blood samples from jugular vein of sows were drawn and collected in disposable material (see below) containing heparin as anticoagulant. Milk samples from the sows of each group were also collected for zinc and AF determination on day 15 of lactation.

Heparined blood samples from 16 randomly chosen piglets (four from each litter) were collected from their heart before euthanasia (Tanax) on day 28. The blood samples of these piglets were used for biochemical tests, zinc and thymulin determinations as well as lymphocytes isolation.

2.2. *Gross anatomy and thymus histopathology*

Necroscopies were performed on 16 piglets. Thymus was collected and weighed. A sample of thymic tissue was frozen in liquid nitrogen for immunofluorescence studies. A second sample was fixed in 10% neutral buffered formalin (pH 7.4), embedded in paraffin wax (Paraplast, Bioptica, Milan, Italy) and 5-μm thick serial sections were stained with haematoxylin–eosin.

2.3. *Biochemical parameters determinations*

Routine laboratory techniques were used for all biochemical parameters considered.

2.4. *HPLC method for AF determination in sow milk*

Aflatoxins B_1 and G_1 and the metabolite M_1 were determined by a two-step procedure, consisting of a previous immunoaffinity (Gregory and Manley, 1981) extraction from centrifuged milk samples, followed by an HPLC chromatography able to quantitatively resolve the single molecular species. In order to increase the sensitivity of fluorimetric detection of aflatoxins B_1 and G_1, we obtained the derivative out of samples from the immunoaffinity column in trifluoroacetic acid (TFA). Detection and quantitative evaluation of aflatoxins G_1, B_1 and M_1 were carried out on the basis of a series of calibration curves obtained by injecting different amount of the pure products.

2.5. *Zinc determination*

2.5.1. *Blood*

To avoid zinc contamination, the plasma samples for zinc determination were collected into fluorinated tubes (no. 115317, LP, Milan, Italy) and then centrifuged 20

min later at $3000 \times g$ for 10 min. Plasma samples were frozen at $-70°C$ and stored until tested. Zinc was determined by AAS against zinc standard references (Fernandez and Khan, 1971).

2.5.2. Milk

On day 15 of lactation, the sow mammary glands were washed with distilled water and the milk samples were collected into fluorinated tubes and stored for 1 or 2 days at 4°C before the test. A dry-asking technique and AAS were used for milk zinc determination. Briefly, 1 ml of HNO_3 3.5% was added to 5 ml of milk, vaporising in a water bath at 70°C and burned at 400°C in muffle. The ashes were reported to volume (5 ml) with HNO_3 2% and then centrifuged at $3000 \times g$ for 10 min at 18°C to remove proteins. Zinc was determined in the supernatants against zinc standard references (Casey, 1977).

2.6. Thymulin (ZnFTS) determination

Splenocytes from young mice carry receptors for SRBCs and can therefore form rosettes when mixed with SRBC. T- and B-rosettes can be discriminated by adding 10 μg/ml azathioprine, which selectively inhibits T-rosette formation. The azathioprine sensitivity of T-cells is strictly dependent on thymic endocrine function: removal of the thymus in mice induces the total disappearance of azathioprine sensitivity, which reappears later when spleen cells from thymectomized mice are incubated with purified ZnFTS. This phenomenon represents the basis for the bioassay of thymulin in biological fluids (Bach et al., 1975). The maximum dilution of plasma samples inducing azathioprine sensitivity in 50% of RFCs from thymectomized mice was taken as the thymulin titer. The percentage of RFCs that became azathioprine-sensitive in the presence of excess thymulin ranges from 50% to 65%.

This technique, extensively described elsewhere (Bach et al., 1975; Fabris et al., 1984), is specific for ZnFTS because the assay is unaffected by other thymic hormones, and the rosette-inducing activity is completely removed by passing plasma samples through an antithymulin immunoabsorbent (Bach et al., 1975). The sensitivity of the bioassay makes it possible to detect 1 pg/ml synthetic thymulin (Sigma, USA). Since in two consecutive blind assays no differences of more than one $\log_2$ was found in all samples, assay was considered reliable (Bach et al., 1975). This bioassay is still required, since questions have arisen over the specificity of the radioimmunoassays (RIAs) developed till now (Fabris and Mocchegiani, 1995). In order to evaluate possible interference from zinc bioavailability, thymulin measurements were performed concomitantly with the in vitro addition of zinc sulphate to the plasma samples at a final concentration of 200 nM. This zinc concentration was chosen on the basis of previous experiments, performed with graded concentrations ranging from 1 pM to 10 μM, which showed that 200 nM was the optimal concentration for unmasking FTS, making it possible to evaluate the total amount of thymulin present (ZnFTS + FTS) in the circulation (Fabris et al., 1984). The apparently low molar concentration of zinc required may be explained by the fact that the bioavailable free zinc is no more than 2–3% of

total plasma zinc, the major quota being bound to proteins (Fabris and Mocchegiani, 1995) which are retained by the 50.000 mol. wt. cut-off membranes (Fabris et al., 1984). The data are expressed in $\log_{-2}$.

