

301163

Acta Veterinaria Hungarica

20.

VOLUME 48, NUMBER 2, 2000

EDITOR-IN-CHIEF

J. MÉSZÁROS

MANAGING EDITOR

A. SZÉKELY

EDITORIAL BOARD

**J. BOKORI, T. GAÁL, B. HARRACH, T. KASSAI,
F. KUTAS, B. NAGY, P. RUDAS, L. SOLTÍ,
S. TUBOLY, J. VARGA, F. VETÉSI, K. VÖRÖS**

INTERNATIONAL ADVISORY BOARD

M. ANKE (Germany), **G. BALJER** (Germany), **G. BREM** (Austria),
E. DECUYPERE (Belgium), **H. GÜRTLER** (Germany),
K. HRUŠKA (Czech Republic), **W. KÖRTING** (Germany),
J. LEIBETSEDER (Austria), **H. LUDWIG** (Germany),
F. NÉMETH (The Netherlands), **A. POSPISCHIL** (Switzerland),
J. PRESCOTT (Canada), **J. M. SÁNCHEZ-VIZCAÍNO** (Spain),
Joan A. SMYTH (UK)



Akadémiai Kiadó, Budapest

ACTA VET. HUNG. AVHUEA 48 (2) 139-251 (2000) HU ISSN 0236-6290

ACTA VETERINARIA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Veterinaria publishes reviews and original papers on veterinary science in English.

Acta Veterinaria is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ
Prielle Kornélia u. 4, H-1117 Budapest, Hungary
<http://www.akkrt.hu>

Manuscripts and editorial correspondence should be addressed to

Acta Veterinaria Hungarica

Veterinary Medical Research Institute of the Hungarian Academy of Sciences
P.O. Box 18, H-1581 Budapest, Hungary

Phone: (36-1) 252-2455 (ed.-in-chief) or
(36-1) 213-9793 (editor)

Fax: (36-1) 252-1069 (ed.-in-chief) or
(36-1) 213-9793 (editor)

E-mail: meszaros@novell.vmri.hu (ed.-in-chief) or
szekely.a@mail.datanet.hu (editor)

Contents, abstracts and editorial information are available through Internet: <http://www.vmri.hu/~acta/>

Subscription information

Orders should be addressed to

AKADÉMIAI KIADÓ
P.O. Box 245, H-1519 Budapest, Hungary
Fax: (36-1) 464-8221
E-mail: kiss.s@akkrt.hu

Subscription price for Volume 48 (2000) in 4 issues US\$ 196, including normal postage, airmail delivery US\$ 20.00.

Language Editor:

András Székely

Desktop typesetting:

Margit Székely (SZÉKELY Translations Ltd.)

Computer Consultant:

Balázs Deák

Acta Veterinaria is indexed in Current Contents.

Acta Veterinaria Hungarica is abstracted/indexed in AGRICOLA, Biological Abstracts, Bibliography of Agriculture, Chemical Abstracts, Current Awareness in Biological Sciences, Current Contents-Agriculture, Biology and Environmental Sciences, Dairy Science Abstracts, Excerpta Medica, Focus On: Veterinary Science and Medicine, Helminthological Abstracts, Index Medicus, Index Veterinarius, International Bibliography of Periodical Literature, La Presse Médicale, Nutrition Abstracts and Reviews, Review of Plant Pathology, Soils and Fertilizers, Veterinary Bulletin.

"This periodical is included in the document delivery program THE GENUINE ARTICLE of the Institute of Scientific Information, Philadelphia. The articles published in the periodical are available through *The Genuine Article* at the Institute for Scientific Information, 3501 Market Street, Philadelphia PA 19104."

© Akadémiai Kiadó, Budapest 2000

DETERMINATION OF RESIDUES OF PYRETHROID AND ORGANOPHOSPHOROUS ECTOPARASITICIDES IN FOODS OF ANIMAL ORIGIN

Mária SZERLETICS TÚRI*, Katalin SOÓS and Emőke VÉGH

*Fodor József National Center of Public Health, National Institute of Food Hygiene and Nutrition, H-1097 Budapest, Gyáli u. 3/a, Hungary

(Received August 19, 1999; accepted February 1, 2000)

Analytical methods were introduced for the determination of residues of ectoparasiticides containing pyrethroid and organophosphate active ingredients in foods. Milk and edible tissues of cows treated with three experimental ectoparasiticides (containing cypermethrin + diazinon, deltamethrin + diazinon and al-famethrin + diazinon, respectively) were assayed for the presence of active ingredient residues. Synthetic pyrethroid residues were not detected in any of the samples processed. Diazinon residues could only be detected in milk samples taken on the first day after treatment (0.005–0.025 mg/kg) and in liver and fat tissue samples taken on the day of slaughtering (0.12 and 0.01 mg/kg, respectively). Permethrin and propetamphos residues were determined in the skin, meat and liver of chickens kept on 'Blotic-B' treated litter and in eggs collected at different times after the treatment of layer houses. Permethrin residues could not be detected in any of the samples (< 0.01 mg/kg). Meat and fat tissues of chickens slaughtered on the day after treatment contained small amounts of propetamphos (0.003 and 0.02 mg/kg, respectively). In the case of chickens kept on the treated litter and slaughtered after one week, active ingredient was not detected in meat, but 0.006 mg/kg propetamphos was present in the fat. The residue content of other samples (liver, egg) was below the detection limit of the applied method at all sampling times. From the food toxicological point of view these pesticide combinations can be used safely if the recommended withdrawal period is observed between ectoparasiticide administration and slaughter.

Key words: Ectoparasiticides, residue analysis, pyrethroids, organophosphorous compounds, food of animal origin

In the last decades several insecticide combinations containing synthetic pyrethroid and organophosphorous compounds as active ingredients were registered and/or tested experimentally for use against ectoparasites of domestic animals in Hungary.

*Fax: +36 (1) 215 1545

During the marketing authorization process and the use of these products it is essential to protect the environment and the food chain. In addition to other basic data and animal toxicity results, the knowledge of residue levels occurring in foods and thus having the potential to contaminate the human organism via food is required already at the stage of submitting a given preparation for food toxicological evaluation. Consumer concerns about residual quantities of veterinary medicines or substances in edible tissues of animals that might endanger human health have drawn the attention to the analysis of food for residues of these compounds (Macrae et al., 1993). Analysis for insecticide residues requires methods that are suitable for serial determinations.

For this purpose, we have adapted and introduced highly reproducible analytical methods of adequate sensitivity for the gas-chromatographic determination of alphamethrin, cypermethrin, deltamethrin and permethrin residues in foods of animal origin.

Materials and methods

Cattle are often subject to attacks by ectoparasites. Diseases and nuisance caused by ectoparasites (lice, mites and ticks) are controlled and prevented by the application of organophosphorous and pyrethroid compounds. Three new insecticide combinations were used experimentally against ectoparasites of cattle. The compositions of these ectoparasiticides were as follow: 4% cypermethrin – 30% diazinon, 2% alphamethrin – 30% diazinon, and 1% deltamethrin – 30% diazinon.

Figure 1 shows the chemical structures of the active ingredients (Tomlin, 1994). The products were sprayed onto the skin of the animals after dilution in water.

The aim of the present study was to determine residue levels of the above-mentioned active ingredients in the milk and edible tissues of treated cows. A further objective was to study the elimination (excretion) of the active ingredients via the milk and to elaborate preliminary food hygienic regulations for the above-mentioned products on the basis of the results obtained.

To determination the residue levels, cows were treated externally with each of the above-mentioned products in a 1:400 aqueous solution by experts of the National Institute of Public Health. The volume of sprayed liquid was one litre for each cow.

Milk samples were taken from the treated cows one day before treatment and on the first, third and fifth days after treatment. One cow treated with alphamethrin + diazinon was slaughtered on the sixth day. We received samples from the muscle (sirloin), liver and adipose tissue (fat) of that animal and tissue samples from an untreated cow on the day of the slaughtering.

Active ingredient	Structure	Chemical (IUPAC) name
cypermethrin pyrethroid		(RS)- α -cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
alpha-cypermethrin = alphamethrin pyrethroid		A racemate comprising (S)- α -cyano-3-phenoxybenzyl (1R)-cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and (R)- α -cyano-3-phenoxybenzyl (1S)-cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
deltamethrin pyrethroid		(S)- α -cyano-3-phenoxybenzyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate
permethrin pyrethroid		3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
diazinon organophosphorous		O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate
propetamphos organophosphorous		(E)-O-2-isopropoxycarbonyl-1-methylvinyl O-methyl-ethylphosphoramidothioate

Fig. 1. Structure of active ingredients of ectoparasiticides

Analytical methods applied for the determination of ectoparasiticide residues consist of the following procedures: initial extraction into organic solvent; liquid-liquid partitions to remove water-soluble coextractives and/or lipid coex-

tractives; cleanup of the extracts by adsorption chromatography; quantitative determination of insecticide residues by gas-liquid chromatographic techniques (Miyamoto et al., 1981).

Figure 2 summarises the particular aspects of the methodology used for the determination of cypermethrin, deltamethrin and alphamethrin residues in milk and in animal tissues.

In case of milk samples, the pyrethroid-containing milk fat was separated by centrifugation. After grinding the samples with anhydrous Na_2SO_4 , active ingredients were extracted from meat, liver, fat and milk fat with n-hexane. The extracts were cleaned up first by partition between n-hexane and acetonitrile and then on Florisil column. Pyrethroid residues were determined by gas chromatography with electron capture detection (GC-ECD) (Szerletics Túri et al., 1987; Mestres and Mestres, 1992). The parameters of gas chromatography are shown in Table 1.

Parallel to the treated samples, control milk and tissue samples and samples spiked with active ingredients were also assayed.

To milk samples taken from an untreated cow, cypermethrin, alphamethrin and deltamethrin were added separately in 0.25–0.5 mg/kg amounts and subsequently the recovery rates (%) were determined. Table 2 shows the mean recoveries. The detection limits of the applied method were 0.005 mg/kg for milk, meat, liver and 0.01 mg/kg for adipose tissue.

The analysis of diazinon residues was performed according to the prescriptions of Hungarian Standard MSZ 14475-44: 1988 (Determination of organophosphorous compounds in foods of animal origin).

Recently, the ectoparasiticide 'Blotic-B' powder has also been used for other applications. This veterinary product of Bábolna Bioenvironmental Centre Ltd. (Budapest) contains 0.25% permethrin and 1% propetamphos as active ingredients. The dusting agent is used in poultry houses for litter treatment. However, the artificially raised dust gets on the feathers of broilers and laying hens and the absorbed active ingredients may produce residues in the tissues.

After the large-scale application of this ectoparasiticide, residues were determined in skin, subcutaneous fat, meat and liver samples taken from chickens kept on the treated litter. Three chickens were slaughtered on the day after treatment, then three chickens each were slaughtered after having been kept on the treated litter for one and three weeks, respectively.

In layer houses nest-boxes were treated with Blotic-B. In two series of experiments, three eggs each were taken at different times after treatment and processed. In the first series of experiments, samples were taken on the day of treatment and 3 and 7 days thereafter. In the second series samples were taken 19, 23, 26, 33 and 49 days after the treatment. The aim was to check whether the active ingredients of the dusting agent could get into the eggs.

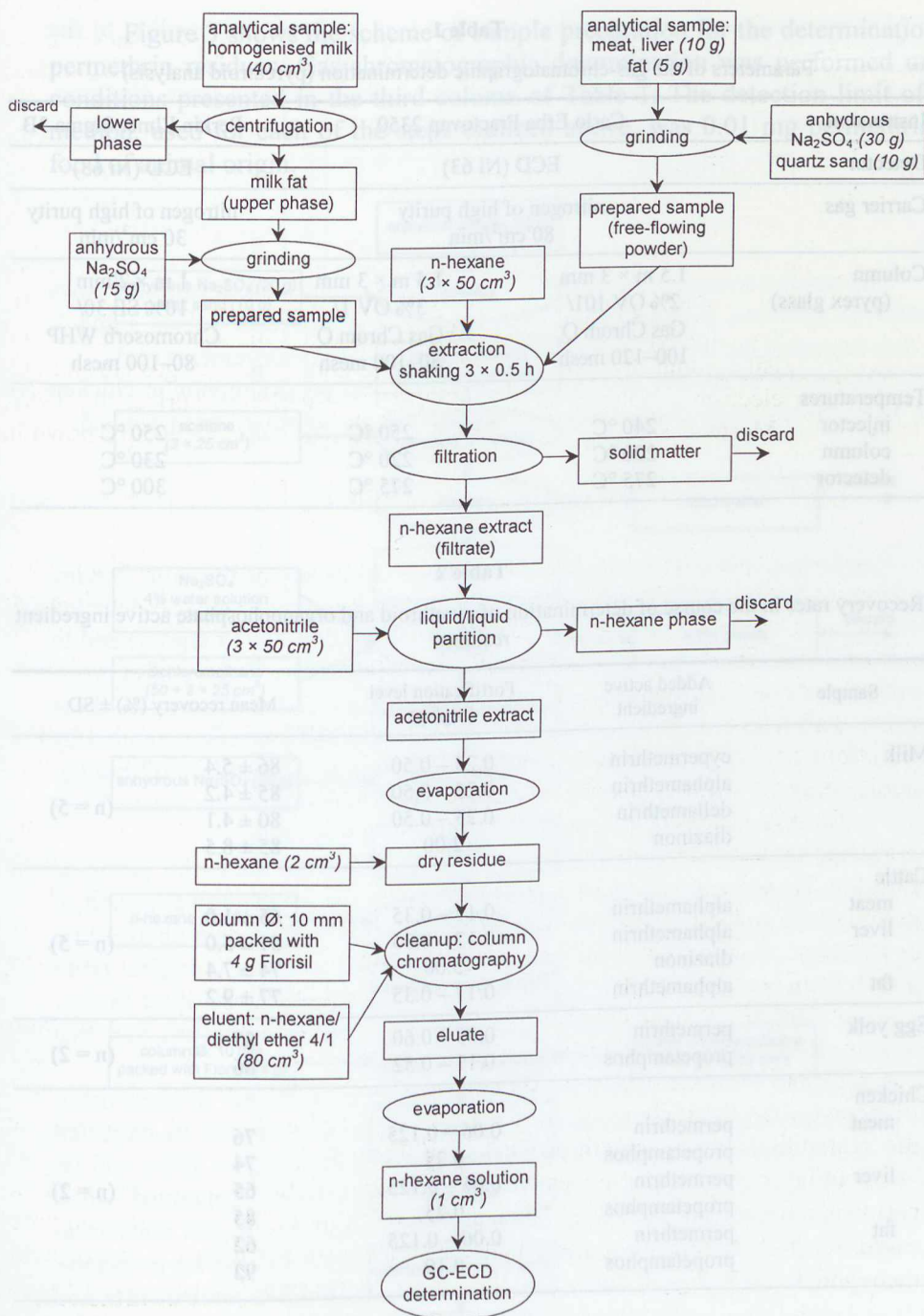


Fig. 2. Analytical method for the determination of pyrethroid (cypermethrin, deltamethrin, alphasulphothrin) residues in milk and animal tissues

Table 1
Parameters of the gas-chromatographic determination (pyrethroid analysis)

Instrument	Carlo Erba Fractovap 2350		Perkin Elmer Sigma 3B
Detector	ECD (Ni 63)		ECD (Ni 63)
Carrier gas	nitrogen of high purity 80 cm ³ /min		nitrogen of high purity 30 cm ³ /min
Column (pyrex glass)	1.5 m × 3 mm 2% OV 101/ Gas Chrom Q 100–120 mesh	1.5 m × 3 mm 3% OV 1/ Gas Chrom Q 80–100 mesh	1 m × 2 mm 10% SE 30/ Chromosorb WHP 80–100 mesh
Temperatures			
injector	240 °C	250 °C	250 °C
column	220 °C	220 °C	230 °C
detector	275 °C	275 °C	300 °C

Table 2

Recovery rates in the course of determination of pyrethroid and organophosphate active ingredient residues

Sample	Added active ingredient	Fortification level (mg/kg)	Mean recovery (%) ± SD
Milk	cypermethrin	0.25 – 0.50	86 ± 5.4
	alphamethrin	0.25 – 0.50	85 ± 4.2
	deltamethrin	0.25 – 0.50	80 ± 4.1
	diazinon	2.00	85 ± 8.5
(n = 5)			
Cattle	alphamethrin	0.17 – 0.35	65 ± 1.0
	alphamethrin	0.17 – 0.35	81 ± 5.0
	diazinon	5.00	74 ± 7.4
	alphamethrin	0.17 – 0.35	77 ± 9.2
(n = 5)			
Egg yolk	permethrin	0.03 – 0.60	87
	propetamphos	0.11 – 0.52	86
(n = 2)			
Chicken	permethrin	0.06 – 0.125	76
	propetamphos	0.25	74
	permethrin	0.06 – 0.125	65
	propetamphos	0.25	85
	permethrin	0.06 – 0.125	62
	propetamphos	0.10	92
(n = 2)			

Figure 3 shows the scheme of sample preparation for the determination of permethrin residues. Gas-chromatographic determination was performed under conditions presented in the third column of Table 1. The detection limit of the method, used for each of the steps outlined above, was 0.01 mg permethrin/kg food of animal origin.

