DOCTORATE (PhD) DISSERTATION THESES

UNIVERSITY OF KAPOSVÁR
FACULTY OF ANIMAL SCIENCE
Department of Animal Physiology and Hygiene

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Doctor of the Hungarian Academy of Sciences

DETERMINATION OF TIME AND DOSE DEPENDENT EFFECT OF FUMONISIN B_1 IN PIGS

DR. GÁBOR TORNYOS

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1. BACKGROUND OF RESEARCH, OBJECTIVES

The various damages of microscopically fungi in feedstuff are well known. In the primary metabolism they use the nutrients in feedstuff and in the secondary metabolism they produce mycotoxins. The chemical structure of mycotoxins are various as well. This is the reason of the different effects of those in organisms. The fumonisin produced by *Fusarium moniliforme* and its related species was discovered in 1988. There are several compounds of different structure among the fumonisin however the fumonisin B_1 is the most important both in human and animal health aspects.

The effects of fumonisin are various in different species. The pulmonary oedema being typical in pigs was described by Doman and Petrás in 1952 without knowing the exact aetiology. In 1990 Harrison et al. found the fumonisin in the background of pulmonary oedema. In Hungary Fazekas et al. found the relationship between pulmonary oedema and fumonisin in 1997. The exact pathomechanism is still not clear.

Several mycotoxins are immunsuppressive, enhance the susceptibility to secondary infectious diseases. As a consequence the medical costs increase and the efficacy of medical treatments decrease. The risk of occurrence of antibiotics in food chain increases as well. Above all mycotoxins and their metabolisms can get into food chain. Most of our information concerning immunsuppressive effect of fumonisins originate from *in vitro* or *in vivo* experiments on laboratory animals. There are very few data on pigs.

The aims of the present work were the followings:

1. To detect time and dose dependent effect of fumonisin B_1 using non invasive digital imaging technique, the computer tomography (CT).

2. To examine the effects of toxin on the immune system by measuring cellular and humoral immunresponse using specific and non specific mitogens, and detecting the antibody titer.

2. MATERIALS AND METHODS

2.1. Experimental animals

Weaned barrows of KA-HYB (Norwegian landrace x Large white) genotype about 10 kg initial bodyweight were used. After a 5-days long accommodation period the pigs were randomly sorted into groups and individually housed. The groups were separated by empty pens to avoid the cross-contamination. *Fusarium moniliforme* fungal culture containing a known amount of fumonisin B_1 was added in different concentrations to the experimental diets. The control group received a toxin-free diet. The diets fed to the animals did not contain detectable amounts of other mycotoxins (T-2, zearalenone, ochratoxin A, DON).

The animals were fed twice a day, drinking water was provided *ad libitum*. The behaviour and the clinical status of the pigs were controlled twice a day. The main points to check were the followings: general status, respiration, the speed of food intake, sorting of the food, the amount of water to be drunk, regurgitation, cyanosis or other unusual signs.

2.2. Experimental design

Four experiments were performed, the length of exposure, the amounts of dosed fumonisin, and the number of pigs are summarized in Table 1.

Table 1: Experimental design

No.of the experiment	Length of exposure	The amount of fumonisin B_1	Number of pigs / group
I.	4 weeks	0 ppm (control)	5
		10 ppm	5
		20 ppm	5
		40 ppm	5
II.	8 weeks	0 ppm (control)	5
		1 ppm	5
		5 ppm	5
		10 ppm	5
III.	20 weeks	0 ppm (control)	6
		1 ppm	6
		5 ppm	6
		10 ppm	6
IV.	10 days	0 ppm (control)	6
		100mg/animal/day	14

2.3. Computer tomography examinations (CT)

The pigs were subjected to computer tomography examination (CT) three times in each experiment (Table 2).

Table 2: Dates of the CT examinations in the different experiments

No. of the experiment	Dates of the CT examinations
I.	Day 0, 2nd and 4th week
II.	Day 0, 4th and 8th week
III.	Day 0, 8th and 20th week
IV.	Day 0, Day 5 and 10

The examinations were performed in anaesthesia, using Somatom plus S 40 type CT equipment (Siemens Erlangen, Germany) in the Institute of Diagnostic, University of Kaposvár. The lung was examined from the cranial end of apical lobe to the caudal end of the diaphragmatic lobe. Looking over the whole image the most characteristic changes were found in the apex of heart.