2.7. Determination of the inactive plasma thymulin

The determination of inactive thymulin (FTS) is based on the capacity of inactive hormone to inhibit the biological effect of synthetic zinc-bound active thymulin (Sigma) (Fabris et al., 1984). The method to detect plasma inhibitory activity of thymulin was originally developed by Bach and Beaurain (1979) and extensively described elsewhere (Fabris et al., 1984). It was later modified to induce specificity for the inactive thymulin, by concomitantly measuring inactive thymulin in the plasma samples without and with the in vitro addition of zinc sulphate (200 nM) up to the plasma samples (Fabris et al., 1984). Inactive thymulin was expressed in pg/ml as the highest concentration of synthetic thymulin (Sigma) inhibited by the plasma samples (Fabris et al., 1984).

2.8. Immunofluorescence studies

Thymulin-containing cells were analysed in 10-μm thymus cryosections. Thymulin-secreting cells were identified by an indirect immunofluorescence anti-thymulin IgG_2 antibody (kindly donated by Dr. Mirelle Dardenne, Paris). The number of thymulin-secreting cells in the thymus of each piglet was assessed by counting 100 microscope fields of 135.000 μm^2 from 3- or 4-serial cryosections obtained at different levels of the organ.

2.9. PHA lymphocyte proliferative response

In order to isolate peripheral blood mononuclear cells (PBMCs), each sample of heparined blood was diluted with an equal volume of phosphate buffer saline (PBS) and layered into Histopaque-1.077 specific gravity (Sigma). After centrifugation ($400 \times g$) of the sample for 30 min at room temperature, the cells at the interface were collected, washed twice in PBS and once in culture medium. For lymphocytes purification, the monocytes were depleted by plastic adherence after 24 h of incubation in RPMI-1640 medium (Seromed, Milan, Italy) supplemented with 10% heat-inactivated foetal calf serum (FCS-I, Seromed), 100 IU/ml penicillin and 100 μg/ml streptomycin (Seromed). The recovered lymphocytes were then seeded (2×10^5 cells per well) into 96-well round-bottom-microtitre plates (Costar, Cambridge, MA) in 200 μl of RPMI with 10% FCS-I containing 1, 5 or 10 μg/ml of phytohaemagglutinin M (PHA) (Sigma). The cultures were incubated for 48 h in a humidified 5% CO_2 atmosphere at 37°C and then the cells were pulsed with 0.25 μCi of ^{3}H-thymidine/well (Amersham Int., UK). After 6 h the radioactivity incorporated in the cells was measured by a betaplate counter (FilterMate 196 Packard, USA). The results were reported as mean count per minute (cpm) of triplicate assays for each piglet, referring to the highest response regardless of

Table 1
Piglets from AF-exposed sows: biochemical parameters in plasma (day 28 from delivery)