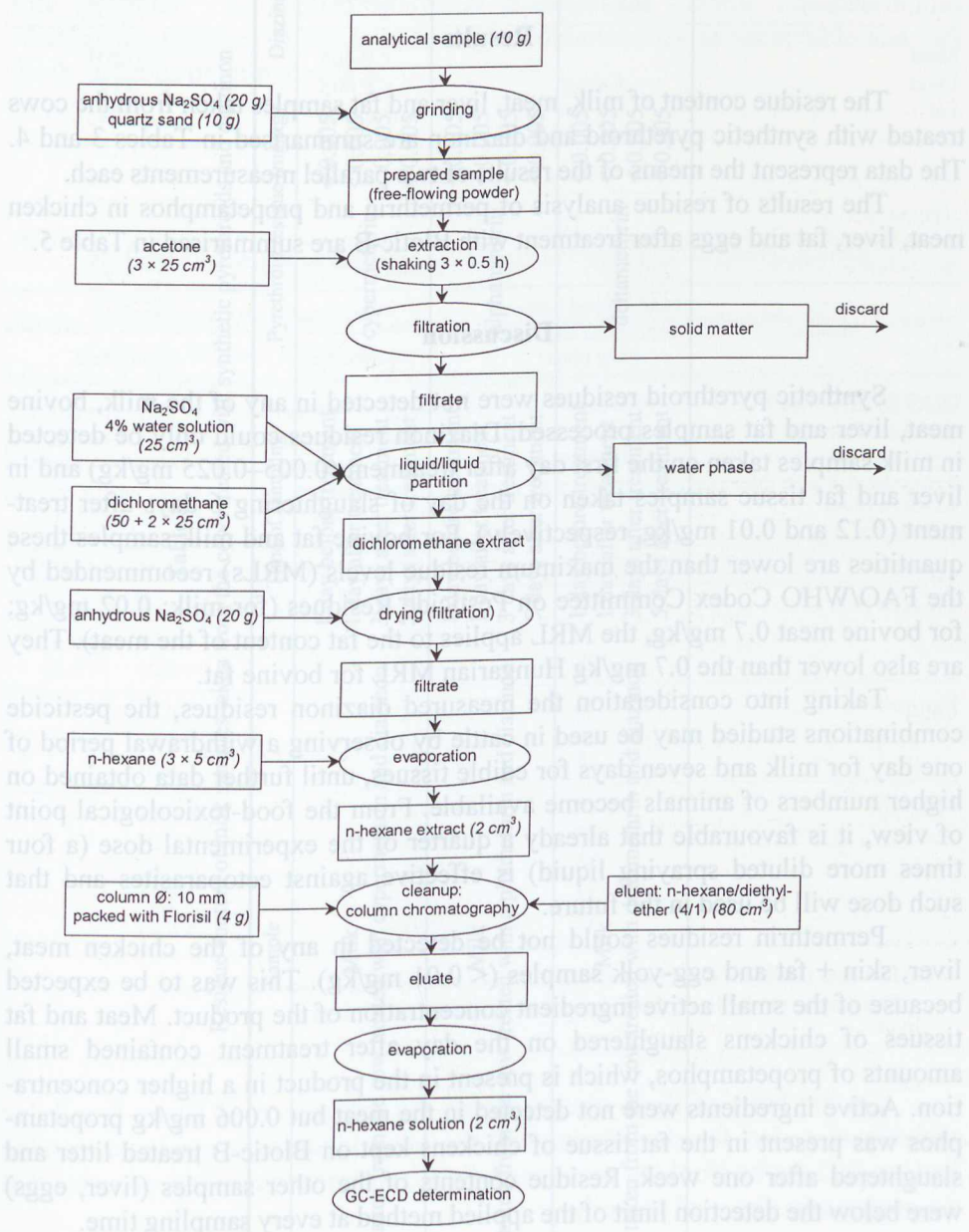


Fig. 3. Analytical method for the determination of permethrin residues in animal tissues and egg yolk

Determination of propetamphos residues was performed according to the prescriptions of Hungarian Standard MSZ 14475-44: 1988.

Parallel to 'treated' samples, control and spiked samples were also analysed in all cases. Table 2 shows the mean recoveries.

Results

The residue content of milk, meat, liver and fat samples taken from the cows treated with synthetic pyrethroid and diazinon are summarised in Tables 3 and 4. The data represent the means of the results of two parallel measurements each.

The results of residue analysis of permethrin and propetamphos in chicken meat, liver, fat and eggs after treatment with Blotic-B are summarised in Table 5.

Discussion

Synthetic pyrethroid residues were not detected in any of the milk, bovine meat, liver and fat samples processed. Diazinon residues could only be detected in milk samples taken on the first day after treatment (0.005–0.025 mg/kg) and in liver and fat tissue samples taken on the day of slaughtering 6 days after treatment (0.12 and 0.01 mg/kg, respectively). For bovine fat and milk samples these quantities are lower than the maximum residue levels (MRLs) recommended by the FAO/WHO Codex Committee on Pesticide Residues (for milk: 0.02 mg/kg; for bovine meat 0.7 mg/kg, the MRL applies to the fat content of the meat). They are also lower than the 0.7 mg/kg Hungarian MRL for bovine fat.

Taking into consideration the measured diazinon residues, the pesticide combinations studied may be used in cattle by observing a withdrawal period of one day for milk and seven days for edible tissues, until further data obtained on higher numbers of animals become available. From the food-toxicological point of view, it is favourable that already a quarter of the experimental dose (a four times more diluted spraying liquid) is effective against ectoparasites and that such dose will be used in the future.

Permethrin residues could not be detected in any of the chicken meat, liver, skin + fat and egg-yolk samples (< 0.01 mg/kg). This was to be expected because of the small active ingredient concentration of the product. Meat and fat tissues of chickens slaughtered on the day after treatment contained small amounts of propetamphos, which is present in the product in a higher concentration. Active ingredients were not detected in the meat but 0.006 mg/kg propetamphos was present in the fat tissue of chickens kept on Blotic-B treated litter and slaughtered after one week. Residue contents of the other samples (liver, eggs) were below the detection limit of the applied method at every sampling time.

Table 3

Residue content of milk samples taken from the cows treated with synthetic pyrethroids and diazinon

Sample	Time of sampling	Pyrethroid residue (mg/kg)	Diazinon residue (mg/kg)
Milk taken from the cow treated with cypermethrin and diazinon	1 day before treatment	< 0.005	< 0.005
	1 day after treatment	< 0.005	0.010
	3 days after treatment	< 0.005	< 0.005
	5 days after treatment	< 0.005	< 0.005
Milk taken from the cow treated with alphamethrin and diazinon	1 day before treatment	< 0.005	< 0.005
	1 day after treatment	< 0.005	0.025
	3 days after treatment	< 0.005	< 0.005
	5 days after treatment	< 0.005	< 0.005
Milk taken from the cow treated with deltamethrin and diazinon	1 day before treatment	< 0.005	< 0.005
	1 day after treatment	< 0.005	0.005
	3 days after treatment	< 0.005	< 0.005
	5 days after treatment	< 0.005	< 0.005

Table 4

Residue content of meat, liver and fat taken from the cow treated with alphamethrin and diazinon against ectoparasites

Sample (animal tissue or organ)	Time interval between ecto- parasiticide administration and slaughter	Alphamethrin residue (mg/kg)	Diazinon residue (mg/kg)
Meat	6 days	< 0.005	< 0.001
Liver		< 0.005	0.12
Fat		< 0.010	0.01

Table 5

Residue content of chicken meat, liver, fat and eggs after treatment with Blotic-B

Sample	Time interval between application and sam- pling (days)	Active ingredient residue (mg/kg)			
		Propetamphos		Permethrin	
Chicken skin + fat (subcutaneous fat)	1	0.020	(0.040)	< 0.01	(< 0.02)
	7	0.006	(0.012)	< 0.01	(< 0.02)
	22	< 0.003	(< 0.006)	< 0.01	(< 0.02)
Chicken meat (breast and leg)	1	0.003	(0.05)	< 0.01	(< 0.17)
	7	< 0.003	(< 0.05)	< 0.01	(< 0.17)
	22	< 0.003	(< 0.05)	< 0.01	(< 0.17)
Chicken liver	1	< 0.003	(< 0.15)	< 0.01	(< 0.5)
	7	< 0.003	(< 0.15)	< 0.01	(< 0.5)
	22	< 0.003	(< 0.15)	< 0.01	(< 0.5)
Egg yolk	0	< 0.01		< 0.01	
	3	< 0.01		< 0.01	
	7	< 0.01		< 0.01	
Egg yolk	19	< 0.01		< 0.01	
	23	< 0.01		< 0.01	
	26	< 0.01		< 0.01	
	33	< 0.01		< 0.01	
	49	< 0.01		< 0.01	

Note: The data represent the means of the results of two parallel measurements each.

The data in parentheses show the results expressed on fat basis.

On the basis of these results, during the registration of Blotic-B a 7-day withdrawal period for slaughtering and the prescription of a maximum residue limit of 0.01 mg/kg (for both active ingredients) were recommended, provided that the preparation was used properly.

In these studies, the tested veterinary preparations containing pyrethroid and organophosphate active ingredients did not give rise to measurable residues or produced only low residue levels in the organs, tissues, milk and eggs of the animals treated. From the food-toxicological point of view these ectoparasitocides may be used safely when withheld for the recommended period of time before slaughter. The applied analytical methods are suitable for determining whether the residues of these veterinary preparations are at acceptable and safe levels in foods of animal origin.

(Received April 19, 1999; accepted October 20, 1999)

References

- Macrae, R., Robinson, R. K. and Sadler, M. J. (eds) (1993): Encyclopaedia of Food Science, Food Technology and Nutrition, Veterinary Medicines. Academic Press Limited, London, pp. 203–208.
- Mestres, R. and Mestres, G. (1992): Deltamethrin: Uses and environmental safety. *Rev. Environm. Contamin. Toxicol.* **124**, 1–18.
- Miyamoto, J., Beynon, K. I., Roberts, T. R., Hemingway, R. J. and Swaine, H. (1981): The chemistry, metabolism and residue analysis of synthetic pyrethroids. *IUPAC Reports on Pesticides* 15. *Pure and Appl. Chem.* **53**, 1967–2022.
- Szerletics Túri, M., Soós, K., Szépvölgyi, J. and Zsinka, Á. (1987): Determination of deltamethrin in the feeds and in the fat of experimental rats (in Hungarian, with English abstract). *Egészségtudomány* **31**, 52–58.
- Tomlin, C. (ed.) (1994): The Pesticide Manual, Incorporating The Agrochemicals Handbook. The British Crop Protection Council and The Royal Society of Chemistry, Tenth Edition, pp. 852, 783, 296, 287, 261, 259.

Key words: Mycotoxin, deoxynivalenol, DON, wheat, high-performance liquid chromatography, HPLC

Deoxynivalenol (DON, vomitoxin) is a trichothecene mycotoxin produced by several *Fusarium* species, most commonly by *Fusarium graminearum* and *Fusarium culmorum* (Szécsi and Bartók, 1995; Miedaner et al., 1997). In Hungary, *F. graminearum* and *F. culmorum* often infect cereals and are the commonest cause of ear fusariosis of wheat (Mesterházy, 1984). The species *F. graminearum* frequently plays a role in the development of fusarium ear moulding of maize (Mesterházy and Vojtíková, 1977).

NATURAL DEOXYNIVALENOL (DON) CONTAMINATION OF WHEAT SAMPLES GROWN IN 1998 AS DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

B. FAZEKAS*, E. T. HAJDU, A. K. TAR and J. TANYI

Veterinary Institute of Debrecen, H-4031 Debrecen, Bornemissza u. 3–7, Hungary

(Received April 19, 1999; accepted October 20, 1999)

A high-performance liquid chromatography – diode array detection (HPLC-DAD) method was developed for determining the deoxynivalenol (DON) content of wheat and other cereals. The samples were extracted with a mixture of acetonitrile and water (84 + 16). Part of the extract was evaporated and purified on Florisil and activated charcoal columns. HPLC separation was performed on a C₁₈ column, using acetonitrile–water (8 + 92) as eluent. Diode array detection (DAD) was performed at 218 and 236 nm, by determination of the UV spectrum. Quantitative analysis was carried out by the external standard method, using the UV spectrum obtained by DAD for confirmation. The recovery rate of DON was 75 ± 3.1% and the detection limit was 0.05 mg/kg DON. Using this method, the DON content of 99 feeding wheat samples grown in the northeastern part of Hungary in 1998 was determined. Eighty-eight percent of the samples originating from three counties contained 0.94 mg/kg DON on the average. The highest individual value was 4.3 mg/kg. DON contamination of wheat was of higher prevalence (100%) and severity (0.27–4.3 mg/kg) in the southeastern county of Békés than in Szabolcs county located in the northeastern part of Hungary (ratio of positive samples: 82%; DON concentration: 0.05–1.3 mg/kg). The higher than usual DON contamination of feeding wheat can be explained by the rainy summer weather. DON contamination of feeding wheat poses a major risk to the production and animal health status of pig herds.

Key words: Mycotoxin, deoxynivalenol, DON, wheat, high-performance liquid chromatography, HPLC

Deoxynivalenol (DON, vomitoxin) is a trichothecene mycotoxin produced by several *Fusarium* species, most commonly by *Fusarium graminearum* and *Fusarium culmorum* (Szécsi and Bartók, 1995; Miedaner et al., 1997). In Hungary, *F. graminearum* and *F. culmorum* often infect cereals and are the commonest cause of ear fusariosis of wheat (Mesterházy, 1984). The species *F. graminearum* frequently plays a role in the development of fusarium ear moulding of maize (Mesterházy and Vojtovics, 1977).

*E-mail: fazekasb@indigo2.oai.hu; Fax: +36 (52) 310 823

DON is a common contaminant of cereals all over the world. Of the domestic animals, the pig is the species most susceptible to it. Pigs affected by DON mycotoxicosis reject feed, vomit, develop enteritis, and their immune system becomes compromised. Poultry species and ruminants have low susceptibility to DON (Ványi, 1990; Rotter and Prelusky, 1996). The consumption of foods made from cereals may be a direct source of human exposure to DON. In 1984 and 1987, respectively, human cases of DON mycotoxicosis occurred after the consumption of mouldy rice in Japan and China and after the consumption of bread made from mouldy flour in India (Rotter and Prelusky, 1996). Affected people showed clinical symptoms including vomiting, abdominal pain, headache and diarrhoea. The foods incriminated in these cases of human mycotoxicosis contained high concentrations of DON (mouldy rice: 0.34–92.8 mg/kg; mouldy flour: 0.35–8.38 mg/kg). Although DON contamination of such extremely high level very rarely occurs, low-level contamination is common.

Several methods are available for determining the DON contamination of feeds. High-performance liquid chromatography (HPLC) is a commonly used method (Chang et al., 1984; Lauren and Greenhalgh, 1987). Recently the use of HPLC with diode array detectors (DAD) has gained ground (Walker and Meier, 1998). The advantage of HPLC-DAD over the conventional UV detector is that the determination of UV spectra allows the analysis of peak 'purity' as well as confirmation.

Cereals are highly susceptible to fusarium ear disease, which is most often caused by the species *F. graminearum* and *F. culmorum* in Hungary (Mesterházy, 1984). In 1998, the vegetation period of wheat was characterised by an unusually high amount of rainfall, which resulted in fusarium ear disease of 'epidemic' proportions in certain areas of the country (Szumics et al., 1998). As the fungus species *F. graminearum* and *F. culmorum* causing the disease produce DON as their principal toxin, feeding wheat samples originating from three eastern counties of Hungary were assayed for DON contamination by the HPLC-DAD method. The purpose of the present studies was twofold: to study the applicability of HPLC-DAD for determining the DON contamination of feeding wheat, and to survey the DON contamination of wheat in order to supply data for assessing the animal health risks involved.

Materials and methods

Wheat samples

A total of 99 feeding wheat samples originating from three eastern counties of Hungary were analysed in the period between 20 July and 1 October 1998. Twenty-eight samples were obtained from Szabolcs-Szatmár-Bereg county, 45 from Hajdú-Bihar county and 26 from Békés county. The wheat samples were submitted to our institute from feed processing and feed mixing plants, animal

holdings and wheat-growing farms. The collected samples were stored at -20°C until the mycotoxin analysis.

Mycotoxin analysis

The extraction and cleanup procedure of the wheat samples was developed by combination and partial modification of the cleanup steps of methods described in the international literature.

(a) *Extraction.* Extract 50 g of the finely ground wheat with 200 ml acetonitrile–water (84 + 16) by shaking on a shaker for 1 h (Chang et al., 1984). Defat 30 ml of the filtrate by shaking with 50 ml n-hexane. Evaporate 20 ml of the acetonitrile phase.

(b) *Cleanup on Florisil column* (Sano et al., 1987; Möller and Gustavsson, 1992). Dissolve the evaporated extract in 1 ml methanol plus 9 ml chloroform. Apply the mixture onto a column filled with 2 g Florisil and previously conditioned with 10 ml methanol and 10 ml of methanol–chloroform mixture (1:9). After sample application, make 40 ml of the methanol–chloroform mixture (1:9) flow through the column at a flow rate of about 2 ml per minute. Discard the conditioning solvents and collect the mixture containing the sample and the elution mixture. Evaporate the solution of about 50 ml volume.

(c) *Cleanup based on liquid-liquid partition.* Dissolve the evaporated residue in 3 ml n-hexane, and shake it three times with an acetonitrile–water mixture (3:4). Collect the acetonitrile phase (about 12 ml) and evaporate it (Rajakylä et al., 1987).

(d) *Cleanup on activated charcoal column.* Dissolve the residue in 2 ml methanol and apply it onto a column filled with 0.5 g activated charcoal, then elute the sample with 20 ml methanol (Lauren and Greenhalgh, 1987). After evaporation, dissolve the residue in 200 μl HPLC-grade methanol–water mixture (1:9), and inject 20 μl into the HPLC for analysis.