According to the severity of the damage four categories were set up: healthy, mildly damaged, medium damaged, seriously damaged. Changes in the severity and all symptoms were also followed.

2.4. Immunological examinations

The effects of low and high doses of fumonisin B_1 on the for immune response were examined both in case of short and long time exposure in experiment III. and IV. (Table 3).

Table 3: Vaccination and blood sampling protocol

	Experiment III.	Experiment IV.
1st blood sampling	90th day	0th day
The beginning of feeding the toxin	1st day	1st day
1st vaccination	90th day	0th day
2nd blood sampling	97th day	6th day
2nd vaccination	104th day	-
3rd blood sampling	111th day	-
4th blood sampling	125th day	-

Blood samples were take from the *v. cava cranialis*. In order to determine immune response animals were vaccinated against Aujeszky disease with 2ml inactivated vaccine (Aujespig K, Phylaxia-Sanofi, Budapest, Hungary). Humoral immune response, e.g. specific antibody titre was measured by virus neutralisation test. Specific and non specific *in vitro* cellular immune response was measured by the lymphocyte stimulation test (LST). To induce the non-specific blastogenesis phytohaemagglutinin-P (PHA-P) was used as mitogen in the first place. In the case when adequate number of lymphocytes could be separated concanavalin A (ConA) and lipopolysaccharid (LPS) were used too. The specific blastogenesis was induced by the inactivated suspension of the Aujeszky virus (SHV-1). Mitotic activity was tested after 72 hours of stimulation by MTT (methyltiazoltetrazolium, Sigma-Aldrich) conversion test, a fast colorimetic method used to determine the dehydrogenase enzyme activity of the lymphocytes, which correlates to the rate of blastogenic transformation. Stimulation index was calculated on the basis of the optical density of the control and the mitogen induced samples.

The LST test and the antibody titre determination were carried out at the Faculty of Veterinary Science, Department of Microbiology and Infectious Diseases.

2.5. Statistical analysis

SPSS 9.0 was used for the analysis.

In case of cellular immune response one-way ANOVA (Dunnet-test, post hoc test) was used to investigate the significant difference between groups. Results of the groups were compared only at the same examination times (effect of the treatment). Differences between data of the control and the treated group were compared by independent t-test in examination IV.

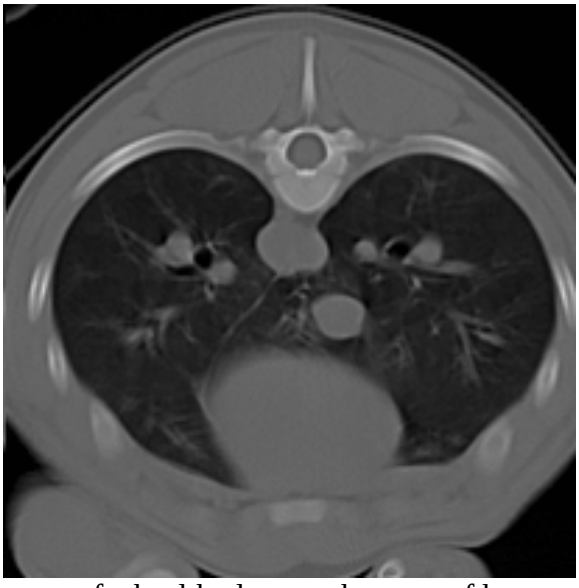
In case of humoral immune response one-way ANOVA (Dunnet-test, post hoc test) was used in the investigation of the significance between the groups at the same examination times. To compare the different data between groups paired t-test was used.

The level of significance was set to the 0.05 level of probability.