Biochemical parameters	Control (n°4)	AF B_1 (800 ppb) (n°4)	AF G_1 (800 ppb) (n°4)	AF $B_1 + G_1$ (400 + 400 ppb) (n°4)
Total protein (g/dl)	6.90 ± 0.31	$6.18 \pm 0.33^{+}$	$6.15 \pm 0.27^{*}$	$6.07 \pm 0.19^{*}$
Albumin (g/dl)	2.50 ± 0.24	$1.91 \pm 0.30^{+}$	$2.13 \pm 0.16^{+}$	$1.83 \pm 0.16^{*}$
Urea (mg/dl)	18.11 ± 4.0	$29.16 \pm 3.62^{+}$	$27.42 \pm 3.85^{+}$	$34.0 \pm 5.0^{*}$
Creatinin (mg/dl)	0.92 ± 0.47	$1.83 \pm 0.39^{+}$	$1.67 \pm 0.4^{\bullet}$	$1.97 \pm 0.41^{*}$
Glucose (mg/dl)	79.3 ± 11.1	73.56 ± 10.62	74.61 ± 8.66	$57.32 \pm 8.31^{+}$
Triglyceride (mg/dl)	65.28 ± 6.31	$80.15 \pm 6.84^{+}$	76.24 ± 7.51	$86.29 \pm 7.36^{*}$
Total cholesterol (mg/dl)	88.14 ± 7.11	$104.21 \pm 6.22^{*}$	$108.73 \pm 9.11^{*}$	$108.32 \pm 8.62^{*}$
Phospholipids (mg/dl)	67.39 ± 7.0	$84.26 \pm 6.0^{+}$	$80.51 \pm 7.69^{+}$	$85.15 \pm 6.54^{+}$
Total lipids (mg/dl)	281.64 ± 31.13	$375.62 \pm 24.31^{\bullet}$	$316.82 \pm 31.29^{\bullet}$	$305.18 \pm 9.29^{\bullet}$
Total bilirubin (mg/dl)	0.17 ± 0.03	$0.32 \pm 0.04^{*}$	$0.27 \pm 0.5^{*}$	$0.38 \pm 0.03^{*}$
GOT (U/l)	18.46 ± 5.16	$26.39 \pm 4.81^{\bullet}$	$24.61 \pm 4.26^{\bullet}$	$36.50 \pm 4.53^{*}$
GPT (U/l)	14.19 ± 4.0	$20.82 \pm 3.72^{\bullet}$	$21.86 \pm 3.87^{+}$	$28.51 \pm 3.89^{*}$
GLDH (U/l)	1.52 ± 1.15	$6.0 \pm 1.26^{*}$	$5.11 \pm 0.89^{*}$	$4.00 \pm 0.91^{*}$
Alkaline phosphatase (U/l)	174.90 ± 41.10	$269.11 \pm 56.21^{+}$	$241.89 \pm 43.21^{*}$	$275.0 \pm 31.30^{*}$

Mean ± SD $^{\bullet}$, $p < 0.05$; $^{+}$, $p < 0.005$; $*$, $p < 0.001$ as compared to control.

mitogen concentration used. The data are expressed in counts per minute per culture [(cpm/culture/min)]. The stimulation index was calculated as follows: cpm in stimulated cells (with PHA) minus cpm in not-stimulated cells (without PHA) divided by cpm in not-stimulated cells (without PHA).

2.10. Statistical analysis

The significance between the means was assessed by paired Student's *t*-test and ANOVA test (one-way). The correlations were determined by the linear square method. The differences were considered significant when $p < 0.05$.

3. Results

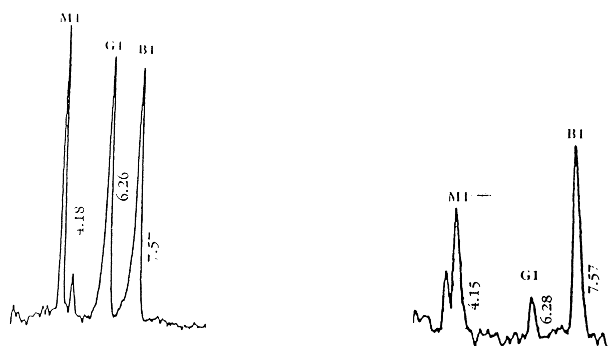
3.1. Effects of aflatoxins on health status and pregnancy of sows and some biochemical parameters in piglets from AF-exposed sows

No changes on health and pregnancy status were recorded in AF-exposed sows. Time parturition was also normal in the control sows as well as in those exposed to AF. No significant differences in litter size and vitality of piglets from sows exposed or not exposed to AF were recorded (data not shown).

In contrast, the piglets from AF ($B_1 + G_1$)-exposed sows showed abnormality in many biochemical parameters considered with various score of significance (Table 1). In particular, significant alterations were found in total protein, albumin, urea, total cholesterol phospholipids, total bilirubin, glutamicodehydrogenase (GLHD) and alkaline phosphatase as compared with the control group (Table 1) ($p < 0.001$ or $p < 0.005$).

3.2. Presence of AF B_1 and G_1 and the metabolite M_1 in the milk of AF-exposed sows

The quantitative HPLC evaluation of B_1, G_1 and the metabolite M_1 in milk samples from AF-exposed sows and those observed in standard aflatoxins showed quite similar



A. Aflatoxin standard chromatogram **B.** Chromatogram of milk of sow exposed to aflatoxin

Fig. 1. Aflatoxins chromatogram by means of HPLC in the milk of sow exposed to AF (B) as compared to aflatoxins standard (A).