(e) *HPLC-DAD analysis.* Separation was performed on a reversed-phase C_{18} column using acetonitrile–water (8:92) as eluent. Detection was carried out on DAD at 218 nm and 236 nm. Quantitative determination was performed with the help of a calibration line constructed at 236 nm. Confirmation of the results was done on the basis of the UV spectrum.

Validation of the method

For the quantitative determination of DON, a calibration line was constructed on the basis of the area under the DON chromatographic peak in the chromatogram taken up on DAD at 236 nm. The calibration line was linear in the concentration range of 0.625 ng/ μl to 20 ng/ μl . The equation of the calibration line was as follows:

$$y = 29.8766x - 0.055$$

The coefficient of correlation proved to be 0.999, indicating the linearity of the calibration line. In the equation, y represents the area under the chromatographic peak, while x represents the DON concentration. The DON concentration of wheat samples was calculated by the following formula:

$$C_{\text{ng/g}} = (A \times T \times D) : (I \times W),$$

where

C = DON concentration of the sample in ng/g, which, when divided by 1000, yields the DON concentration expressed in mg/kg units;

A = ng DON in 20 μl , i.e. the injected volume, of the analysed solution;

T = volume of the solution from which the injected volume was taken (200 μl);

D = dilution factor;

I = volume of the injected solution (20 μl);

W = quantity of sample equivalent with solution T in g (5 g).

Substituting the specific values in the equation, it is modified as follows:

$$C_{\text{ng/g}} = A \times 2 \times D \quad (A = 20 x)$$

The values thus obtained were corrected with the average toxin recovery rate.

Determination of the DON recovery rate: DON standard was added to DON-free wheat in 0.3 and 0.6 mg/kg concentration. The DON concentration of the samples was determined by 5 parallel measurements each, and the recovery rate was calculated. To determine the reproducibility of the method, the relative difference between parallel measurements was established.

During qualitative identification, the UV spectrum was determined on DAD. The maximum occurred at 218 nm (Fig. 2). The chromatogram was taken up at 218 and 236 nm. Quantitative determination was usually performed on the basis of the peak obtained at 236 nm, as at 218 nm certain samples showed some disturbing peaks close to the DON peak that were due to contamination. The UV spectrum of the peak corresponding to DON was considered identical with that of the standard if it gave a value higher than 950 on a scale of 1000.

HPLC apparatus

A Hewlett-Packard 1050 type gradient pump and a HP 1050 type Diode Array Detector were used for the analysis. The pump and the detector were controlled with the help of a ChemStation software and the data obtained on the detector were processed. Identification of the UV spectra was also done with the ChemStation software.

Analytical column

A LiChrospher RP 18 type, 250 × 4 mm column of 5 µm granule size was used for the separation.

Chemicals

The DON standard was obtained from Sigma. It was dissolved in HPLC-grade methanol and stored at -20 °C. Working dilutions were made from the stock solution using a mixture of methanol and water (1:9). Acetonitrile used for the instrumental analysis was of HPLC RS grade. All other chemicals were of analytical grade.

Results

Validation of the method

Using the analytical method presented here, the recovery rate of DON proved to be $75 \pm 3.1\%$. When determining the reproducibility of the method, the relative difference between parallel measurements did not exceed 10%. The detection limit of DON was 0.05 mg/kg.

Figure 1 shows the HPLC chromatogram of wheat contaminated with DON. It can be seen that DON was well separated from the contaminating components. Figure 2 presents the UV spectra of the DON standard and the sample peak having the same retention time as that of the standard. The two spectra are identical (degree of identity: 998), which proves that the standard and the sample peak are identical (confirmation).

DON contamination of wheat samples

The DON contamination of feeding wheat samples is summarised in Table 1. It can be seen that the DON contamination of feeding wheat markedly varied by county. The ratio of samples contaminated with DON was high in all the three counties surveyed; however, the average contamination level of samples from Békés county was about three times higher than that of samples from Szabolcs county. Samples from Hajdú county had an intermediate position in this respect. The overall ratio of DON-contaminated samples in the three counties was 88% (total number of samples: 99; samples contaminated with DON: 87; negative samples: 12), whereas the average level of contamination was 0.94 mg/kg. Figure 3 shows the distribution of DON contamination according to concentration.

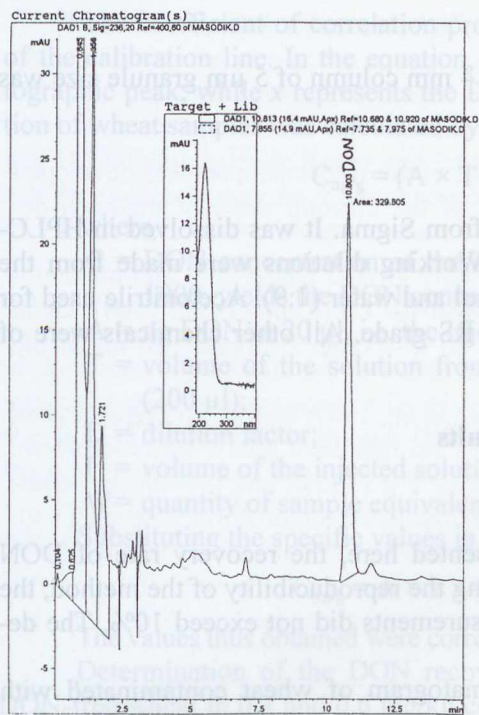


Fig. 1. HPLC-DAD chromatogram of a wheat sample (236 nm). Level of DON contamination: 0.59 mg/kg

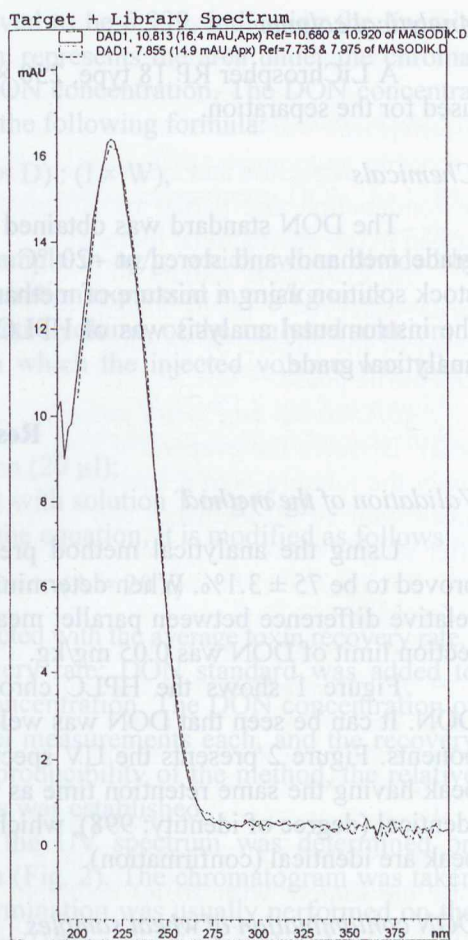


Fig. 2. UV spectra of the DON standard and of the DON peak of wheat sample on DAD. Identity: 998 (confirmation)

Table 1
DON contamination of feeding wheat samples in 1998

	Positive %	Minimum (mg/kg)	Maximum (mg/kg)	Average (mg/kg)
Szabolcs county	82	< 0.05	1.3	0.6
Hajdú county	84	< 0.05	2.8	0.7
Békés county	100	0.27	4.3	2.0

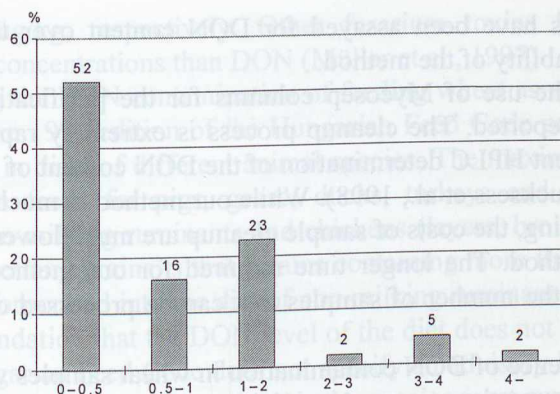


Fig. 3. Distribution of the DON contamination of wheat samples by concentration range (mg/kg)

Discussion

With the method applied in this study, the extraction of DON was performed by the generally used acetonitrile–water mixture (84:16). The further phases of sample cleanup were also carried out according to the conventional methods. The sample was first purified on a Florisil column. Florisil columns have successfully been used for the cleanup of trichothecene toxins also by other authors (Sano et al., 1987; Möller and Gustavsson, 1992). The evaporated residue eluted from the Florisil column readily dissolved in n-hexane, from which DON was extracted with aqueous acetonitrile solution while most of the contaminants was retained in the n-hexane phase. Similar cleanup procedures have been used by other authors for the isolation of trichothecenes (Rajakylä et al., 1987; Möller and Gustavsson, 1992). Sample cleanup was completed on an activated charcoal column that bound most of the remaining contaminants while DON was readily eluted with methanol. A similar cleanup step has been used successfully by others for the determination of DON and nivalenol (Lauren and Greenhalgh, 1987).

The relatively time-consuming cleanup steps resulted in a pure sample, as proven by the wheat sample chromatogram shown in Fig. 1. There are no interfering peaks to disturb toxin determination; thus, identification of the toxin can be confirmed by the UV spectrum taken up on DAD in addition to the retention time. This method has successfully been used for determining the DON content of other cereals and processed products (e.g. wheat flour, wheat bran, maize, barley and rye) as well as compound feeds. The recovery rate was somewhat lower as compared to other methods, which can be explained by the longer cleanup procedure. However, recovery rate and reproducibility have a low standard deviation, which indicates that the method is reliable. The low-level presence of matrix enables the attainment of a low detection limit. More than one

thousand samples have been assayed for DON content over the years, which shows the applicability of the method.

Recently the use of Mycosep columns for the purification of trichothecenes has been reported. The cleanup process is extremely rapid and efficient, and allows efficient HPLC determination of the DON content of flour, wheat and bran samples (Trucksess et al., 1998). While our method is much more laborious and time-consuming, the costs of sample cleanup are much lower as compared to the Mycosep method. The longer time required for our method, however, undoubtedly limits the number of samples that can be processed during a specific period of time.

The prevalence of DON contamination in wheat samples was almost identical in the three counties surveyed, and came close to 100%. That high prevalence of DON contamination was likely to be related to the rainy weather that favours the spread of fusaria and mycotoxin production. The DON-contamination level of wheat grown in Békés county situated in the southeastern part of the country markedly exceeded that of wheat from the northeastern Szabolcs county in terms of both the average and the maximal value. That difference can probably be explained by the typically warmer and moister weather of the more southerly Békés county. In addition to differences in the weather conditions, the chemical control of fusarium infection may also have differed substantially between the individual counties.

In Hungary, DON was first detected in maize but later on it was established primarily as a contaminant of wheat. In a survey of the wheat crop harvested in 1985, 82.6% of the samples contained DON in a concentration of 0.2 to 1.6 mg/kg (Ványi, 1990), while in 1992 DON contamination was found in 77.6% of the samples analysed (Kovács and Ványi, 1994). Differences in the level of DON contamination of wheat samples grown in different counties have been observed previously. According to a 1987 survey, in Békés county 80% of the wheat crop contained 0.2 to 6.1 mg/kg DON while in Szabolcs county 50% of the wheat crop was contaminated with DON in a concentration of 0.2 to 1.5 mg/kg (Ványi, 1990). The DON-contamination level found in this study is similar to the values measured by Ványi in 1987. The higher ratio of DON-positive samples found in this study can be explained by the lower detection limit of the method used by us.

Wheat is regularly monitored for DON contamination all over the world (Tanaka et al., 1988; Trucksess et al., 1995; Müller et al., 1997). In recent years the largest European survey was the one in which the fusarium toxin contamination of wheat harvested in southwest Germany was monitored by gas chromatography-mass spectrometry (GC-MS) and by HPLC over a 6-year period. In samples assayed for 11 fusarium toxins DON occurred the most commonly and in the highest concentration. The ratio of DON-contaminated samples was 68–95%, while the average and the maximum DON concentration was 0.152–1.692 and

1.187–20.538 mg/kg, respectively. Other fusarium toxins had much lower prevalence and concentrations than DON (Müller et al., 1997).

Assessing the DON contamination of feeding wheat as a risk factor is not an easy task. The 1990 edition of the Hungarian Feed Code published advisory levels for DON in diets of different animal species. The maximal advisory level is 0.4 mg/kg in feeds for pigs, geese, ducks, turkeys and pet animals and 2.0 mg/kg in non-milking ruminants and chickens (layers, broilers). The recommendation concerning grains is that 'grains containing more than 4 mg/kg DON may only be incorporated into the diet of non-milking ruminant animals, with the added recommendation that the DON level of the diet does not exceed the above limit'. The Hungarian Feed Code does not specify an advisory level for DON on feeding wheat destined for swine, which is the species most susceptible to DON. It is rather difficult to extrapolate values specified for pig feeds to feeding wheat, as feed constituents other than wheat (e.g. maize) may also be contaminated with DON. According to the current guidelines of the Food and Drug Administration (FDA), the advisory level is 5 mg/kg DON on grains and grain by-products destined for swine, with the added recommendation that these ingredients not exceed 20% of their diet. The maximum allowed DON level stated for pig diets by the Hungarian Feed Code (0.4 mg/kg) is stricter than the FDA recommendation. In our opinion, stating and complying with a lower advisory level would facilitate the prevention of the damage caused by DON toxicosis.

There are no regulatory limits specified for the DON contamination of feeding wheat in Hungary. It is well known, however, that the DON limit specified for exported wheat is 0.5 mg/kg. The importers strictly require compliance with that limit. In this study, almost half of the wheat samples had a DON content exceeding 0.5 mg/kg, indicating that these batches failed to meet the export quality requirements. The fact that the samples originated from batches destined for domestic use suggests that the more contaminated batches were processed within the country.

Summarising the results of the present studies, it can be established that the rainy weather dominating the vegetation period of wheat in 1998 resulted in such excessive DON contamination of the feeding wheat that rarely occurs under the Hungarian conditions. In our experience, that DON contamination presented an increased production and animal health risk in the pig herds, which was confirmed also by our diagnostic findings. Namely, since August 1998 disease entities indicative of DON toxicosis have been diagnosed in much more pig herds than in the earlier years. These disease entities could be brought into connection with the high DON contamination of the pig diets or of the wheat and triticale incorporated into them (Fazekas, 1999).

References

- Chang, H. L., DeVries, J. W., Larson, P. A. and Patel, H. H. (1984): Rapid determination of deoxynivalenol (vomitoxin) by liquid chromatography using modified Romer column cleanup. *J. AOAC Int.* **67**, 52–54.
- Fazekas, B. (1999): Fusariotoxicozes in swine (in Hungarian with English abstract). *Magyar Állatorvosok Lapja* **121**, 340–343.
- Kovács, F. and Ványi, A. (1994): Moulds, mycotoxins, food quality and public health (in Hungarian with English abstract). *Magyar Állatorvosok Lapja* **49**, 325–328.
- Lauren, D. R. and Greenhalgh, R. (1987): Simultaneous analysis of nivalenol and deoxynivalenol in cereals by liquid chromatography. *J. AOAC Int.* **70**, 479–483.
- Mesterházy, Á. (1984): *Fusarium* species of wheat in South Hungary, 1970–1983. *Cer. Res. Comm.* **12**, 167–170.
- Mesterházy, Á. and Vojtovics, M. (1977): Study of the *Fusarium* infection of maize in 1972–1975 (in Hungarian). *Növénytermelés* **26**, 367–378.
- Miedaner, T., Gang, G., Scholling, A. and Geiger, H. H. (1997): Aggressiveness and mycotoxin production of populations of *Fusarium culmorum* and *F. graminearum* in winter rye. *Cer. Res. Comm.* **25**, 467–470.
- Möller, T. E. and Gustavsson, H. F. (1992): Determination of type A and B trichothecenes in cereals by gas chromatography with electron capture detection. *J. AOAC Int.* **75**, 1049–1053.
- Müller, H. M., Reimann, J., Schumacher, U. and Schwadorf, K. (1997): *Fusarium* toxins in wheat harvested during six years in an area of southwest Germany. *Nat. Toxins* **5**, 24–30.
- Rajakylä, E., Laasasenaho, K. and Sakkers, P. J. D. (1987): Determination of mycotoxins in grain by high-performance liquid chromatography and thermospray liquid chromatography – mass spectrometry. *J. Chromatography* **384**, 391–402.
- Rotter, B. A. and Prelusky, D. B. (1996): Toxicology of deoxynivalenol (vomitoxin). *J. Toxicol. Environ. Health* **48**, 1–34.
- Sano, A., Matsutani, S., Suzuki, M. and Takatani, S. (1987): High-performance liquid chromatographic method for determining trichothecene mycotoxins by post-column fluorescence derivatization. *J. Chromatogr.* **410**, 427–436.
- Szécsi, Á. and Bartók, T. (1995): Trichothecene chemotypes of *Fusarium graminearum* isolated from corn in Hungary. *Mycotoxin Res.* **11**, 85–92.
- Szumics, L., Bedő, Z. and Vida, Gy. (1998): *Fusarium* is the culprit (in Hungarian). *Magyar Mezőgazdaság* pp. 10–12.
- Tanaka, T., Hasegawa, A., Yamamoto, S., Lee, U. S., Sugiura, Y. and Ueno, Y. (1988): Worldwide contamination of cereals by the *Fusarium* mycotoxin nivalenol, deoxynivalenol and zearalenone. 1. Survey of 19 countries. *J. Agric. Food Chem.* **36**, 979–983.
- Trucksess, M. W., Page, S. W., Wood, G. E. and Cho, T. H. (1998): Determination of deoxynivalenol in white flour, whole wheat flour, and bran by solid-phase extraction / liquid chromatography: interlaboratory study. *J. AOAC Int.* **81**, 880–886.
- Trucksess, M. W., Thomas, F., Young, K., Stack, M. E., Fulgueras, W. J. and Page, S. W. (1995): Survey of deoxynivalenol in U. S. 1993 wheat and barley crops by enzyme-linked immunosorbent assay. *J. AOAC Int.* **78**, 631–636.
- Ványi, A. (1990): Deoxynivalenol fusariotoxicozis. In: Téren, J., Draskovics, I. and Novák, E. K. (eds) *Mycotoxins, toxigenic fungi, mycotoxicozes* (in Hungarian). MÉTE, Budapest, pp. 194–205.
- Walker, F. and Meier, B. (1998): Determination of the *Fusarium* mycotoxins nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol and 15-O-acetyl-4-deoxynivalenol in contaminated whole wheat flour by liquid chromatography with diode array detection and gas chromatography with electron capture detection. *J. AOAC Int.* **81**, 741–748.