3. RESULTS

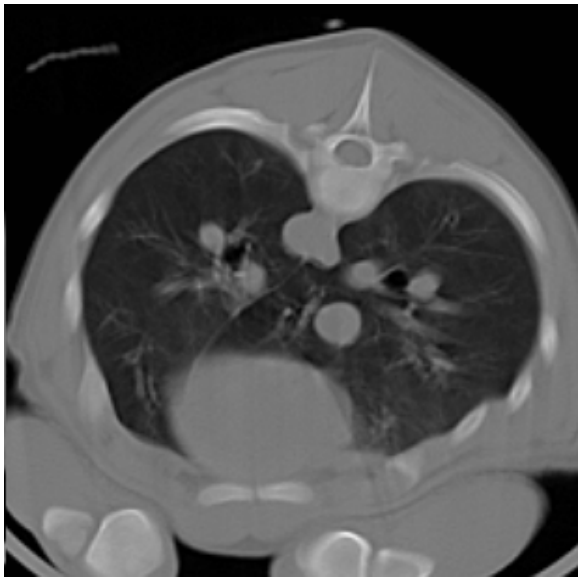
3.1. Results of the CT examinations

In healthy pigs (Picture 1) the lung is homogeneously dark grey, the bigger blood vessels and the apex of heart are well seen. The bigger bronchi sometimes can be seen as branches.



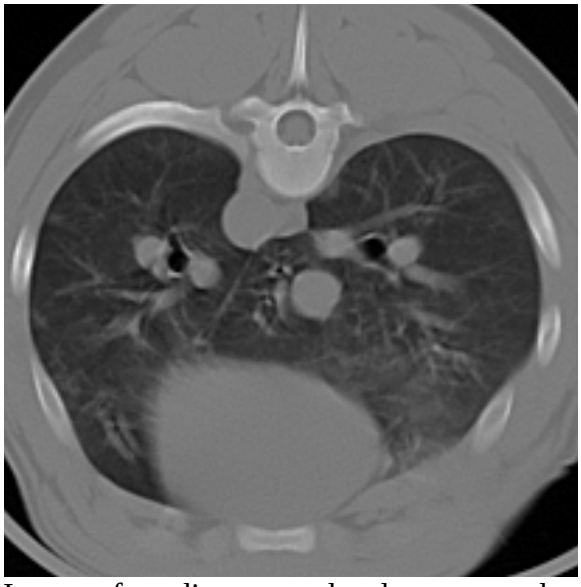
Picture 1: Image of a healthy lung at the apex of heart

In the first 8 weeks pulmonary oedema with dose dependent severity was found. In the case of mild staged oedema (Picture 2.) the lung was homogenous: the distal parts of the lung were darker. In this image more characteristic changed could be seen in the right lobe. Mild damages concerned only the areas next to the apex of heart.



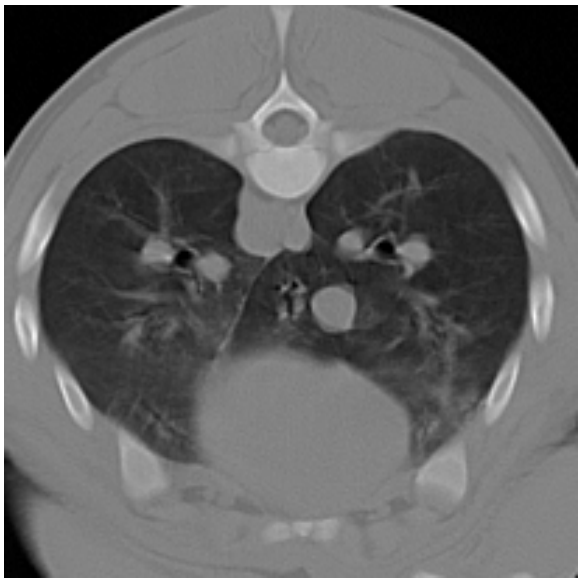
Picture 2: Image of mild staged pulmonary oedema in pig at the apex of heart

In case of medium staged pulmonary oedema (Picture 3) the damaged areas were already larger, spreading above the apex of heart. Not the whole lung was affected, areas under the spine were still healthy.