Table 2
Body weight, thymus weight (absolute and relative) in piglets from AF-exposed sows and in healthy controls

	Control (n°4)	B_1 (n°4)	G_1 (n°4)	$B_1 + G_1$ (n°4)
Body weight (kg)	6.51 ± 0.42	$5.32 \pm 0.63^+$	5.66 ± 0.39	5.25 ± 0.44●
Absolute thymus weight (g)	18.22 ± 0.12	$11.17 \pm 0.05*$	$14.15 \pm 0.27*$	$9.45 \pm 0.15*$
Relative thymus weight (g) (ratio thymus weight/body weight)	0.28 ± 0.03	0.21 ± 0.02●	$0.25 \pm 0.05*$	0.18 ± 0.04●

Mean ± SD $^+$, $p < 0.05$; ●, $p < 0.005$; $*$, $p < 0.0005$ as compared to control.

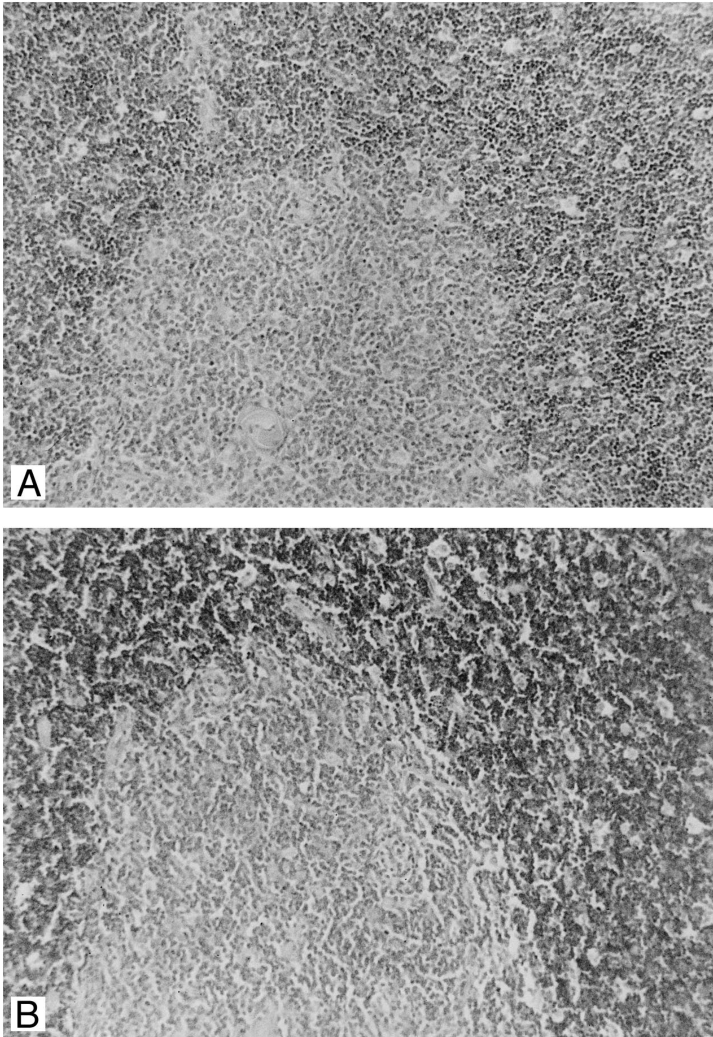


Fig. 2. Thymus morphology in piglets from AF (B_1) (A), G_1 (B)-exposed sows (magnitude = 200×). The toxic effect of the single aflatoxin on thymocytes depletion is more marked when exposed to AF B_1 (A). A mild thymocytes depletion is observed when exposed to AF G_1 (B).

peaks at defined retention times (Fig. 1A,B). HPLC chromatograms did not exhibit any detectable peak at the retention times in the milk of the control sows as compared to standard aflatoxins (data not shown).

3.3. Effect of aflatoxins on the body and thymus weight

There was a reduction of the body weight in piglets of AF-exposed sows vs. controls; the decrease was more marked when B_1 and G_1 were associated ($p < 0.01$). Thymus weight was reduced either as absolute or as relative weight values when compared to