EFFECTS OF DIETARY PROTEIN AND CARBOHYDRATE SOURCE ON RUMEN FERMENTATION AND NUTRIENT FLOW IN SHEEP

Hedvig FÉBEL*, Szilvia HUSZÁR and Ildikó ZSOLNAI HARCZI

Research Institute of Animal Breeding and Nutrition, H-2053 Herceghalom, Hungary

(Received July 1, 1999; accepted October 20, 1999)

The effects of decreasing levels of rumen degradable protein (RDP) and nonstructural carbohydrate (NSC) (Diet 1: 74% RDP and 38% NSC; Diet 2: 57% RDP and 32% NSC; Diet 3: 48% RDP and 23% NSC) were studied in cannulated sheep. Total volatile fatty acid (VFA) content rose in response to increasing NSC content. The molar ratio of acetate to propionate was the narrowest for Diet 1. Ruminal concentrations of ammonia and urea increased in response to the rising level of RDP. Flow of organic matter (OM) to the duodenum was increased for Diet 3, which resulted in the lowest apparent and true ruminal digestion of OM. Duodenal flow of total nitrogen (N) increased as RDP content decreased. The highest quantity of undegraded feed protein in duodenal digesta was measured in sheep fed Diet 3. Microbial N flow and microbial efficiency were unaffected by the diets. These results indicate that an NSC level lower than 25% and an RDP content lower than 50% did not exert any negative effect on microbial N production. This phenomenon supports the theory that if the level of RDP is lowered with a concomitant decrease in NSC, uncoupled fermentation cannot be observed.

Key words: Degradation, rumen fermentation, microbial efficiency, non-structural carbohydrate, rumen degradable protein, sheep

Microbial growth and protein synthesis are dependent on the amount of ammonia and amino acid derived from the ruminal degradation of feed protein and on the presence of an adequate supply of energy from carbohydrate fermentation. If the ration contains a sufficient amount of rumen degradable protein (RDP), microbial protein synthesis is determined by the source and amount of carbohydrate. The chemical structure of carbohydrate available to microorganisms can be different and therefore microbial growth can be modified. A higher microbial protein yield was observed in an *in vitro* experiment (Stokes et al., 1991a), when the proportion of nonstructural carbohydrate (NSC) was increased. Rode et al. (1985) measured the highest microbial nitrogen (N) flow at the duodenum when 62% concentrate was fed. The higher grain proportion and therefore higher NSC intake of animals had a negative effect on rumen function, resulting

*E-mail: hfebel@atk.hu; Fax: +36 (23) 319 133

in a decreased microbial protein yield (Stokes et al., 1991b). The complexity of microbial growth and energy supply is shown by the fact that several authors (Chamberlain and Thomas, 1979; Mathers and Miller, 1981) observed a reduction in microbial growth at concentrate levels exceeding 30%. Hoover (1987) noted that, in those studies, the concentrate was increased without a concomitant increase in RDP, therefore the NSC to RDP ratio widened. The imbalance in NSC to RDP may have been responsible for the reduction in microbial growth.

The objective of our experiments was to study ruminal digestion and microbial efficiency in that case when the NSC and RDP contents of diets were decreased proportionately, and therefore the NSC to RDP ratios were similar. Diet 1 contained a high amount of RDP (extracted sunflower meal) and NSC (corn meal). The carbohydrate source was changed in Diet 2 and 3 by replacing corn meal with an increasing amount of structural carbohydrate, meadow hay. Corn gluten meal (for its lower ruminal protein degradation) was added to Diets 2 and 3 to achieve a lower RDP content in rations 2 and 3.

Materials and methods

Six Hungarian Merino wethers (average body weight: 62 ± 5.3 kg) were fitted with ruminal cannulas and T-type cannulas in the duodenum. The duodenal cannula was placed approximately 5 cm posterior to the pylorus (Aguilar and Depeters, 1988). The sheep were kept in individual slatted-floor pens where water and salt blocks were freely available.

The ingredients and chemical composition of experimental diets are listed in Table 1. Diets were assigned in a Latin square design.

The nylon bag (*in sacco*) procedure was used to estimate the rumen crude protein (CP) degradability of the diets (Ørskov and McDonald, 1979). The NSC content of each diet was determined by difference calculations (Stokes et al., 1991b): $100 - [\text{CP} + (\text{neutral detergent fibre (NDF)} - \text{NDF bound protein}) + \text{ether extract} + \text{ash}]$.

All sheep were limit-fed their respective diet at a dry matter (DM) intake of $60 \text{ g DM/bw}^{0.75}$ (Van Es and Van Der Meer, 1980). Sheep were fed their respective diets twice daily in equal portions at 08:00 and 16:00.

The experimental periods lasted 18 days, with day 1 to 13 for adaptation to diet and day 14 to 18 for sample collection. The duodenal nutrient flow was measured and duodenal digesta samples were collected, as was described previously (Fébel et al., 1995).

Feed and freeze-dried duodenal samples were analyzed for DM, OM and N content according to the standards specified for the determination of the nutritive value of feeds (Hungarian Standard MSz 6830, 1986). The methods of Van Soest and Robertson (1985) were used for the determination of NDF, acid detergent fibre

(ADF) and cellulose of feed and digesta. Ammonia concentration was analyzed by the Berthelot procedure (Chaney and Marbach, 1962). For amino acid (AA) analysis, each duodenal sample was hydrolysed with 6N HCl at 110 °C for 24 h. AAs were separated in an AMINOCHROM II type instrument by ion-exchange column chromatography. Purine contents of duodenal samples were analyzed to estimate microbial N flows using microbial purines/N ratio as a reference (Zinn and Owens, 1986). For Cr analysis digesta samples were prepared by the method of Christian and Coup (1954), and measured by an ICP-OES inductive plasma emission spectroscope (ARL 3410 ICP spectrometer). Polyethylene glycol (PEG) concentration was determined by a turbidimetric method according to Hyden (1956).

Table 1
Dietary ingredients and diet composition

Item	Diet 1	Diet 2	Diet 3
<i>Ingredients, %</i>			
Meadow hay	30.00	50.00	70.00
Corn meal	44.23	23.50	17.25
Extracted sunflower meal	24.00	8.00	—
Corn gluten meal	—	7.50	12.25
Barley	—	9.44	—
Limestone	1.27	1.06	—
Premix	0.50	0.50	0.50
<i>Composition, g/kg DM</i>			
Organic matter	932.9	932.7	936.3
Crude protein	181.3	177.0	177.5
NDF	359.7	438.6	508.0
ADF	178.7	220.4	271.5
Cellulose	147.7	178.3	222.5
Hemicellulose	191.1	218.2	236.5
Nonstructural carbohydrate (NSC)	381.9	317.2	233.4
Rumen degradable protein (RDP), %	74	57	48
Ratio of NSC to RDP	2.85	3.14	2.74

Ruminal samples were collected on days 17 and 18 before feeding and 3 h post-feeding. Rumen fluid pH was measured immediately after sample collection. Ammonia and urea were determined as described by Chaney and Marbach (1962). Ruminal samples for volatile fatty acid (VFA) determination were preserved with 25% metaphosphoric acid and were analyzed by gas chromatography (Supelco Inc., 1975). Rumen volume and ruminal fluid dilution rate were estimated by the method of Dehareng and Godeau (1989).

On the last day of each period, ruminal fluid was collected before feeding and 3 and 6 h thereafter for bacterial separation. Composite ruminal fluid was centrifuged at $1000 \times g$ for 20 min to remove feed particles. Bacteria were separated

from the supernatant fluid by multiple centrifugation at $30,000 \times g$ for 15 min. The pellet containing bacteria was freeze-dried. Bacterial samples were assayed for DM, OM, N and purine content by the methods described above.

Comparison of the results and statistical evaluation were performed using multifactorial analysis of variance (ANOVA program) and *t*-test (Sváb, 1981).

Results

Results on ruminal fermentation before and 3 h post-feeding are presented in Table 2. When sheep were fed the high RDP and NSC ration (Diet 1) a significant increase in total VFA content was observed in the ruminal fluid 3 h after feeding. At that sampling time lower rumen pH was measured, which is related to the high NSC diet used. Sheep fed the high RDP and NSC ration (Diet 1) had lower acetate and higher propionate in the rumen fluid. Acetate to propionate ratio was the lowest for Diet 1. Isobutyrate, butyrate, isovalerate and valerate concentrations increased when more RDP and NSC sources were fed (Diet 1). Ruminal ammonia and urea concentrations decreased gradually as diets of higher rumen undegradable protein content were fed.

OM passage to the small intestine was higher in sheep fed the ration with the lowest level of RDP and NSC (Diet 3). Therefore, the lowest percentage of OM digested in the rumen was obtained in this group (Table 3). The intake of fibre fractions was higher for Diets 2 and 3 than for Diet 1, which was reflected by the higher NDF, ADF, cellulose and hemicellulose content of duodenal digesta. However, digestion of these carbohydrates was unaffected by the RDP and NSC level of the rations.

Replacement of extracted sunflower meal by corn gluten meal led to increases in total N (Table 4). Feed N (nonmicrobial N) flow increased from 2.7 g/day (Diet 1) to 14 g/day (Diet 3). Both apparent and true degradation of N significantly increased in sheep fed Diet 1. Microbial efficiency was similar across treatments.

Duodenal flow of total, essential and nonessential AA increased as the level of RDP decreased (Table 5). Significant increases were observed in outflows of isoleucine, leucine, valine, alanine, glutamine, proline and serine in sheep fed Diet 3.

Dilution rate and rumen volume were not significantly different among treatments (Table 6). Rumen volume was the highest (9.06 l) for sheep receiving the high structural carbohydrate ration (Diet 3).

Table 2

Effect of diets on rumen pH, VFA, ammonia and urea before and 3 h after feeding

Item	Before feeding			3 h after feeding		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
pH	6.27 \pm 0.26	6.22 \pm 0.16	6.34 \pm 0.15	5.63 ^a \pm 0.18	5.73 ^b \pm 0.13	5.95 ^{ab} \pm 0.24
Total VFA, mmol/l	91.87 \pm 20.09	99.57 \pm 12.09	101.73 \pm 9.70	132.72 ^a \pm 15.47	135.28 ^b \pm 17.50	108.80 ^{ab} \pm 10.85
Acetate (A), %	59.05 ^{ab} \pm 2.49	64.05 ^{ac} \pm 2.30	70.24 ^{bc} \pm 1.14	60.71 ^{ab} \pm 2.09	62.44 ^{ac} \pm 1.46	68.44 ^{bc} \pm 1.80
Propionate (P), %	21.04 ^{ab} \pm 2.22	17.97 ^{ac} \pm 1.61	15.85 ^{bc} \pm 1.25	21.31 ^{ab} \pm 2.06	20.68 ^{ac} \pm 1.61	16.67 ^{bc} \pm 1.64
A:P	2.85 ^{ab} \pm 0.38	3.60 ^{ac} \pm 0.45	4.46 ^{bc} \pm 0.42	2.89 ^{ab} \pm 0.38	3.04 ^{ac} \pm 0.29	4.15 ^{bc} \pm 0.46
Isobutyrate, %	3.00 ^{ab} \pm 0.37	2.19 ^a \pm 0.49	2.09 ^b \pm 0.25	2.73 ^{ab} \pm 0.29	2.09 ^a \pm 0.43	1.96 ^b \pm 0.29
Butyrate, %	13.16 ^a \pm 1.77	12.95 ^b \pm 1.88	9.26 ^{ab} \pm 0.97	12.02 ^a \pm 1.24	12.16 ^b \pm 1.56	10.40 ^{ab} \pm 0.96
Isovalerate, %	2.53 ^{ab} \pm 0.46	1.86 ^a \pm 0.31	1.88 ^b \pm 0.52	1.78 ^{ab} \pm 0.54	1.30 \pm 0.23	1.40 ^b \pm 0.36
Valerate, %	1.22 ^{ab} \pm 0.17	0.98 ^{ac} \pm 0.22	0.67 ^{bc} \pm 0.17	1.45 ^a \pm 0.17	1.34 ^b \pm 0.20	0.97 ^{ab} \pm 0.24
Ammonia, mmol/l	17.48 ^{ab} \pm 4.33	13.85 ^{ac} \pm 2.27	10.97 ^{bc} \pm 1.95	25.04 ^{ab} \pm 4.14	19.14 ^{ac} \pm 4.34	11.06 ^{bc} \pm 1.79
Urea, mmol/l	7.76 ^{ab} \pm 2.42	5.99 ^{ac} \pm 1.55	4.71 ^{bc} \pm 0.90	11.06 ^{ab} \pm 2.02	9.05 ^{ac} \pm 2.68	4.76 ^{bc} \pm 1.08

^{a,b,c}Values within rows with a common letter in their superscripts differ significantly ($P < 0.05$)

Table 3

Intakes, flows and digestibilities of OM and fibre fractions in response to diet

	Diet 1	Diet 2	Diet 3
Organic matter (OM)			
Intake, g/day	1210.0 ± 117.4	1253.0 ± 60.7	1244.0 ± 98.0
Duodenal flow, g/day	449.8 ^{ab} ± 25.0	526.3 ^{ac} ± 26.3	585.8 ^{bc} ± 25.0
Ruminal digestion			
Apparent, %	62.7 ^{ab} ± 2.7	57.9 ^{ac} ± 2.0	52.9 ^{bc} ± 2.6
True, %	77.0 ^{ab} ± 2.1	70.4 ^a ± 3.4	65.8 ^b ± 2.5
NDF			
Intake, g/day	479.5 ± 46.5	581.8 ± 38.9	659.3 ± 49.3
Duodenal flow, g/day	175.9 ^{ab} ± 13.9	231.0 ^a ± 10.3	255.9 ^b ± 17.1
Ruminal digestion, %	62.9 ± 5.3	60.2 ± 2.9	61.4 ± 4.7
ADF			
Intake, g/day	242.4 ± 23.5	296.3 ± 21.9	356.1 ± 26.1
Duodenal flow, g/day	105.5 ^{ab} ± 9.1	135.4 ^a ± 5.6	155.5 ^b ± 10.1
Ruminal digestion, %	56.1 ± 5.5	54.1 ± 4.3	56.4 ± 4.4
Cellulose			
Intake, g/day	198.4 ± 19.1	239.7 ± 18.3	290.3 ± 21.1
Duodenal flow, g/day	79.9 ^{ab} ± 7.2	101.3 ^a ± 7.4	112.5 ^b ± 9.1
Ruminal digestion, %	59.3 ± 5.9	57.5 ± 5.1	61.2 ± 1.7
Hemicellulose			
Intake, g/day	237.1 ± 23.0	285.5 ± 17.1	303.1 ± 23.1
Duodenal flow, g/day	70.4 ^{ab} ± 7.4	95.6 ^a ± 6.4	100.4 ^b ± 11.1
Ruminal digestion, %	69.9 ± 5.7	66.5 ± 2.1	66.7 ± 2.6

^{a,b,c}Values within rows with a common letter in their superscripts differ significantly ($P < 0.05$)**Table 4**

Nitrogen intake and partitioning as affected by diets

N	Diet 1	Diet 2	Diet 3
Intake, g/day	38.7 ± 3.8	38.3 ± 3.8	37.5 ± 2.9
Flow to the duodenum, g/day			
Total N	22.2 ^{ab} ± 1.9	26.6 ^{ac} ± 1.8	31.8 ^{bc} ± 0.9
Non-ammonia N	20.7 ^{ab} ± 1.7	25.2 ^{ac} ± 1.3	30.6 ^{bc} ± 0.9
Microbial N	17.9 ± 1.4	16.3 ± 1.4	16.6 ± 0.9
Dietary N	2.7 ^{ab} ± 0.6	9.0 ^{ac} ± 1.2	14.0 ^{bc} ± 0.8
Apparent digestion, % of intake	42.7 ^{ab} ± 4.5	30.6 ^{ac} ± 3.5	15.3 ^{bc} ± 1.3
True digestion, % of intake	92.7 ^{ab} ± 1.2	76.5 ^{ac} ± 2.1	62.7 ^{bc} ± 2.3
Microbial N g/kg OM truly digested	19.6 ± 1.5	18.2 ± 0.9	20.2 ± 1.4

^{a,b,c}Values within rows with a common letter in their superscripts differ significantly ($P < 0.05$)

Table 5
Duodenal flow of amino acids (g/day)

Amino acids	Diet 1	Diet 2	Diet 3
Total	114.7 ^{ab} ± 9.4	136.7 ^{ac} ± 9.9	162.9 ^{bc} ± 8.9
Essential	51.4 ^{ab} ± 3.9	57.6 ^{ac} ± 3.2	69.2 ^{bc} ± 5.2
Nonessential	63.2 ^{ab} ± 5.8	79.1 ^{ac} ± 6.9	93.7 ^{bc} ± 4.2
Isoleucine ¹	5.1 ^a ± 0.5	5.5 ^b ± 0.8	6.9 ^{ab} ± 0.3
Leucine ¹	10.6 ^{ab} ± 1.7	16.3 ^{ac} ± 2.3	20.4 ^{bc} ± 2.1
Valine ¹	6.1 ^a ± 0.8	7.1 ^b ± 0.8	8.6 ^{ab} ± 0.7
Alanine ²	8.3 ^{ab} ± 1.2	12.6 ^{ac} ± 2.2	16.2 ^{bc} ± 1.3
Glutamic acid ²	20.9 ^{ab} ± 3.0	29.4 ^{ac} ± 3.3	34.8 ^{bc} ± 3.6
Proline ²	5.0 ^a ± 0.8	6.3 ± 1.7	7.5 ^a ± 1.2
Serine ²	5.5 ^a ± 0.7	6.8 ± 1.0	7.6 ^a ± 0.6

^{a,b,c}Values within rows with a common letter in their superscripts differ significantly ($P < 0.05$)

¹Essential; ²Nonessential

Table 6
Dilution rate and rumen volume in response to diets

	Dilution rate (%/h)	Rumen volume (l)
Diet 1	7.56 ± 1.58	7.34 ± 1.60
Diet 2	9.95 ± 1.08	7.46 ± 0.90
Diet 3	8.22 ± 1.91	9.06 ± 0.85

Discussion

Stokes et al. (1991b) reported that ruminal digestion of OM increased when the NSC level was higher than 24% and ruminally degradable protein higher than 9% of DM. The percentage of OM truly digested in the rumen was also increased as corn-based concentrate was increased from 20 to 76% of the diet (Rode et al., 1985). In contrast to these studies, Cameron et al. (1991) found no change in the ruminal digestibility of OM when the starch content of the diet was increased from 34 to 40%.