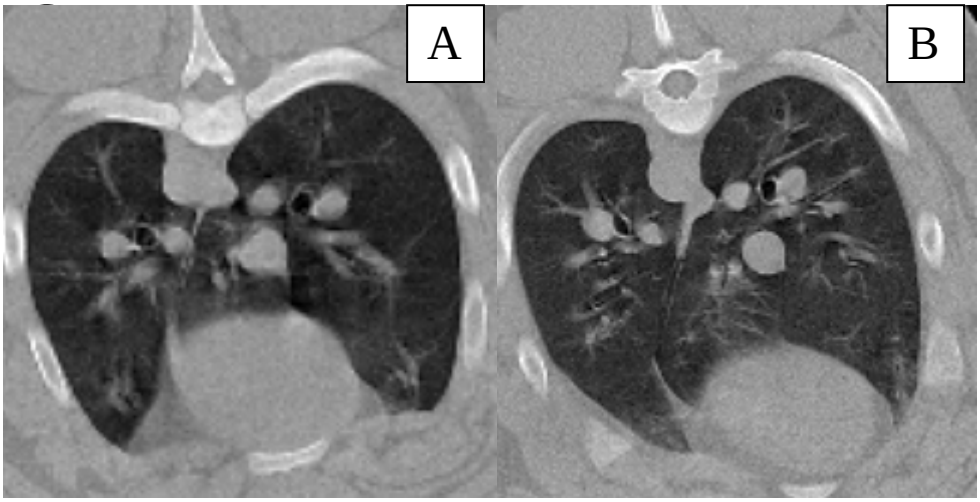
Picture 3. : Image of medium staged pulmonary oedema in pig at the apex of heart

In case of the most serious damages (Picture 4) the whole lung was affected, the most characteristic oedema was found near to the distal parts (see the right side where the lung is nearly homogeneously grey and the structure of the lung is blunted).



Picture 4. : Image of serious staged pulmonary oedema in pig at the apex of heart

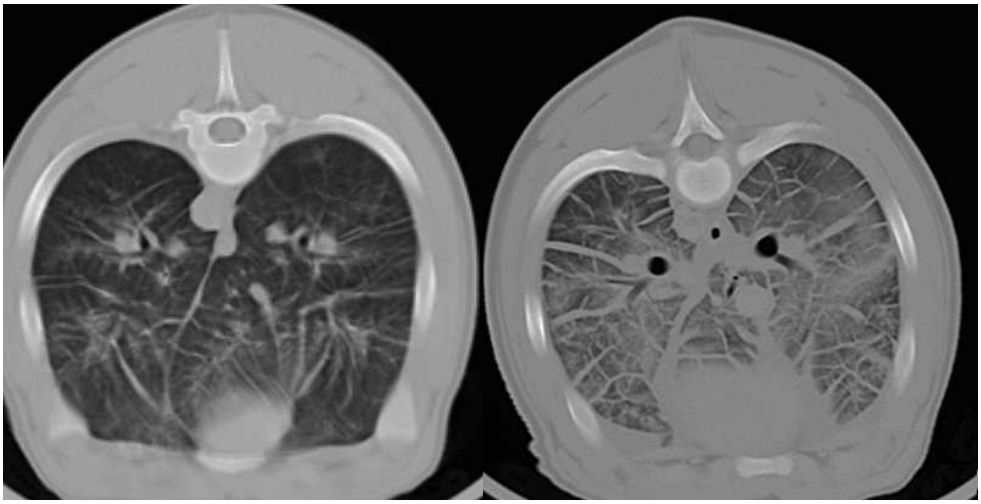
When feeding the toxin at a low dose pulmonary oedema was not found at the end of the 20th week, because it changed into fibrosis by that time.



Picture 5. : Image of healthy (a) and fibrotic (b) lung in pigs

The image shows lined shadows appearing next to the opacity. The peribronchial areas got widened and their density became more intensive. The fact of the fibrosis was supported by the pathological and histopathological examinations too.

In the fourth experiment, where the effect of fumonisin in high dose was examined the pigs received 100 mg toxin per day (7,2-9,1 mg/b.w. kg). Giving the same amount of toxin to pigs different damages in lung could be seen (Picture 6). On the left image the pulmonary oedema is limited mostly to the ventral areas. On the right image the signs of oedema are more significant and the signs of interstitial oedema can also be seen.



Picture 6: The damages in two pigs caused by the same amount of fumonisin B_1 on the fifth day

3.2. Results of the LST probe

3.2.1. The effect of 1, 5 and 10 ppm fumonisin B_1 exposition for 140 days

At the first two examination time (Table 4) there were no significant differences among groups. At the third and fourth blood sampling however there were significant differences between the control and the experimental groups. The immune response didn't depend on the amount of toxin because there were no significant difference among the treated experimental groups.