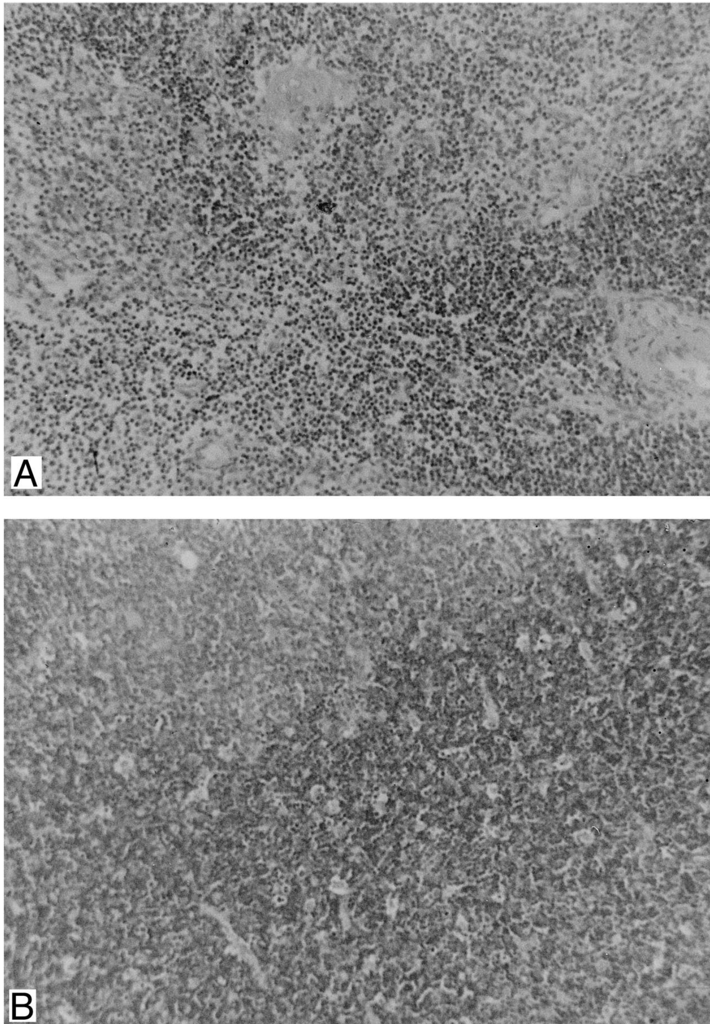


Fig. 3. Thymus morphology in piglets from AF ($B_1 + G_1$)-exposed sows (A) and in healthy control piglets (B) (magnitude = 200×).

controls ($p < 0.01$) (Table 2). Such decrements occurred despite no significant variations in the diet content in sows either exposed or not to aflatoxins during pregnancy or lactation were observed (data not shown). A significant correlation existed when the data of the body weight from piglets of sows exposed or not to aflatoxins were plotted against the corresponding values of the absolute thymus weight from the same piglets ($r = 0.90$; $p < 0.01$).

3.4. Thymus histopathology

Thymus histology showed a depletion of the thymus cortical lymphocytes in piglets from AF B_1 and G_1-exposed sows (Fig. 2A,B). A more marked depletion was observed in piglets from sows exposed to AF B_1 (Fig. 2A) and AF B_1 + AF G_1 (Fig. 3A), as compared with healthy controls (Fig. 3B). No histological lesions were found in abdominal or thoracic organs or in the Central Nervous System (CNS) in piglets exposed to maternal aflatoxicosis (data not shown).

3.5. Plasma zinc concentrations in piglets from AF-exposed sows and zinc levels in plasma and in milk of sows exposed or not exposed to AF

Plasma zinc levels were markedly reduced in piglets from AF-exposed sows as compared to healthy controls ($p < 0.0005$) (Table 3A). No significant differences in plasma zinc levels were observed among the various aflatoxin considered (Table 3A). No variations in plasma zinc levels were observed in AF-exposed sows as compared with the values of healthy age-matched controls at day 90 of pregnancy and at day 15 of lactation (Table 3 B). An increasing, but not significant, trend of zincaemia was present, however, during lactation as compared with the values observed during the pregnancy in both sows exposed and not exposed to aflatoxins (Table 3B). With regard to the zinc content in the milk, a marked increase was observed in all sows (exposed or not to

Table 3
Plasma zinc levels in piglets from AF-exposed sows (A), in AF-exposed sows (B) and milk zinc content in AF-exposed sows at day 15 of lactation (B)

A	Piglets (28th day of lactation)			
	Control (n°4)	B_1 (n°4)	G_1 (n°4)	$B_1 + G_1$ (n°4)
Zinc plasma levels (μg/dl)	119.2 ± 9.7	56.0 ± 11.1 *	65.2 ± 9.0 *	53.0 ± 6.8 *

Mean ± SD *, $p < 0.0005$ as compared to control

B	Sows (90th day of pregnancy)			
	Control (n°6)	B_1 (n°6)	G_1 (n°6)	$B_1 + G_1$ (n°6)
Zinc plasma levels (μg/dl)	67.50 ± 9.5	67.6 ± 10.2	68.5 ± 9.6	65. 8 ± 10.7
Zinc milk concentration (μg/dl)	–	–	–	–
Day 15 of lactation				
Zinc plasma levels (μg/dl)	85.0 ± 12.6	83.6 ± 11.2	85.7 ± 10.6	86.7 ± 11.7
Zinc milk concentration (μg/dl)	299.0 ± 63.7	236.2 ± 68.7	358.2 ± 46.5	246.2 ± 72.7

aflatoxins) as compared with plasma zinc values at day 15 of lactation, with no significant differences in zinc milk concentrations among all sows considered (Table 3B).