The more extensive OM degradation (Diet 1) resulted in a higher concentration of VFA. This result agrees with the data of Stokes et al. (1991b). The trend for change in the molar proportions of acetate and propionate reflects the carbohydrate composition of the diets (Stokes et al., 1991b). Nocek and Polan (1984) observed the highest acetate and the lowest propionate in calves fed a ground hay ration.

Ruminal pH below 6 may have a detrimental effect on microbial growth and cellulolytic activity (Feng et al., 1993). Although the low pH observed with Diet 1 appeared biologically significant, fibre digestion was not inhibited. In this study,

the ruminal digestibilities of NDF, ADF, cellulose and hemicellulose were similar for all diets. These results agree with the data of Stokes et al. (1991b), who also found no changes in ruminal degradation of NDF and ADF when NSC and RDP levels of the ration were decreased.

In agreement with the data of Stokes et al. (1991b), the greatest feed N flow to the duodenum occurred with Diet 3, which included a protein source more resistant to ruminal degradation. Titgemeyer et al. (1989) reported that soybean meal (extensive ruminal degradation of its AA) addition caused the greatest increase in ruminal ammonia concentration, while blood meal (large amount of ruminally nondegraded N) resulted in a nonsignificant increase.

Since N and DM intakes of the animals were similar, therefore the larger quantities of total N, non-ammonia N and dietary N that passed to the duodenum when Diet 3 was fed could be attributed to the less extensive degradation of protein in corn gluten meal as compared to extracted sunflower meal. Similar DM and OM intakes of sheep were also indicated by the rumen turnover. Different carbohydrate and protein sources did not have an effect on ruminal dilution rate. This result agrees with the data of Cameron et al. (1991), who reported that ruminal dilution rate was not altered when starch was added to the diet. In contrast, Feng et al. (1993) indicated that liquid passage rate was decreased by high NSC.

In the experiment of Titgemeyer et al. (1989) the addition of corn gluten meal supplied a greater amount of total AA to the duodenum than did soybean meal. Ruminal escape values for individual AAs showed that corn gluten meal addition resulted in more methionine, isoleucine, leucine and tyrosine. In case of isoleucine and leucine a similar trend was observed in our study but other branched-chain AAs, valine and proline were also less degraded. Earlier studies revealed the biochemical connection between AA degradation and concentration of the minor VFA. Isobutyrate and isovalerate are produced in the rumen by oxidative deamination and decarboxylation of the amino acids valine, leucine and isoleucine (Allison and Bryant, 1963). Valerate is produced from amino acids such as proline (Dehority et al., 1958). Smaller degradation of leucine, isoleucine, valine and proline was reflected by individual VFA analysis. Molar proportions of isobutyrate, isovalerate and valerate were lower when sheep were fed Diet 3.

In our experiment, duodenal flow of microbial N was equal in the treatments. This result is in conflict with the data of others (McCarthy et al., 1989; Stokes et al., 1991b), which indicated that the flow of microbial N to the duodenum was increased when the diet contained more fermentable starch or NSC. However, earlier studies indicated that the rising forage content of the ration resulted in a higher microbial yield. In sheep, the maximum bacterial yield was measured if the forage intake constituted 70% of the ration. Forage intakes below, but especially those above that level were found to result in a lower yield (Chamberlain and Thomas, 1979; Mathers and Miller, 1981). After increasing the forage level of the ration from 0% to 25% and from 10% to 40% in steers and dairy cows,

respectively, a higher microbial yield was measured (Cole et al., 1976). Microbial N flow to the duodenum was lower when the RDP level of the diet was decreased (Stokes et al., 1991a; Mansfield et al., 1994).

Feng et al. (1993) reported that microbial efficiency was decreased when cows were fed 39% NSC compared with 29% NSC diets. In an *in vitro* study Stokes et al. (1991a) observed increased microbial efficiency if the fibre level was decreased. In contrast, Chamberlain and Thomas (1979) and Mathers and Miller (1981) measured lower microbial efficiency when the concentrate level of the ration exceeded 30%. In these experiments RDP remained constant when the concentrate intake and thus the NSC content were increased, which meant a wider NSC:RDP ratio. If the ratio of protein and carbohydrate is inadequate, microbial growth will be limited due to energetic uncoupling. Microbial efficiency was decreased when corn starch was replaced by different protein sources (soybean meal, blood meal, fish meal, corn gluten). True microbial efficiency was found to decline 0.037 ± 0.014 g N/kg OM fermented for each gram of supplemental N added to the diet at the expense of corn starch (Titgemeyer et al., 1989). This decrease was explained by the decreased ATP yield from the degradation of protein compared to carbohydrate in corn starch (Demeyer and Van Nevel, 1986).

Our results indicate that, despite the low NSC and RDP content of the ration, the efficiency of microbial protein synthesis was not impaired if NSC and RDP were decreased proportionately. The feeding schedule in the current trial meant that Diet 1, in spite of the higher ruminal OM and N degradation, did not support a greater microbial efficiency. For an efficient synthesis of microbial biomass it is desirable that both energy and the N source become available at a fairly steady rate. With increasing protein degradability (Diet 1) the concentration of ammonia in the rumen and its diurnal variation increased. The rate of ammonia release exceeded the rate of incorporation into the microbes. Although with the more easily degradable protein (Diet 1) the major part of the N entering the small intestine was of microbial origin, the efficiency of microbial protein production did not increase, and its value was similar across treatments. This can be explained by the fact that Diet 3 with higher structural carbohydrate content was more resistant to degradation in the rumen resulting in a more steady rate of fermentation. Although the ration of the lowest NSC and RDP level contained a lower amount of readily available substrates, through a more stabilised fermentation Diet 3 caused N and energy to be transformed into microbial protein quite efficiently.

Acknowledgement

This work was supported by a grant (OTKA T 019204) from the Hungarian Scientific Research Fund.

References

- Aguilar, A. A. and Depeters, E. J. (1988): Modification for cannulation of duodenum with flexible, T-shaped cannula in goats. *Small Ruminant Res.* **1**, 73–79.
- Allison, M. J. and Bryant, M. P. (1963): Biosynthesis of branched chain fatty acids by rumen bacteria. *Arch. Biochem. Biophys.* **101**, 269–277.
- Cameron, T. H., Klusmeyer, T. H., Lynch, G. L., Clark, J. H. and Nelson, D. R. (1991): Effects of supplemental starch and urea on rumen fermentation and nutrient flow to the duodenum in lactating Holstein cows fed diets containing fish meal. *J. Dairy Sci.* **74**, 1321–1336.
- Chamberlain, D. G. and Thomas, P. C. (1979): Ruminal nitrogen metabolism and the passage of amino acids to the duodenum in sheep receiving diets containing hay and concentrates in various proportions. *J. Sci. Food Agric.* **30**, 677–686.
- Chaney, A. L. and Marbach, E. P. (1962): Modified reagents for determination of urea and ammonia. *Clin. Chem.* **8**, 130–132.
- Christian, R. R. and Coup, M. R. (1954): Measurement of feed intake by grazing cattle and sheep. VI. The determination of chromic oxide in faeces. *New Zealand J. Sci. and Tech. Sec. A.* **36**, 328–330.
- Cole, N. A., Johnson, R. R., Owens, F. N. and Males, J. R. (1976): Influence of roughage level and corn processing method on microbial protein synthesis by beef steers. *J. Anim. Sci.* **43**, 497–503.
- Dehareng, D. and Godeau, J. M. (1989): Polyethylene glycol dilution curves: a comparison of apparent ruminal liquid volumes and dilution rates estimated from either ruminal or duodenal fluid samples of Friesian cows. *J. Anim. Physiol. a. Anim. Nutr.* **62**, 268–276.
- Dehority, B. A., Johnson, R. R., Bentley, O. G. and Moxon, A. L. (1958): Studies on the metabolism of valine, proline, leucine and isoleucine by rumen microorganisms *in vitro*. *Arch. Biochem. Biophys.* **78**, 15–27.
- Demeyer, D. and Van Nevel, C. (1986): Influence of substrate and microbial interaction on efficiency of rumen microbial growth. *Reprod. Nutr. Dev.* **26**, 161–180.
- Feng, P., Hoover, W. H., Miller, T. K. and Blauwiekel, R. (1993): Interactions of fiber and non-structural carbohydrates on lactation and ruminal function. *J. Dairy Sci.* **76**, 1324–1333.
- Fébel, H., Romváry, A., Zsolnai Harezi, I. and Huszár, Sz. (1995): Effect of avoparcin on rumen fermentation and duodenal nutrient flow in sheep. *Acta Vet. Hung.* **43**, 229–246.
- Hoover, W. H. (1987): Potential for managing rumen fermentation. *Cornell Nutr. Conf.*, Ithaca, NY.
- Hungarian Standard MSz 6830 (1986): Determination of the nutritive value of feeds. Chemical tests and calculations (in Hungarian).
- Hyden, S. (1956): Turbidimetric method for the determination of higher polyethylene glycols in biological materials. *Kungl. Lantbrukshögskolans Annaler* **22**, 139–145.
- Mansfield, H. R., Endres, M. I. and Stern, M. D. (1994): Influence of non-fibrous carbohydrate and degradable intake protein on fermentation by ruminal microorganisms in continuous culture. *J. Anim. Sci.* **72**, 2464–2474.
- Mathers, J. C. and Miller, E. L. (1981): Quantitative studies of food protein degradation and the energetic efficiency of microbial protein synthesis in the rumen of sheep given chopped lucerne and rolled barley. *Br. J. Nutr.* **45**, 587–604.
- McCarthy, R. D., Jr., Klusmeyer, T. H., Vicini, J. L., Clark, J. H. and Nelson, D. R. (1989): Effects of source of protein and carbohydrate on ruminal fermentation and passage of nutrients to the small intestine of lactating cows. *J. Dairy Sci.* **72**, 2002–2016.
- Nocek, J. E. and Polan, C. E. (1984): Influence of ration form and nitrogen availability on ruminal fermentation patterns and plasma of growing bull calves. *J. Dairy Sci.* **67**, 1038–1042.
- Ørskov, E. R. and McDonald, I. (1979): The estimate of protein degradability in the rumen from incubation measurements weighed according to the rate of passage. *J. Agric. Sci.* **92**, 499–503.

- Rode, L. M., Weakley, D. C. and Satter, L. D. (1985): Effect of forage amount and particle size in diets of lactating dairy cows on site of digestion and microbial protein synthesis. *Can. J. Anim. Sci.* **65**, 101–111.
- Stokes, S. R., Hoover, W. H., Miller, T. K. and Mansky, R. P. (1991a): Impact of carbohydrate and protein level on bacterial metabolism in continuous culture. *J. Dairy Sci.* **74**, 860–870.
- Stokes, S. R., Hoover, W. H., Miller, T. K. and Blauweikel, R. (1991b): Ruminant digestion and microbial utilization of diets varying in type of carbohydrate and protein. *J. Dairy Sci.* **74**, 871–881.
- Supelco Inc. (1975): G. C. Separation of VFA C2-C5. Bull. 749 Supelco Inc., Bellefonte, PA.
- Sváb, J. (1981): Biometric Methods in Agricultural Research (in Hungarian). Mezőgazdasági Kiadó, Budapest.
- Titgemeyer, E. C., Merchen, N. R. and Berger, L. L. (1989): Evaluation of soybean meal, corn gluten meal, blood meal and fish meal as sources of nitrogen and amino acids disappearing from the small intestine of steers. *J. Anim. Sci.* **67**, 262–275.
- Van Es, A. J. H. and Van der Meer, J. M. (1980): Methods of analysis for predicting the energy and protein value of feeds for farm animals. Workshop on methodology of analysis of feed-stuffs for ruminants, Lelystad, May 27–29.
- Van Soest, P. J. and Robertson, J. B. (1985): Analysis of forages and fibrous foods. AS 613 Manual, Dep. Anim. Sci., Cornell Univ., Ithaca, NY.
- Zinn, R. A. and Owens, F. N. (1986): A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. *Can. J. Anim. Sci.* **66**, 157–166.

linked immunosorbent assay (ELISA) in the groups of PM+ and apparently non-infected lambs, respectively.

Key words: Diagnosis, ELISA, sheep, *Taenia hydatigena*, 68 KDa protein

In India there are 45.71 million sheep and 110.21 million goats, which contribute 4.95 million and 0.53 million of rupees, respectively, to the national economy through their meat and wool (Sastri et al., 1995). Due to inadequate pasture and crowded grazing habits that huge population of small ruminants comes across many diseases that affect the meat industries. Infection with larval stages of taenid worms (nematodes) is of paramount significance and responsible for huge economic losses to livestock owners. To minimise losses due to *cysticercus tenuicollis* infection an accurate and reliable serologic assay is needed. However, efforts to develop immunoassays to identify metacystode infections are greatly hindered by the unavailability of antigenic materials and the cross-reactivity inherent in heterogeneous antigen materials. Attempts have been made for the diagnosis of *Taenia hydatigena* cysticercosis in sheep and goats by different serological tests (Varma et al., 1973, 1974, 1975; Dacud and Herbert, 1982; Pathak et al., 1983; Deka and Gaur, 1990; Boga et al., 1995) with variable results. This is because animals with natural cestode infections usually have low cyst burdens and consequently low antigenic response to crude material.

POTENTIAL DIAGNOSTIC TEST FOR EXPERIMENTAL AND NATURAL OVINE *TAENIA HYDATIGENA* CYSTICERCOSIS

M. R. PANDA, S. GHOSH* and T. K. VARMA

Division of Parasitology, Indian Veterinary Research Institute, Izatnagar 243 122,
India

(Received November 1, 1999; accepted February 1, 2000)

An ion-exchange chromatographic fraction of *Taenia hydatigena* metacystode was evaluated for use in the immunodiagnosis of ovine cysticercosis. Analysis of the fraction by sodium dodecyl sulphate - polyacrylamide gel electrophoresis revealed the presence of a 68 KDa protein. Antibodies against the isolated protein were detected in 7 out of 10 experimentally infected lambs. The diagnostic potential of the 68 KDa protein was further confirmed by testing sera from naturally infected post-mortem positive (PM+) and from apparently healthy groups of animals. Eighty % and 8% of animals were found positive by enzyme-linked immunosorbent assay (ELISA) in the groups of PM+ and apparently non-infected lambs, respectively.

Key words: Diagnosis, ELISA, sheep, *Taenia hydatigena*, 68 KDa protein

In India there are 45.71 million sheep and 110.21 million goats, which contribute 4.95 million and 0.53 million of rupees, respectively, to the national economy through their meat and wool (Sastry et al., 1995). Due to inadequate pasture and crowded grazing habits that huge population of small ruminants comes across many diseases that affect the meat industries. Infection with larval stages of taeniid worms (metacestodes) is of paramount significance and responsible for huge economic losses to livestock owners. To minimise losses due to cysticercus tenuicollis infection an accurate and reliable serologic assay is needed. However, efforts to develop immunoassays to identify metacestode infections are greatly hindered by the unavailability of antigenic materials and the cross-reactivity inherent in heterogeneous antigen materials. Attempts have been made for the diagnosis of *Taenia hydatigena* cysticercosis in sheep and goats by different serological tests (Varma et al., 1973, 1974, 1975; Daoud and Herbert, 1982; Pathak et al., 1983; Deka and Gaur, 1990; Bogh et al., 1995) with variable results. This is because animals with natural cestode infections usually have low cyst burdens and consequently low antigenic response to crude material.