Table 4: Phytohaemagglutinin-P (PHA-P) induced non specific cellular immune response measured by LST

	Control group	1 ppm	5 ppm	10 ppm
90th day	1,03 ± 0,01	1,09 ± 0,07	0,93 ± 0,12	0,99 ± 0,07
97th day	0,94 ± 0,07	1,03 ± 0,11	1,04 ± 0,07	1,05 ± 0,16
111th day	1,26 ± 0,10[a]	1,07 ± 0,07[b]	1,02 ± 0,08[b]	1,02 ± 0,07[b]
125th day	1,21 ± 0,11[a]	1,06 ± 0,03[b]	1,02 ± 0,05[b]	1,05 ± 0,06[b]

([a,b] significant difference within a row, P<0,05)

Cellular responses to specific antigen (inactivated Aujeszky virus, SHV-1) indicated better activity (Table 5). It showed that vaccination had been successful but no significant differences were found between groups.

Table 5: Suid herpesvirus-1 (SHV-1) induced specific cellular immune response measured by LST

	Control group	1 ppm	5 ppm	10 ppm
90th day	0,98 ± 0,03	0,99 ± 0,08	0,97 ± 0,04	1,01 ± 0,06
97th day	1,07 ± 0,12	1,06 ± 0,05	1,07 ± 0,07	1,08 ± 0,10
111th day	1,29 ± 0,10	1,11 ± 0,10	1,22 ± 0,8	1,19 ± 0,11
125th day	1,30 ± 0,10	1,17 ± 0,09	1,21 ± 0,07	1,25 ± 0,16

3.2.2. The effect of 100 mg/animal fumonisin B_1 exposition for 10 days

In this trial, responses to PHA-P were the most explicit. None of the mitogens except Con A showed significant difference between the groups. The relatively small changes in the LST values induced by the specific antigen (SHV-1) showed that the animals were not able to accommodate within this short time.

Table 6: Specific (SHV-1) and non specific (Pha-P, LPS, Con A) mitogens induced cellular immune response measured by LST

Mitogen	Time of examination	Control group	Experimental group
Pha-P	0^{th} day	1,64 ± 0,21	1,60 ± 0,14
	6^{th} day	1,66 ± 0,16	1,61 ± 0,12
LPS	0^{th} day	1,22 ± 0,07	1,20 ± 0,07
	6^{th} day	1,21 ± 0,06	1,19 ± 0,05
Con A	0^{th} day	1,02 ± 0,05	1,05 ± 0,06
	6^{th} day	$1,06 \pm 0,03^{a}$	$1,00 \pm 0,03^{b}$
SHV-1	0^{th} day	1,06 ± 0,05	1,04 ± 0,06
	6^{th} day	1,10 ± 0,10	1,08 ± 0,08

(^{a,b} significant difference within a row, P<0,05)

3.3. Results of the humoral immune response

In order to examine the humoral immune response antibody titers in blood after vaccination were meassured. Antibody titer increased significantly after the 2^{nd} vaccination in all groups, except in case of 1 ppm toxin exposure, where the increase was not significant, because of the significantly lower response compared to the other three groups (Table 7). It relates to the fact that the members of this group had slower immune response (see also Day 97).

Table 7: The summary of the results of humoral immune response (mean ± SD).

	Control group	1 ppm	5 ppm	10 ppm
90^{th} day	$2,04 \pm 0,36^{A}$	$2,00 \pm 0,49^{A}$	$2,20 \pm 0,49^{A}$	$2,17 \pm 0,64^{A}$
97^{th} day	$2,88 \pm 1,00^{A}$	$1,83 \pm 0,57^{A}$	$3,05 \pm 1,11^{A}$	$3,30 \pm 1,03^{A}$
111^{th} day	$7,04 \pm 0,65^{a,B}$	$4,00 \pm 1,00^{b,A,B}$	$8,50 \pm 2,38^{aB}$	$8,27 \pm 1,31^{aB}$
125^{th} day	$6,20 \pm 0,84^{C}$	$6,72 \pm 1,38^{B}$	$8,00 \pm 2,16^{B}$	$8,17 \pm 1,17^{B}$

(^{a,b} significant difference within a row, P<0,05)
(^{A,B,C} significant difference within a column, P<0,05)

4. CONCLUSIONS

4.1. Computer tomography examinations

On the basic of the present examinations it could be concluded, that CT is suitable for detecting changes in the lung caused by fumonisin B_1 in pigs. Signs of pulmonary oedema are well characterised, while fibrosis (in accordance with literature) is not so characteristic.