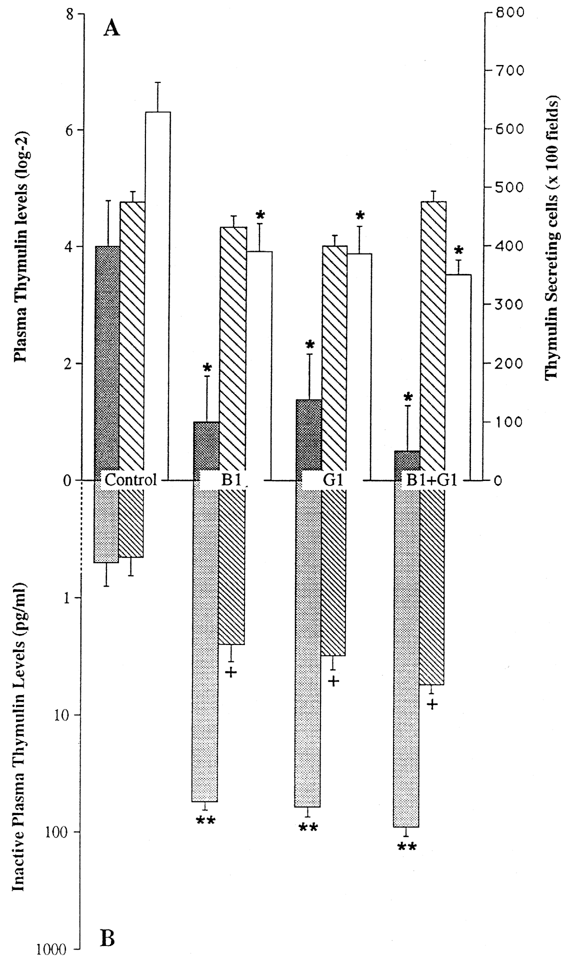


Fig. 4. (A) Active thymulin levels (■), total thymulin quota (box filled with diagonal lines) and number of thymulin-secreting cells (□) in piglets from AF (B_1, G_1 and B_1+G_1)-exposed sows at the time of euthanasia (day 28 from birth). Data from piglets from not AF-exposed sows (control) are reported. (B) shows inactive plasma thymulin levels (box with mildly-splotched marks) in piglets from AF (B_1, G_1 and B_1+G_1)-exposed sows and in healthy controls. The in vitro addition of zinc up to the plasma samples induces a significant reduction of inactive thymulin (box with heavily splotched marks) as compared to the values with no in vitro zinc addition (box with a fair amount of splotched marks) with similar values observed in healthy controls. The B_1+G_1 aflatoxins association seems to induce a more marked thymic endocrine impairment as compared to aflatoxins alone (A and B). $*$, $p<0.001$ as compared to healthy controls (A) (paired Student's *t*-test and ANOVA). $**$, $p<0.0001$ as compared to healthy controls (B) (paired Student's *t*-test and ANOVA). $^{+}$, $p<0.01$ as compared to the column (box with mildly-splotched marks) with no in vitro zinc addition (B) (paired Student's *t*-test and ANOVA).

Table 4
PHA lymphocytes responsiveness and stimulation index (SI) in piglets from AF-exposed sows

	+PHA (cpm/min)	−PHA	SI
Control	11734 ± 1212	30 ± 13	390 ± 90
Aflatoxin B_1	$7315 \pm 1141^{+}$	$176 \pm 56^{+}$	$41 \pm 19^{\bullet}$
Aflatoxin G_1	$6671 \pm 1206^{+}$	$113 \pm 67^{+}$	$58 \pm 15^{\bullet}$
Aflatoxin $B_1 + G_1$	$8835 \pm 1279^{+}$	$193 \pm 116*$	$45 \pm 10^{\bullet}$

$*$, $p < 0.05$; $^{+}$, $p < 0.001$; $^{\bullet}$, $p < 0.01$ as compared to control.
n° = 4 piglets for each group.
SI = ((cpm + PHA) − (cpm − PHA))/(cpm − PHA).