*Corresponding author: E-mail: sghosh@ivri.up.nic.in; Fax: 0091-581-50284

The diagnostic potential of homologous whole cyst antigen and its different fractions has been established by the use of different serological tests (Daoud and Herbert, 1982; Varma et al., 1986; Deka and Gaur, 1991; Georgieva, 1991). However, very limited attempts have been made to purify the whole cyst antigen for the diagnosis of ovine cysticercosis. Therefore, the present work was undertaken to evaluate the antigen obtained from *T. hydatigena* cysticerci of caprine origin in the immunodiagnosis of ovine cysticercosis.

Materials and methods

Sheep sera

Positive and negative reference sera. The serum of a sheep heavily infected with cysticerci of *T. hydatigena* was chosen as positive reference serum. Negative sera were collected from uninfected animals maintained in the experimental shed of the Division of Parasitology and their negativity was confirmed by post-mortem examination.

Experimental sera. Gravid segments of *T. hydatigena* adult worm of ovine isolate were collected from the faeces of donor pups. These were cleaned with several changes in deionised water and were processed to obtain mature eggs. They were stored at 4 °C in normal salt saline (NSS). Ten 6-month-old lambs were orally administered 3000 eggs and were stall-fed. Blood samples were collected from the experimentally infected lambs on days 0, 7, 10, 15, 20, 30 and 40 postinfection. The sera were separated and stored at -20 °C until use.

Field sera

Post-mortem positive group. Ten lamb sera were collected separately from the local abattoir. These animals were found infected with 3–8 cysticerci (3.5–5.5 × 3.7–6.8 cm in diameter) attached to the liver and omental fat as determined by post-mortem examination.

Noninfected group. Twenty-five serum samples were also collected from apparently noninfected, healthy animals.

Hyperimmune sera

Hyperimmune sera were raised in New Zealand White rabbits following the method of Deka and Gaur (1990) with some modifications. In brief, antigen (3 mg) was emulsified in equal volumes of Freund's complete adjuvant (FCA) or incomplete Freund's adjuvant (IFA) and inoculated subcutaneously on day 0 with FCA and intramuscularly on days 7 and 14 with IFA. Rabbits were bled intracardially on day 7 after the last inoculation. The antibody titre of the sera was measured by ELISA.

Antigen

T. hydatigena cysticerci were collected in NSS from naturally infected goats, and were thoroughly cleaned from host tissue covering. Ten ml of cooled 0.5 M NaCl (pH 7.0) was added to each 50 cysticerci that were disrupted in an ice-bath using tissue homogenizer. The homogenate was centrifuged at 3500 g for 10 min at 4 °C and the supernatant was again centrifuged until two distinct layers were seen. The upper layer was carefully removed and designated as whole cyst antigen (WCGAg) and was stored at -20 °C after adding 1 mM/L phenylmethane sulphonyl fluoride and 0.02% merthiolate. Protein content was determined by the method of Lowry et al. (1951).

Chromatography

For gel filtration chromatography, WCGAg was equilibrated with 0.01 M phosphate buffered saline (PBS), pH 7.3 and was concentrated by polyethylene glycol 20000. Thirty mg WCGAg in 3 ml was applied to a 100 × 2.5 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Sweden) at room temperature and fractions were collected at the flow rate of 18 ml/h of 0.01 M PBS containing 0.02% merthiolate. One hundred twenty-five fractions in 3-ml volume were collected. Individual fractions were tested by Ouchterlony's immunodiffusion method (Ouchterlony, 1958) using hyperimmune sera. Positive fractions were individually separated by 12% polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Antigenic fractions F₅ to F₁₁ and F₁₂ to F₂₃ were pooled separately and designated as pool A and pool B, respectively.

For further purification of pool A antigens, 4 mg sample in 4 ml of 0.01 M PBS, pH 7.3 was equilibrated with 20 mM Tris-HCl, pH 7.4. The sample was then applied to a 30 × 1.2 cm column of DEAE-Sepharose (Pharmacia Fine Chemicals, Sweden) previously equilibrated with 20 mM Tris-HCl, pH 7.4, at room temperature. Fractions were collected at a flow rate of 30 ml/h with continuous gradient of 20 mM Tris-HCl containing 0.1 to 0.6 M NaCl. Fractions from each of the peaks were pooled, concentrated, the antigenic fractions were identified by immunoprecipitation and were designated as A₁, A₂ and A₃. Similarly, a 4-mg sample of pool B was fractionated with 20 mM Tris-HCl containing 0.1 to 0.5 M NaCl and the antigenic fractions were identified as B₁, B₂ and B₃. All the fractions were stored at -20 °C until use.

Enzyme-linked immunosorbent assay (ELISA)

Microtitration plates (Nunc) were coated with A₁, A₂, A₃, B₁, B₂ and B₃ antigens diluted (10 µg/ml⁻¹) in carbonate buffer, pH 9.6, and incubated overnight at 4 °C (Harlow and Lane, 1988). Plates were washed 4 times with PBS plus Tween-20 (PBST), pH 7.2, and were blocked with bovine serum albumin in 1 × PBS. After washing the plates, 100 µl of test sera was diluted (1:50) with 1 ×

PBS, added to each well and incubated at room temperature for 2 h. The plates were again washed, 100 μ l of anti-sheep IgG (1:1500 dilution, Sigma Chemical Company, USA) peroxidase conjugate was added and the plates were kept at room temperature for 2 h. After washing, 100 μ l of O-phenylenediamine (OPD) (Sigma Chemical Company) (40 mg OPD in 100 ml of phosphate citrate buffer, pH 5.0 and 40 μ l of H₂O₂) was added and the plates were kept in darkness for 30 min. Optical density was measured at 492 nm using a Titertek Multiscan plate reader (Flow Labs, UK). The data were expressed as S/P values, as follows:

$$S/P = \mu S - \mu N / \mu P - \mu N,$$

where:

μS = mean absorbance of the duplicate of the sample;

μN = mean absorbance of the duplicate of the negative reference sera;

μP = mean absorbance of the duplicate of the positive reference sera.

Cut off S/P values were calculated as mean S/P values of the negative sera + 2 standard deviations (2 cut off).

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

The diagnostic antigen was resolved by SDS-PAGE on a 1.5-mm thick gel using a discontinuous system (Laemmli, 1970). The stacking gel was 3% acrylamide in 0.5 M Tris, pH 6.8 with 0.25% SDS and the resolving gel was 12% acrylamide in 1.5 M Tris, pH 8.8 with 0.25% SDS. Gels were stained with Coomassie Brilliant Blue R-250 for identification of marker proteins, which ranged from 29 to 205 KDa (Bangalore Genei Ltd., India).

Results

Gel filtration and ion-exchange chromatography

WCGAg proteins eluted from the G-100 Sephadex column yielded two peaks with 78% recovery of the applied proteins. The immunoprecipitation test showed a strong precipitation reaction with fractions number 5 to 23. After SDS-PAGE separation it was revealed that the ascending fractions (5 to 11) were composed of eight polypeptides of 29, 37.2, 43, 56.2, 72.2, 75, 105 and 187 Kda molecular weights. Six polypeptides of 29, 37.2, 43, 56.2, 68 and 72.2 KDa molecular weights were resolved in the descending antigenic fractions (12–23).

Pool A sample (fractions 5 to 11) eluted from DEAE-Sepharose by 0.1 to 0.6 M NaCl gradient yielded 65% recovery divided among six fractions as shown in Fig. 1. Precipitation reaction using 12 μ l of recovered proteins each separately showed precipitin reaction in the fractions collected by 0.2, 0.3 and

0.4 M NaCl and designated as A₁, A₂ and A₃, respectively. Similarly, about 80% recovery was obtained from pool B sample divided in seven fractions as shown in Fig. 2. The precipitin line was detected in the fractions collected by 0.2, 0.3 and 0.4 M NaCl, which were labelled as B₁, B₂ and B₃, respectively.

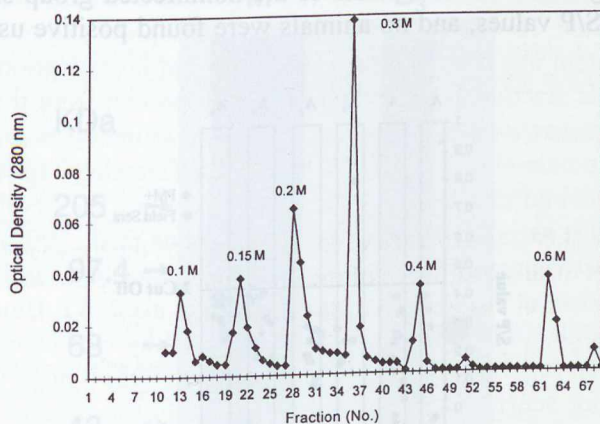


Fig. 1. Elution profile of pool A proteins from ion-exchange column

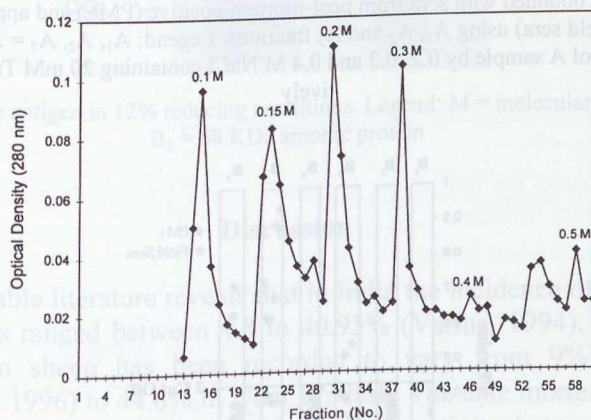


Fig. 2. Elution profile of pool B proteins

ELISA

Out of 10 experimentally infected lambs, 7 (70%) gave significantly elevated S/P values using B₃. However, 0, 1, 0, 1 and 4 animals only showed significant reactions using A₁, A₂, A₃, B₁ and B₂, respectively.

Figures 3 and 4 show the efficiency of different antigenic fractions in the diagnosis of infection using 2 cut off value. The sensitivity of the test using B₃

was 80% in the PM+ group, while employing other antigens it was 0–20% only. In the other group of 25 animals no visible cysticerci were located on the visceral organs. However, in this apparently noninfected group of animals the possibility of a recent infection with other taeniid eggs cannot be ruled out completely. Employing B₃ antigen 8% of the animals of the noninfected group showed significantly elevated S/P values, and no animals were found positive using other anti-

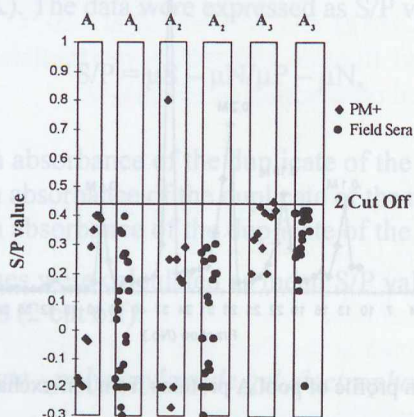


Fig. 3. S/P values obtained with sera from post-mortem positive (PM+) and apparently healthy groups of sheep (field sera) using A₁, A₂ and A₃ fractions. Legend: A₁, A₂, A₃ = Anionic fractions separated from pool A sample by 0.2, 0.3 and 0.4 M NaCl containing 20 mM Tris-HCl, respectively

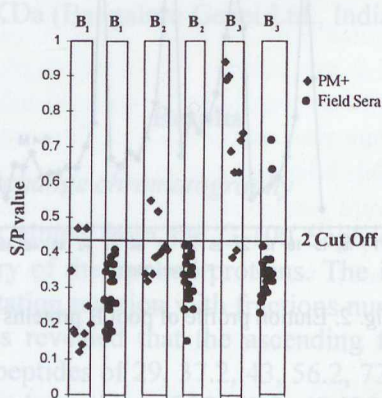


Fig. 4. S/P values obtained with sera from post-mortem positive (PM+) and apparently healthy groups of sheep (field sera) using B₁, B₂ and B₃ fractions. Legend: B₁, B₂, B₃ = Anionic fractions separated from pool A sample by 0.2, 0.3 and 0.4 M NaCl containing 20 mM Tris-HCl, respectively

SDS-PAGE separation of antigen

Electrophoretic separation of B₃ showed the presence of a single protein band of 68 KDa (Fig. 5).

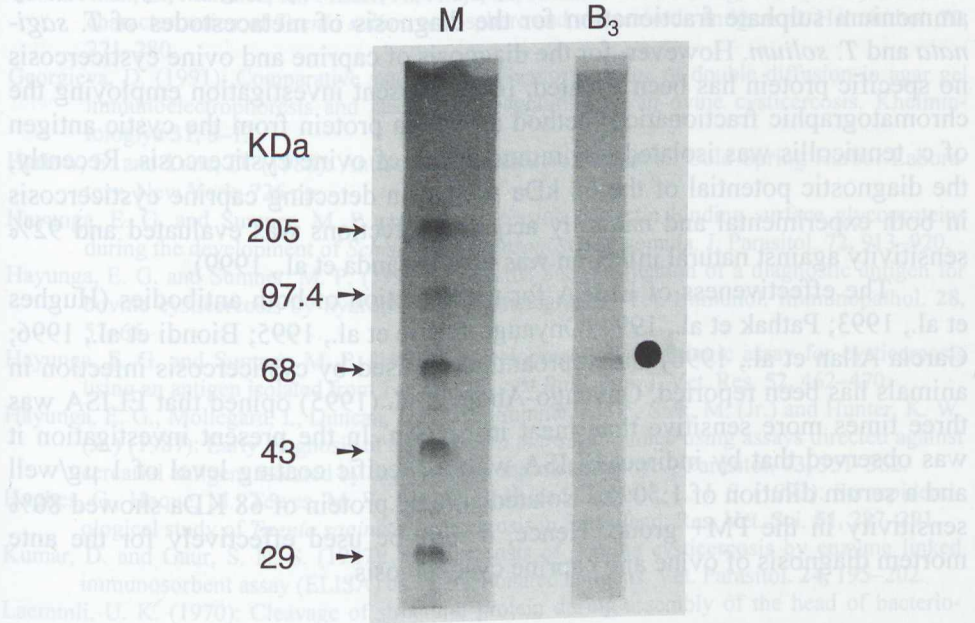


Fig. 5. Diagnostic antigen in 12% reducing conditions. Legend: M = molecular weight marker; B₃ = 68 KDa anionic protein

Discussion

The available literature reveals that in India the incidence of *T. hydatigena* infection in dogs ranged between 4.8 to 40.95% (Varma, 1994). Consequently, the incidence in sheep has been recorded to vary from 9% in Rajasthan (Swarnkar et al., 1996) to 44.6% in Uttar Pradesh. The ante mortem diagnosis of cestodosis has usually been hindered by cross-reactivity between antigens of even distantly related helminths (Craig and Rickard, 1980; Aronstein et al., 1986; Hayunga et al., 1987) and by the heterogeneity of even well-defined antigenic preparations (Hayunga and Sumner, 1986). Efforts to reduce cross-reactivity have focussed on purification of different antigens by fractionation and identification of candidate species-specific diagnostic molecules. Amongst the antigens tested, cystic antigen has been found promising (Kumar and Gaur, 1987). Previously, cystic antigens have been purified by different physicochemical procedures. For example, Rhoads et al. (1985) isolated high molecular weight (65 to 77 KDa) and low molecular weight (9.5 to 16 KDa) protein fractions for the di-

agnosis of bovine cysticercosis. By fast protein liquid chromatography Deka and Gaur (1990) purified cyst fluid, scolex and membrane antigens of *T. hydatigena* metacestodes and found cyst fluid antigen to be the most reactive. Hayunga and Sumner (1991a, b) isolated a 12 kDa protein from *T. hydatigena* cyst fluid by ammonium sulphate fractionation for the diagnosis of metacestodes of *T. saginata* and *T. solium*. However, for the diagnosis of caprine and ovine cysticercosis no specific protein has been isolated. In the present investigation employing the chromatographic fractionation method a 68 kDa protein from the cystic antigen of *c. tenuicollis* was isolated for immunoassay of ovine cysticercosis. Recently, the diagnostic potential of the 68 kDa protein in detecting caprine cysticercosis in both experimental and naturally acquired infections was evaluated and 92% sensitivity against natural infection was noted (Panda et al., 1999).

The effectiveness of ELISA for the detection of both antibodies (Hughes et al., 1993; Pathak et al., 1994; Onyango-Abuje et al., 1995; Biondi et al., 1996; Garcia Allan et al., 1996) and coproantigens raised by cysticercosis infection in animals has been reported. Onyango-Abuje et al. (1995) opined that ELISA was three times more sensitive than meat inspection. In the present investigation it was observed that by indirect ELISA with a specific coating level of 1 µg/well and a serum dilution of 1:50 the isolated anionic protein of 68 KDa showed 80% sensitivity in the PM+ group. Hence, it may be used effectively for the ante mortem diagnosis of ovine and caprine cysticercosis.

Acknowledgement

Sincere thanks are due to the Director, Indian Veterinary Research Institute for providing laboratory facilities.

References

- Aronstein, W. S., Lewis, S. A., Norden, A. P., Dalton, J. P. and Strand, M. (1986): Molecular identity of a major antigen of *Schistosoma mansoni* which cross-reacts with *Trichinella spiralis* and *Fasciola hepatica*. *Parasitology* **92**, 133–151.
- Biondi, G. F., Mucciolo, R. G., Nunes, C. M. and Richtzenhain, L. J. (1996): Immunodiagnosis of swine cysticercosis by indirect ELISA employing a heterologous antigen from *Taenia crassiceps* metacestode. *Vet. Parasitol.* **64**, 261–266.
- Bogh, H. O., Lind, P., Sonderby, B. V., Kyvsgaard, N. C., Maeda, G. E., Henriksen, S. A. and Nansen, P. (1995): Immunodiagnosis of *Taenia saginata* in cattle using hydrophobic antigens from *Taenia hydatigena* metacestode cyst fluid. *Appl. Parasitol.* **36**, 226–238.
- Craig, P. S. and Rickard, M. D. (1980): Evaluation of crude antigen prepared from *Taenia saginata* for the serological diagnosis of *T. saginata* cysticercosis in cattle using the enzyme-linked immunosorbent assay (ELISA). *Z. Parasitenkunde* **61**, 287–297.
- Daoud, I. S. and Herbert, I. V. (1982): Isolation of two lipoprotein antigens from the metacestodes of *Taenia hydatigena* (Pallas, 1966) and *Taenia multiceps* (Leske, 1780) and their evaluation in sero-diagnosis. *Vet. Parasitol.* **11**, 155–164.