We found high differences between individuals concerning sensitivity to the same dose of toxin. So severity of the disease was different within the groups as well, whereas toxin intake and time of the exposure was very similar or the same.

When animals are fed a diet which contains the toxin in a low concentration for a long period – which often occurs in practice - pulmonary oedema changes to fibrosis which is already an irreversible pathological alteration. This should be taken into consideration when pulmonary fibrosis is detected at the slaughterhouses to keep the toxin out the food chain. These results draw the attention also to the human helath aspect of low FB_1 exposure.

4.2. Immunological examinations

Data in the literature concerning the effect of FB_1 on the immune system are controversial according to species, sex, mode of toxin application, dose etc.

Summarising the results of the present immunological experiments it could be concluded that fumonisin B_1 did not have significant negative effect on the immune responses in pigs. This was supported by the fact that in the most experiments there were no significant differences between the control and the experimental groups, no dose depended effect could be observed. Experiments should be repeated with higher number of animals. In some cases we found that majority of the animals had been in stage of slight immunodeficiency which had probably resulted from climatic factors (sultry summer weather) or from continuous blood sampling as a stressor.

Our experiment provide useful results mainly for the practice, indicating that long term exposure to FB_1 in low concentration (1-10 ppm), occurring frequently in Hungary, does not denote significant hazard concerning immune response of weaned pigs. However, animals were kept in proper nutritional and hygienic conditions. Other environmental

immunomodulatory effects may have synergic or additional effects or may modulate the effect of FB_1.

5. NEW EXPERIMENTAL RESULTS

1. Fumonisin B_1 had no significant effect on the humoral and cellular specific and non specific immune response when fed in high dose in short period (100 mg/animal/day for 8 days), or in low concentration even for a longer period (1, 5 and 10 ppm for 3-4 months).

2. The computer tomography is suitable to detect the pulmonary damages caused by fumonisin B_1 in pigs.

3. Fumonisin B_1 caused pathological alterations depend highly on individual sensitivity.

4. Long time exposure of fumonisin B_1 causes pulmonary fibrosis in pigs.

6. SCIENTIFIC PAPERS AND LECTURES ON THE SUBJECT OF THE DISSERTATION

6.1. Scientific papers in English:

1. Zomborszky-Kovács, M., Vetési, F., Kovács, F., Bata, Á., Tóth, Á., Tornyos, G.: Examination of the harmful effect to foetuses of fumonisin B_1 in pregnant sows. Teratogenesis, Carcinogenesis and Mutagenesis, 2000. 20. 293-299.
2. Tóth, Á., Zomborszky-Kovács, M., Tornyos, G., Szalai, N., Kübler, K.: Effect of low doses of the mycotoxin fumonisin B_1 on the body mass gain, feed intake and feed conversion rate of pigs. Agriculture, 2000. 6. 149-148.
3. Zomborszky-Kovács, M., Kovács, F., Horn, P., Vetési, F., Repa, I., Tornyos, G., Tóth, Á.: Investigations into time and dose-dependent effect of fumonisin B_1 in order to determine tolerable limit values in pigs. Livestock Product. Science, 2000. 76. 251-256.
4. Tornyos, G., Zomborszky-Kovács, M., Rusvai, M., Horn, H., Kovács, F.: Effect of fumonisin B_1 on immune response of weaned pigs. Acta Agraria Kaposvariensis, 2002. 6. 293.
5. Tornyos, G., Kovács, M., Rusvai, M., Fodor, J., Horn, P., Kovács, F.: Effect of dietary fumonisin B_1 on certain immune parameters of weaned pigs. Acta Veterinaria Hungarica, 2003. 51. 2. 171-179.

6.2. Scientific papers in Hungarian:

1. Tóth Á., Zomborszkyné K. M., Tornyos G., Szalai N., Kübler K.: Kis mennyiségű fumonizin-B_1 mikotoxin kiegészítés hatása a sertések testsúlygyarapodására, takarmányfelvételére és –értékesítésére Állattenyésztés és takarmányozás, 2001. 50. 3. 265-273.

6.3. Lectures in Hungarian:

Zomborszkyné Kovács M., Vetési F., Tóth Á., Tornyos G.: *A Fusarium moniliforme* által termelt toxin hatásának vizsgálata vemhes kocákban és újszülött malacokban. IV. Ifjúsági Tudományos Fórum, Keszthely, 1998. március 19.