3.6. Active, total and inactive thymulin plasma levels in piglets from AF-exposed sows

Plasma active thymulin values (ZnFTS) were markedly reduced in piglets from AF-exposed sows when compared with healthy controls, whereas the total quota of thymulin (ZnFTS + FTS) was within the normal range. In particular, the piglets from AF ($B_1 + G_1$)-exposed sows showed a more marked reduction in active thymulin plasma levels (Fig. 4A). Concomitantly high plasma levels of inactive thymulin (FTS) were found in piglets from AF-exposed sows (Fig. 4B). The 'in vitro' addition of zinc up to the plasma samples induced a significant reduction of inactive thymulin concentrations with similar values observed in healthy control piglets (Fig. 4B). The number of thymulin-secreting cells was also significantly reduced in all piglets from AF-exposed sows as compared with healthy control piglets ($p < 0.001$). The decrement was more evident in piglets from AF ($B_1 + G_1$)-exposed sows (Fig. 4A). Significant inverse correlations existed between plasma zinc levels and thymic hormone saturable fractions (ratio total thymulin/active thymulin) in piglets from AF B_1-, AF G_1- or AF ($B_1 + G_1$)-exposed sows ($r = 0.90$, $p < 0.001$; $r = 0.78$; $p < 0.01$; $r = 0.88$, $p < 0.001$, respectively).

3.7. Lymphocytes PHA responsiveness in piglets from AF-exposed sows

Significant reduction of PHA mitogen responsiveness in all piglets from AF-exposed sows was observed as compared with healthy controls ($p < 0.001$). The reduction was more marked in AF ($B_1 + G_1$) association (Table 4). AF B_1 and AF G_1 or their association induced a marked reduction of SI as compared to healthy controls ($p < 0.01$), with no differences between two aflatoxins considered and their association (Table 4). However, a difference between aflatoxins and healthy controls without PHA stimulation was observed ($p < 0.05$), because of aflatoxins themselves to be mitogenic (Griffiths et al., 1996, Dimitri and Gabal, 1996).

4. Discussion

The thymic endocrine defect in piglets from AF-exposed sows, associated with depletion of thymulin-secreting cells and cortical thymocytes, is largely dependent by

the low peripheral zinc bioavailability. In addition to a marked zinc deficiency, these piglets show high concentrations of inactive thymulin (FTS), which are normalised to healthy control values after 'in vitro' addition of zinc up to the plasma samples.

Thymic involution, associated with depletion of thymocytes and with reduction of both thymus and body weights, is an usual event in animals exposed to AF (Panangala et al., 1986; Harvey et al., 1988). Aflatoxins are also present in the milk of AF-exposed sow (Wogan, 1977). In agreement with these studies, we have found AF in the milk of AF-exposed sow and decrements of thymus and body weights in piglets. However, such a phenomenon is not dependent by the thymus ontogenesis because our experimental model is carried out from day 60 of pregnancy, whereas complete thymus ontogenesis occurs in piglets at day 40 of pregnancy (Tizard, 1992). Thus, the observed reduced thymus and body weights may be largely due to the presence of AF in sow milk. Despite the difficulty to measure the accurate amount of milk suckled by piglets, the presence of a significant correlation between thymus and body weights in all piglets from AF-exposed and AF-not-exposed sows may be in line with this interpretation. Moreover the thymic defect may be AF dose and time-dependent, as shown both in vivo and in vitro models (Panangala et al., 1986; Harvey et al., 1988; Neldon-Ortiz and Qureshi, 1992; Silvotti et al., 1995; Marin et al., 1996, Griffiths et al., 1996; Dimitri and Gabal, 1996). However the possible cause/s of the thymic involution in piglets from AF-exposed sows are still unclear and undefined. Stress factors coupled with decreased protein synthesis have been suggested (Harvey et al., 1988). In addition stress factors induce a loss of zinc by means of urine or by means of intestinal malabsorption. (Prasad, 1985). Zinc plays a pivotal role for good immune responses, including thymic functions, both in man and animals (Chandra, 1983). Zinc intestinal malabsorption and stress factors are usual events in AF-exposed animals (Miller et al., 1981). Zinc may, therefore, be crucial for thymic involution in piglets from AF-exposed sows. A marked reduction of plasma zinc levels is observed in these piglets despite the high zinc content in the sow milk, largely because of the particular hormonal condition in pregnancy and in nursing (Keen and Hurley, 1989). A marginal zinc deficiency has been suggested one of the possible causes of the thymic involution in aging (Fabris and Mocchegiani, 1995). Indeed zinc is required for the biological activity of a thymic hormone, i.e. thymulin (ZnFTS), which is responsible to the performance of cell-mediated immunity (Dardenne et al., 1982). The zinc-unbound form (FTS) is inactive with an inhibitory action on the active form (ZnFTS) (Fabris et al., 1984). The in vitro addition of zinc up to the plasma samples unmasks the inactive form showing the total amount (active ZnFTS + inactive FTS) of thymulin molecules produced (Fabris et al., 1984). The in vitro addition of zinc up to the plasma samples of our piglets unmasks the inactive form of thymulin showing no differences in total thymulin quota as compared with healthy control piglets. This finding suggests that the thymic endocrine impairment is not an intrinsic process, but it may depend by the low zinc peripheral bioavailability to saturate all thymulin molecules produced, as shown in other marginal zinc deficiencies (Fabris and Mocchegiani, 1995). The existence of inverse correlations between plasma zinc levels and thymic hormone saturable fractions (ratio total thymulin/active thymulin) in piglets from AF (B_1, G_1 and $B_1 + G_1$)-exposed sows may be in line with this interpretation.