- Deka, D. K. and Gaur, S. N. S. (1990): Counter current immunoelectrophoresis in the diagnosis of *Taenia hydatigena* cysticercosis in goats. *Vet. Parasitol.* **37**, 223–228.
- Deka, D. K. and Gaur, S. N. S. (1991): Diagnosis of *Taenia hydatigena* cysticercosis in goats by immunoelectrophoresis. *J. Vet. Parasitol.* **5**, 35–38.
- Garcia Allan, C., Martinez, N., Flisser, A., Aluja, A., Allan, J. C. and Craig, P. S. (1996): Immunocharacterization of *Taenia solium* oncosphere and metacestode antigens. *J. Helminthol.* **70**, 271–280.
- Georgieva, D. (1991): Comparative study of the diagnostic value of double diffusion in agar gel immunoelectrophoresis and passive haemagglutination in ovine cysticercosis. *Khelmin-tologiya* **31**, 8–13.
- Harlow, E. and Lane, D. (1988): *Antibodies. A Laboratory Manual.* Cold Spring Harbor Laboratory, New York. 726 pp.
- Hayunga, E. G. and Sumner, M. P. (1986): Expression of lectin binding surface glycoproteins during the development of *Schistosoma mansoni* schistosomula. *J. Parasitol.* **72**, 913–920.
- Hayunga, E. G. and Sumner, M. P. (1991a): Isolation and purification of a diagnostic antigen for bovine cysticercosis by hydrophobic chromatography. *Vet. Immunol. Immunopathol.* **28**, 57–65.
- Hayunga, E. G. and Sumner, M. P. (1991b): Development of a serologic assay for cysticercosis using an antigen isolated from *Taenia* spp. cyst fluid. *Am. J. Vet. Res.* **52**, 462–470.
- Hayunga, E. G., Mollegard, I., Duncan, J. F. (Jr.), Sumner, M. P., Stek, M. (Jr.) and Hunter, K. W. (Jr.) (1987): Early diagnosis of *Schistosoma mansoni* in mice using assays directed against cercarial antigens isolated by hydrophobic chromatography. *J. Parasitol.* **73**, 351–362.
- Hughes, G., Hoque, M., Tewes, M. S., Wright, S. H. and Harrison, L. J. S. (1993): Seroepidemiological study of *Taenia saginata* cysticercosis in Swaziland. *Res. Vet. Sci.* **51**, 287–291.
- Kumar, D. and Gaur, S. N. S. (1987): Serodiagnosis of porcine cysticercosis by enzyme linked immunosorbent assay (ELISA) using fractionated antigens. *Vet. Parasitol.* **24**, 195–202.
- Laemmli, U. K. (1970): Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature* **227**, 630–668.
- Lowry, D. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- Onyango-Abuje, J. A., Hughes, G., Opicha, M., Nginyi, J. M., Rugutt, M. K., Wright, S. H. and Harrison, L. J. S. (1995): Diagnosis of *Taenia saginata* cysticercosis in Kenyan cattle by antibody and antigen ELISA. *Vet. Parasitol.* **61**, 221–230.
- Ouchterlony, O. (1958): Diffusion in gel methods for immunological analysis. In: Kallos, P. (ed.) *Progress in Allergy*, Vol. 5, Karger, Basel. pp. 1–78.
- Panda, M. R., Ghosh, S. and Varma, T. K. (1999): Enzyme-linked immunosorbent assay using anionic protein for the diagnosis of *Cysticercus tenuicollis* infection in goats. *Riv. iber. Parasitol.* (in press).
- Pathak, K. M. L., Gaur, S. N. S. and Gupta, R. S. (1983): Antigenic component of *Taenia hydatigena* cyst fluid and their relevance to diagnosis of ovine cysticercosis by immunoelectrophoresis. *Trop. Vet. Anim. Sci. Res.* **1**, 247–248.
- Pathak, K. M. L., Allan, J. C., Ersfeld, K. and Craig, P. S. (1994): A western blot and ELISA assay for the diagnosis of *Taenia solium* infection in pigs. *Vet. Parasitol.* **53**, 209–217.
- Rhoads, M. L., Murrell, K. D., Dilling, G. W., Wong, M. M. and Baker, N. F. (1985): A potential diagnostic reagent for bovine cysticercosis. *J. Parasitol.* **7**, 779–787.
- Sastry, N. S. R. (1995): *Livestock sector of India.* National Book Distribution Company, Lucknow, India. 222 pp.
- Swarnkar, C. P., Singh, D., Srivastava, C. P., Bhagwan, P. S. K. and Dimri, U. (1996): A retrospective study on ovine gastrointestinal helminthoses under semi-arid conditions. *J. Vet. Parasitol.* **10**, 15–21.
- Varma, T. K. (1994): Helminth parasites in stray dogs in district Gurgaon (Haryana). *Indian J. Parasitol.* **18**, 65–68.

- Varma, T. K., Kulshrestha, S. B., Rao, B. V. and Kumar, S. (1973): Conglutinin complement absorption test for the serodiagnosis of *Cysticercus tenuicollis* infection in sheep and goats. J. Helminthol. **47**, 191–197.
- Varma, T. K., Kulshrestha, S. B. and Rao, B. V. (1974): Indirect haemagglutination test in the diagnosis of cysticercosis caused by *Cysticercus tenuicollis* infection in sheep and goats. Riv. iber. Parasitol. **35**, 103–111.
- Varma, T. K., Kulshrestha, S. B. and Rao, B. V. (1975): Sero-diagnostic studies on larval and strobilar phase of *Taenia hydatigena* with *in vitro* test. Riv. iber. Parasitol. **36**, 287–294.
- Varma, T. K., Malviya, H. C. and Ahluwalia, S. S. (1986): Serodiagnosis of infection with metacystodes of *Taenia solium* in pigs and taeniasis in man and dogs by indirect haemagglutination assay. Indian J. Anim. Sci. **56**, 621–627.
- Hayanga, E. G. and Sumner, M. P. (1986): Expression of latent binding surface glycoprotein during the development of *Leishmania schisomus*. J. Parasitol. **72**, 913–920.
- Hayanga, E. G. and Sumner, M. P. (1987): Isolation and purification of a diagnostic antigen for *Leishmania schisomus* by immunoelectrophoresis. J. Immunol. **138**, 1000–1005.
- Hayanga, E. G. and Sumner, M. P. (1988): Development of a serologic assay for *Leishmania schisomus* using an antigen isolated from *Leishmania* sp. J. Parasitol. **62**, 462–470.
- Hayanga, E. G., Mollgaard, L., Duncan, J. T. O., Sumner, M. P. and Hunter, K. W. (1989): Early diagnosis of *Leishmania* infection in mice using highly purified antigen. J. Parasitol. **69**, 551–557.
- Hayanga, E. G., Hunter, K. W., Sumner, M. P. and Hunter, K. W. (1990): Serological study of *Leishmania schisomus* in Swaziland. Res. Vet. Sci. **61**, 387–391.
- Kumar, D. and Gaur, S. N. (1987): Serodiagnosis of porcine cysticercosis by enzyme linked immunosorbent assay (ELISA) using fractionated antigen. Vet. Parasitol. **24**, 153–162.
- Laemmli, U. K. (1970): Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature **227**, 680–688.
- Lowy, D. H., Rosebrough, N. J., Farr, A. J. and Bradford, R. J. (1951): Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193**, 265–275.
- Onyiah-Apudji, J. A., Hughes, G., Quinlan, M., Nwanyi, I. M., Rugani, M. K., Wright, S. H. and Thornton, J. T. (1997): Diagnosis of *Leishmania schisomus* in Kenyan cattle by indirect immunofluorescence assay. J. Parasitol. **67**, 231–236.
- Ouchterlony, O. (1958): Diffusion in gel methods for immunological analysis. In: Kallio, P. (ed). Progress in Allergy. Vol. 3. Karger, Basel, pp. 1–78.
- Panda, M. R., Ghosh, S. and Varma, T. K. (1999): Enzyme-linked immunosorbent assay using anti-porcine protein for the diagnosis of *Cysticercus tenuicollis* infection in goats. Riv. iber. Parasitol. **40**, 100–105.
- Patankar, K. M. L., Gaur, S. N. and Gupta, R. S. (1987): Antigenic component of *Leishmania schisomus* and their relevance to diagnosis of ovine cysticercosis by immunoelectrophoresis. J. Parasitol. **67**, 247–250.
- Patankar, K. M. L., Allan, J. C., Fritsch, K. and Gaur, S. N. (1984): A western blot and ELISA assay for the diagnosis of *Leishmania schisomus* infection in pigs. Vet. Parasitol. **13**, 309–315.
- Rogstad, M. L., Blum, K. D., Blum, G. W., Wane, M. M. and Baker, J. E. (1987): A serological diagnostic reagent for porcine cysticercosis. J. Parasitol. **77**, 779–787.
- Saxena, N. S. R. (1993): A pocket sector of India. National Book Distribution Company, Lucknow.
- Swaminath, C. P., Singh, D., Saxena, C. P., Singh, R. S. and Dhillon, J. (1986): A sero-diagnostic study on ovine cysticercosis by immunoelectrophoresis and ELISA. J. Parasitol. **66**, 21–25.
- Varma, T. K. (1994): Helminth parasites in stray dogs in district Garo (Haryana). J. Parasitol. **18**, 65–68.

A SURVEY OF CHICKENS FOR VIABLE TOXOPLASMS IN CROATIA

Viktorija KUTIČIĆ¹* and TH. WIKERHAUSER²

¹Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine,
University of Zagreb, P.O. Box 190, 10000 Zagreb, Croatia; ²Croatian Academy
of Sciences and Arts, Zrinski trg 11, 10000 Zagreb, Croatia

(Received September 15, 1999; accepted February 1, 2000)

Brain tissues of 716 slaughtered domestic chickens (524 broilers and 192 hens) were bioassayed for viable toxoplasms. Each tissue was homogenized and subcutaneously injected into 4 SPF mice. Six weeks later the mice were euthanized and their brains microscopically examined for *Toxoplasma gondii* tissue cysts. Three (0.4%) out of a total of 716 birds were positive. All positive cases were hens. This is the first isolation of *T. gondii* from chickens in Croatia.

Key words: Chicken, brain, *Toxoplasma*, bioassay, Croatia

Viable toxoplasms in edible tissues of food animals represent a health hazard to human consumers and, if ingested by cats, they may cause contamination of the environment with *T. gondii* oocysts. Toxoplasms were isolated from naturally infected chickens in various countries such as Germany (Boch, 1980), Iran (Ghorbani et al., 1990), Brazil (Peixoto and Lopes, 1990) and the Czech Republic (Literak and Hejliček, 1993; Hejliček and Literak, 1994). In the former Yugoslavia they were isolated in Serbia (Simitch et al., 1961) and apparently demonstrated in stained smears from muscular tissues of hens in Bosnia (Živković and Arežina, 1991). The aim of the present study was to assess the prevalence of viable toxoplasms in slaughtered commercial chickens in Croatia.

Materials and methods

The mouse inoculation test originally described by Dubey et al. (1984) was applied for the isolation of toxoplasms. Bioassays were performed with brain tissues which had been found by several authors (Boch, 1980; Biancifiori et al., 1986; Dubey et al., 1993; Kaneto et al., 1997) to be a common site of *T. gondii* in chickens. The brains were collected from slaughtered chickens that had

*E-mail: kuticic@vef.hr; Fax: +385 1/24 41 390

been raised on small private farms and fed commercial poultry feeds supplemented with maize.

The chickens were slaughtered at an industrial abattoir and their brains were tested within 3 days after slaughter. A total of 716 birds were examined, 524 of them broilers and 192 hens. Each brain sample was homogenized with sterile sand and saline and subcutaneously injected into four SPF mice, at a dose of 1 ml/mouse. Six weeks later the mice were euthanatized with chloroform and the fresh preparations of their brains were microscopically examined for *T. gondii* tissue cysts.

Results

By mouse inoculation test, performed with brain tissues of a total of 716 domestic chickens, viable toxoplasms were isolated from 3 birds (0.4%).

From the positive samples 3, 2 and 2 test mice, respectively, out of 4 inoculated, harboured *T. gondii* tissue cysts. The examined lot consisted of 524 broilers and 192 hens. All positive cases were hens.

Discussion

In the present study viable toxoplasms were isolated from chickens in Croatia for the first time. The infection rate was low, similar to that reported by some other European workers. So Simitch et al. (1961) reported an infection rate of 0.25% in Serbia (former Yugoslavia), Boch (1980) 0.3% in Germany, and Literak and Hejliček (1993) 0.35% in the Czech Republic. On the other hand, a high infection rate of 30% was observed in Brazil by Peixoto and Lopes (1990) and of 5.6% in Iran by Ghorbani et al. (1990). Also, a high infection rate of 10.1% was apparently observed in Bosnia by Živković and Arežina (1991), but their method of investigation did not include bioassays.

In this survey, positive cases were found among hens only. We presume that the longer life span of the hens, as compared to that of the broilers, gives the former more opportunity to get in contact with *T. gondii* oocysts.

Our results suggest that the low infection rate, coupled with the fact that chicken brains are not commonly eaten uncooked by humans, means that chicken brain tissue is not a serious threat to human health. Furthermore, cats do not typically catch chickens and eat chicken heads and are therefore not likely to get infected through chicken brains.

Acknowledgements

This study was supported in part by a grant from the Ministry of Sciences and Technology of the Republic of Croatia (053075).

References

- Biancifiori, F., Rondini, C., Grelloni, V. and Frescura, T. (1986): Avian toxoplasmosis: experimental infection of chicken and pigeon. *Comp. Immunol. Microbiol. Infect. Dis.* **9**, 337–346.
- Boch, J. (1980): Die Toxoplasmose des Haustiere: Vorkommen, Diagnose und Bedeutung. *Berl. Münch. Tierärztl. Wschr.* **93**, 385–391.
- Dubey, J. P., Murrell, K. D. and Fayer, R. (1984): Persistence of encysted *Toxoplasma gondii* in tissues of pigs fed oocysts. *Am. J. Vet. Res.* **45**, 1941–1943.
- Dubey, J. P., Ruff, M. D., Camargo, M. E., Shen, S. K., Wilkins, G. L., Kwok, O. C. and Thulliez, P. (1993): Serologic and parasitologic responses of domestic chickens after oral inoculation with *Toxoplasma gondii* oocysts. *Am. J. Vet. Res.* **54**, 1668–1672.
- Ghorbani, M., Gharavi, M. J. and Kahn moui, A. (1990): Serological and parasitological investigations on *Toxoplasma* infection in domestic fowls in Iran. *Iranian J. Public Health* **19**, 9–17.
- Hejliček, K. and Literak, I. (1994): Toxoplasmosis in slaughter poultry (domestic fowls) (in Czech). *Veterinarstvi* **44**, 64–65.
- Kaneto, C. N., Costa, A. J., Paulillo, A. C., Moraes, F. R., Murakami, T. O. and Meireles, M. V. (1997): Experimental toxoplasmosis in broiler chicks. *Vet. Parasitol.* **69**, 203–210.
- Literak, I. and Hejliček, K. (1993): Incidence of *Toxoplasma gondii* in populations of domestic birds in the Czech Republic. *Avian Pathology* **22**, 275–281.
- Peixoto, C. M. S. and Lopes, C. W. G. (1990): Isolation of *Toxoplasma gondii* Nicolle & Manceaux, 1909 (Apicomplexa: Toxoplasmatinae) in naturally infected chickens (in Portuguese). *Arquivos da Universidade Federal Rural do Rio de Janeiro* **13**, 105–111.
- Simitch, T., Bordjochki, A., Petrovitch, Z., Tomanovitch, B. and Savin, Ž. (1961): Toxoplasmosis of birds. I. Natural infection of domestic poultry with *Toxoplasma gondii* in Yugoslavia (in French). *Arch. Inst. Pasteur d'Algerie* **39**, 135–139.
- Živković, J. and Arežina, Lj. (1991): Evidence and hygienic significance of *Toxoplasma gondii* sporozoa in the meat of hens (in Croatian). *Vet. Stanica* **22**, 323–330.

PHARMACOKINETICS, URINARY EXCRETION AND DOSAGE REGIMEN OF DIMINAZENE IN CROSSBRED CALVES

Gurmeet KAUR, R. K. CHAUDHARY* and A. K. SRIVASTAVA

Department of Pharmacology and Toxicology, College of Veterinary Science,
Punjab Agricultural University, Ludhiana-141004, India

(Received April 12, 1999; accepted October 20, 1999)

The pharmacokinetics, urinary excretion and dosage regimen of diminazene were investigated in crossbred male calves following a single intramuscular dose ($3.5 \text{ mg} \times \text{kg}^{-1}$). Following intramuscular administration, the pharmacokinetics of diminazene was described with a one-compartment open model. The absorption rate constant and absorption half-life were $9.86 \pm 3.06 \text{ h}^{-1}$ and $0.121 \pm 0.40 \text{ h}$, respectively. The value of elimination half-life was $107.5 \pm 8.50 \text{ h}$. The apparent volume of distribution was $0.74 \pm 0.07 \text{ L} \times \text{kg}^{-1}$. Systemic availability following intramuscular administration was 91.7%. Approximately 65% of the administered dose of diminazene was eliminated in the urine within 24 h of its intramuscular administration. Diminazene was bound to plasma proteins to the extent of approximately 32%. The satisfactory intramuscular dosage regimen of diminazene for calves would be $2.24 \text{ mg} \times \text{kg}^{-1}$ followed by $1.5 \text{ mg} \times \text{kg}^{-1}$ at 7 days.