The cause of this thymic endocrine impairment may be due because zinc is also

required for the biological activity of more than 200 enzymes, which bind zinc with a different binding affinity (K_d), ranging from 10^{-3} M to 10^{-10} M (Mills, 1989). Thus a shift of zinc towards enzymes with higher K_d than thymulin ($K_d = 10^{-7}$ M) (Gastinel et al., 1984), or at least similar K_d, may occur and, consequently, reduced biological activity of zinc-bound thymulin and impaired peripheral immune efficiency.

This phenomenon occurs in marginal zinc deficiencies, such as infectious diseases. A restoration of thymic and peripheral immune functions is achieved after zinc supplementation (Fabris and Mocchegiani, 1995). Such a mechanism might also occur in piglets exposed to maternal aflatoxicosis. Alkaline phosphatase has a kd for zinc (Mills, 1989) similar to thymulin. An increment of alkaline phosphatase is observed in these piglets. Thus a shift of zinc towards alkaline phosphatase, rather than thymulin, might occur in aflatoxicosis with deficits involving both thymic and peripheral immune functions. Indeed, in agreement with other studies (Scott et al., 1991; Griffiths et al., 1996; Dimitri and Gabal, 1996), decreased peripheral immune efficiency (lymphocytes mitogen responsiveness) is observed in these piglets. Thus the bioavailability of zinc may play a key role for the efficiency of the entire immune system in aflatoxicosis.

Moreover, zinc is also required to activate oxidative enzymes in microsomes. A zinc deficiency affects the stability of the microsomial P-450 enzyme system (Xu and Bray, 1991), which is required by the microsomial fraction of hepatocytes to metabolise AF by means of epoxides formation (Forrester et al., 1990). The epoxides are excreted by urine and bile. However an accumulation of epoxides may occur in various tissues when the epoxides formation is higher than the capacity of detoxification of the organism. Consequently, epoxides are, in turn, toxic (Forrester et al., 1990; Ball et al., 1995). Such a phenomenon occurs during an acute and with high doses administration of aflatoxins (800 ppm) (Cagne et al., 1968). The dose of aflatoxin herein used is low (800 ppb) and the treatment is chronic. The toxic action of epoxides may be, therefore, excluded in our piglets. In contrast, an action of zinc in order to activate the P-450 enzyme system may be taken into account. Such an assumption may be consistent because the hepatic levels of P-450 enzyme system are regulated by glucocorticoids (Elshourbagy and Guzelizen, 1980), which, in turn, induce a loss of zinc in the urine (Prasad, 1985). An abnormal increment of adrenal weight with a thickening of the adrenal cortex occurs in piglets from AF-exposed sows (A. Corradi, unpublished observations), suggesting possible abnormal circulating levels of glucocorticoids. While, on one hand it may justify a reduced zincaemia in these piglets, on the other hand it might suggest a major recall of zinc at cellular microsomial level in order to better stabilise the P-450 enzyme, which is under the control of glucocorticoids. This quota of zinc, however, may not be sufficient. Thus a good homeostasis among zinc, glucocorticoids and P-450 enzyme system may be proposed in aflatoxicosis in order to prevent or to detoxify the organism. This proposal might suggest the possible existence of an interplay among zinc, glucocorticoids and the P-450-enzyme system, as it occurs for zinc and glucocorticoids in aging (Fabris and Mocchegiani, 1995).

Another intriguing point is related with the discovery showing toxins to induce thymocytes apoptosis (McConkey et al., 1988), and zinc, in turn, prevents thymocytes apoptosis (Martin et al., 1991). Thus, following also this last observation, a zinc treatment in these piglets may be carefully suggested in order to improve both thymic

functions and peripheral immune efficiency and to better detoxify the organism by means of the P-450 enzyme system.

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