Key words: Pharmacokinetics, diminazene, urinary excretion, intramuscular, dosage

Diminazene is regarded as an ideal drug for the control of trypanosomosis in livestock (Gilbert and Newton, 1982; Madachi et al., 1995). For determining the proper dosage regimen of a drug, pharmacokinetic studies are essential and such studies are relevant in the animal species and environment in which the drug is to be employed clinically. Accordingly, the pharmacokinetics of diminazene has been studied in rabbits (Gilbert and Newton, 1982), sheep (Aliu and Odegaard, 1985), goats (Mamman and Peregrine, 1994), cattle (Klatt and Hajdu, 1976; Aliu et al., 1993; Mamman et al., 1993) and buffaloes (Singh, 1997). Although diminazene has been widely applied in crossbred bovine calves for many years, knowledge of its detailed pharmacokinetics in this species is lacking. The present investigation was, therefore, undertaken to determine the pharmacokinetics, urinary excretion and optimal dosage regimen of diminazene in crossbred calves. In addition, the *in vitro* plasma protein binding of diminazene was also determined.

*Corresponding author; E-mail: ivispau@satyam.net.in; Fax: 91-161-400945

Material and methods

Six healthy crossbred male calves (75 to 105 kg) were used. The animals were kept under uniform conditions and were fed available green fodder. Wheat straw and water were provided *ad libitum*. The animals were kept in metabolic stalls of standard size. A freshly prepared solution (16%) of diminazene diacetate (Berenil, Hoechst India Ltd.) was administered intramuscularly at a dose of $3.5 \text{ mg} \times \text{kg}^{-1}$ body weight. Blood samples were collected into heparinized tubes from the jugular vein of each animal at 1, 2.5, 5, 7.5, 10, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 30, 48, 72, 96, 120, 144 and 168 h after administration of diminazene. To calculate the systemic availability of diminazene, one month after the intramuscular study the drug was injected into the same animals by intravenous route at the same dose rate and blood samples were collected at the same time intervals. Plasma was separated after centrifugation at 3000 rpm for 15 min at room temperature. Urine samples were collected at 2, 4, 6, 8, 10, 12, 18 and 24 h after administration of drug. Diminazene content of plasma and urine was estimated by a spectrophotometric method (Klatt and Hajdu, 1976). The assay could detect $0.76 \mu\text{g} \times \text{ml}^{-1}$ of diminazene in plasma and urine. The standard curve of diminazene is linear from 1.0 to $25 \mu\text{g} \times \text{ml}^{-1}$. The method reproduces the results within an error of < 4 per cent. The plasma concentration-time profile of diminazene for each animal was used to determine pharmacokinetics. Various pharmacokinetic parameters were calculated by method of Baggot (1977) and Gibaldi and Perrier (1982). T/P ratio was calculated by the following equation:

$$\text{T/P} = \frac{1}{\text{fc}} - 1,$$

where fc is fraction of administered dose present in the central compartment and was calculated as

$$\text{fc} = \frac{\beta}{\text{Kel}},$$

where β is the overall elimination rate constant and Kel is elimination from the central compartment.

$$\text{Kel} = \frac{\text{Cpo}}{\text{AUC}_{0-\infty}}$$

Cpo = expected concentration of drug in plasma at zero time. $\text{AUC}_{0-\infty}$ = area under plasma concentration time curve. Plasma protein binding of diminazene was determined by equilibrium dialysis technique (Kunin et al., 1959).

Results and discussion

The semilogarithmic plot of plasma concentration of diminazene in crossbred male calves as a function of time after intramuscular administration is shown in Fig. 1. Following administration there was an appreciable concentration of drug ($2.64 \pm 0.19 \mu\text{g} \times \text{ml}^{-1}$) at 1 min. Such a high concentration may be due to the use of more concentrated solution, i.e. 16 per cent. The peak plasma level of diminazene ($7.42 \pm 0.11 \mu\text{g} \times \text{ml}^{-1}$) was detected at 45 min. Diminazene could be detected in plasma ($0.96 \pm 0.10 \mu\text{g} \times \text{ml}^{-1}$) up to 7 days. The evolution of plasma levels revealed that diminazene followed the one-compartment open model:

$$C_p = B e^{-\beta t} - A e^{-K_a t},$$

where C_p is diminazene concentration at time t , A and B are zero-time intercepts of initial and terminal phases of plasma concentration–time curve, K_a and β are the absorption and elimination rate constants, respectively, and e represents the base of natural logarithm. To describe pharmacokinetics, one-compartment open model has also been used for diminazene in buffaloes (Singh, 1997). However, Aliu et al. (1993) found that in heifers the three-compartment model was the most suitable.

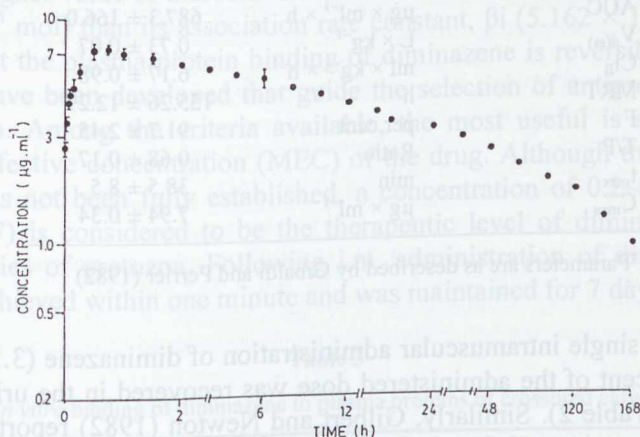


Fig. 1. Semilogarithmic plot of plasma levels of diminazene after a single intramuscular dose of $3.5 \text{ mg} \times \text{kg}^{-1}$ body weight in crossbred calves. The values given are the mean \pm SE of 6 animals.

Data are analysed according to the one-compartment open model

The pharmacokinetic parameters of diminazene in crossbred calves following intramuscular administration are presented in Table 1. The high value of K_a ($9.86 \pm 3.06 \text{ h}^{-1}$) and the low value of $t_{1/2K_a}$ ($0.121 \pm 0.40 \text{ h}$) suggest that diminazene promptly enters the systemic circulation from extravascular injection. The elimination half-life of diminazene in crossbred calves was $107.5 \pm 8.5 \text{ h}$.

Lower values of $t_{1/2\beta}$ have been reported in adult cattle and buffalo. The value of $t_{1/2\beta}$ was 63 h in adult cattle (Klatt and Hajdu, 1976) and 67.9 h in buffalo (Singh, 1997). However, a higher value of elimination half-life (145.48 h) has been reported in heifers (Aliu et al., 1993) following the i.m. administration of diminazene. The systemic availability after intramuscular administration was high (91.7%). Systemic availability was calculated on the basis of the area under the plasma concentration–time curve of the drug after i.m. and i.v. administration. The calculated apparent volume of distribution ($0.74 \pm 0.07 \text{ L} \times \text{kg}^{-1}$) indicates that there is excellent penetration of diminazene through biological membranes. The value of AUC and the T/P ratio were $687.3 \pm 166.0 \mu\text{g} \times \text{ml}^{-1} \times \text{h}$ and 0.68 ± 0.17 , respectively.

Table 1

Pharmacokinetic parameters of diminazene in crossbred calves following intramuscular administration in a single dose of $3.5 \text{ mg} \times \text{kg}^{-1}$ body weight

Parameter ^a	Units	Mean \pm SE
Ka	h^{-1}	9.86 ± 3.06
$t_{1/2\text{Ka}}$	h	0.121 ± 0.40
β	h^{-1}	0.0066 ± 0.0005
$t_{1/2\beta}$	h	107.5 ± 8.50
AUC	$\mu\text{g} \times \text{ml}^{-1} \times \text{h}$	687.3 ± 166.0
$V_{d(ss)}$	$\text{L} \times \text{kg}^{-1}$	0.73 ± 0.07
Cl_B	$\text{ml} \times \text{kg}^{-1} \times \text{h}^{-1}$	6.17 ± 0.98
MRT	h	155.26 ± 12.29
F	per cent	91.7 ± 2.45
T/P	Ratio	0.68 ± 0.17
t_{max}	min	38.5 ± 8.5
C_{max}	$\mu\text{g} \times \text{ml}^{-1}$	7.94 ± 0.34

^aParameters are as described by Gibaldi and Perrier (1982)

After a single intramuscular administration of diminazene ($3.5 \text{ mg} \times \text{kg}^{-1}$) about 65 per cent of the administered dose was recovered in the urine of calves within 24 h (Table 2). Similarly, Gilbert and Newton (1982) reported excretion of about 65 per cent of the total administered dose of diminazene in rabbits within 24 h of its administration. Contrary to this, low values such as 8.26 and 26.8 per cent of renal excretion for diminazene have been reported in heifers (Aliu et al., 1993) and dogs (Onyeyili and Anika, 1989), respectively. This difference may be due to the fact that the diazotization method detects the intact molecule as well as the metabolite. However, Aliu et al. (1993) and Onyeyili and Anika (1989) used HPLC methods that detected the intact molecule and its metabolite separately.

Table 2

Urinary excretion of diminazene in crossbred calves following a single intramuscular administration ($3.5 \text{ mg} \times \text{kg}^{-1}$)

Time interval (h)	Cumulative per cent of drug excreted
0-2	13.0 ± 5.23
0-4	13.6 ± 5.11
0-6	23.8 ± 2.85
0-8	31.9 ± 7.47
0-10	34.8 ± 6.32
0-12	40.6 ± 8.07
0-18	49.1 ± 10.7
0-24	65.3 ± 11.0

Values given are the mean \pm SE of results obtained from six animals

The results obtained on the plasma protein binding of diminazene are shown in Table 3. The drug was bound to plasma proteins to the extent of 32.3 ± 4.67 per cent. The plasma protein binding of diminazene in heifers has been reported to be in the range 38 to 91 per cent (Aliu et al., 1993). The comparatively higher value of dissociation rate constant of protein-drug complex, K_{β} ($3.364 \times 10^{-4} \text{ mol}$) than its association rate constant, βi ($5.162 \times 10^{-9} \text{ mol} \times \text{g}^{-1}$) indicates that the plasma protein binding of diminazene is reversible. A number of criteria have been developed that guide the selection of antiprotozoal agents by clinicians. Among the criteria available, the most useful is said to be the minimum effective concentration (MEC) of the drug. Although the MEC of diminazene has not been fully established, a concentration of $0.2\text{--}0.5 \mu\text{g} \times \text{ml}^{-1}$ (Singh, 1997) is considered to be the therapeutic level of diminazene against various species of protozoa. Following i.m. administration of diminazene, the MEC was achieved within one minute and was maintained for 7 days.

Table 3

In vitro binding of diminazene to plasma proteins of crossbred calves

Experiment no.	Per cent of drug bound to plasma protein	βi ($\text{mol} \times \text{g}^{-1}$)	K_{β} (mol)
1	36.8 ± 5.23	7.303×10^{-9}	8.917×10^{-4}
2	35.5 ± 10.1	7.421×10^{-10}	9.895×10^{-5}
3	23.2 ± 7.61	7.435×10^{-9}	1.87×10^{-5}
Overall mean	32.3	5.162×10^{-9}	3.364×10^{-4}
\pm SE	4.67	2.210×10^{-9}	2.786×10^{-4}

βi and K_{β} are calculated as described by Pilloud (1973)

The ultimate objective of the pharmacokinetic study of a chemotherapeutic agent is to determine a satisfactory dosage regimen. Therefore, based on the pharmacokinetic data, the suitable maintenance (D') dose is calculated by the formula:

$$D' = \text{MEC} \cdot V_d (e^{\beta\tau} - 1)$$

The priming dose (D) is calculated by omitting -1 from the above equation. In this calculation the MEC of diminazene was taken as $1 \mu\text{g} \times \text{ml}^{-1}$, keeping in view the safety factor. By taking $V_{d(\text{area})}$ and β values from Table 1 and the dosage interval (τ) as 7 days, the priming and maintenance doses were calculated to be 2.24 and $1.5 \text{ mg} \times \text{kg}^{-1}$, respectively. However, the dosage can be adjusted depending upon the nature of disease and the therapeutic objectives.

References

- Aliu, Y. O. and Odegaard, S. (1985): Pharmacokinetics of diminazene in sheep. *J. Pharmacol. Biopharma.* **13**, 173–184.
- Aliu, Y. O., Mamman, M. and Peregrine, A. S. (1993): Comparative pharmacokinetics of diminazene in female Boran (*Bos indicus*) cattle. *J. Vet. Pharmacol. Therap.* **16**, 291–300.
- Baggot, J. D. (1977): Principles of drug disposition in domestic animals. In: *The Basis of Veterinary Clinical Pharmacology*. 1st edition. W. B. Saunders Publishing Co., Philadelphia. pp. 144–189.
- Gibaldi, M. and Perrier, D. (1982): Appendix C: Method of Residuals. In: *Pharmacokinetics*. Marcel Dekker, New York. pp. 433–444.
- Gilbert, R. J. and Newton, B. A. (1982): Pharmacokinetics and efficacy of the trypanocide diminazene aceturate (Berenil) in rabbits. *Vet. Rec.* **111**, 397.
- Klatt, P. and Hajdu, P. (1976): Pharmacokinetic investigations on diminazene and rolitetracycline in comparison to combination of both. *Vet. Rec.* **99**, 372–374.
- Kunin, C. M., Drombush, A. C. and Finland, M. (1959): Distribution and excretion of four tetracycline analogues in normal young man. *J. Clin. Invest.* **38**, 1950–1963.
- Madachi, R. E., Murilla, G. A., Omukubh, J. N. and Congolati, V. (1995): Disposition of diminazene aceturate (Berenil) in trypanosoma infected pregnant lactating cows. *Vet. Parasit.* **58**, 215–225.
- Mamman, M. and Peregrine, A. S. (1994): Pharmacokinetics of diminazene in plasma and cerebrospinal fluid of goats. *Res. Vet. Sci.* **57**, 253–255.
- Mamman, M., Aliu, Y. O. and Peregrine, A. S. (1993): Comparative pharmacokinetics of diminazene in non infected Boron (*Bos indicus*) cattle and Boron cattle infected with *T. congolense*. *Antimicrob. Agents Chemother.* **37**, 1050–1055.
- Onyeyili, P. A. and Anika, S. M. (1989): The influence of *T. congolense* infection on the disposition kinetics of diminazene aceturate in dogs. *Vet. Res. Commun.* **13**, 231–236.
- Pilloud, M. (1973): Pharmacokinetics, plasma protein binding and dosage regimen of oxytetracycline in cattle and horses. *Res. Vet. Sci.* **15**, 224–230.
- Singh, P. (1997): Therapeutic efficacy and alteration in disposition kinetics of diminazene in experimental trypanosomiasis in buffalo calves. M.V.Sc. Thesis submitted to Punjab Agric. Univ., Ludhiana.

FORMATION OF A SECONDARY CORPUS LUTEUM AFTER ULTRASOUND-GUIDED FOLLICULAR ASPIRATION IN COWS

G. S. AMIRIDIS^{1*}, Lindsay ROBERTSON², I. A. JEFFCOATE², Sophia BELIBASAKI³,
J. S. BOYD² and P. J. O'SHAUGHNESSY²

¹Department of Reproduction and Obstetrics, Faculty of Veterinary Medicine, University of Thessaly, Thessaloniki, 43100 Karditsa, Greece; ²Department of Preclinical Studies, Glasgow University Veterinary School, Glasgow, Scotland, UK; ³Veterinary Research Institute, Thessaloniki, Greece

(Received December 14, 1999; accepted February 1, 2000)

This paper reports the observed formation of a secondary corpus luteum (CL) in the presence of the cyclic corpus luteum, on the ovaries of a cow after ultrasound-guided follicular aspiration for oocyte recovery. The secondary structure, although smaller and lighter (4.97g vs. 6.02g) than the natural one, had the typical macroscopic appearance of a corpus luteum. Histological examination of the structure using electron microscopy revealed typical structural features of a natural CL. Mean tissue progesterone concentration was significantly lower in the secondary CL (31.15 ± 3.11 compared with 58.29 ± 6.32 $\mu\text{g/g}$ tissue of the cyclic CL) and oestradiol-17 β significantly higher than in the natural CL (108 ± 11.6 compared with 74.2 ± 7.81 pg/g tissue). P450_{sec} and P450_{17 α} mRNA was detected in both structures while P450_{arom} and full-length mRNA FSH receptor were detected only in the secondary structure.

Key words: Corpus luteum, follicular aspiration, P450_{17 α} , P450_{arom}, cow

Current understanding of the process of luteinisation requires that the follicle is exposed to a pre-ovulatory LH surge (Niswender and Nett, 1994), which triggers structural and biochemical differentiation of the granulosa and theca cells and alters the steroidogenic activity of the follicle (Fortune and Hansel, 1985; Wiltbank, 1994). The decrease in oestradiol and androstenedione secretion which follows the LH surge occurs in concert with a decline in the activity of the enzymes P450_{17 α} and P450_{arom} (Vos and Fortune, 1993). Conversely, increased levels of mRNA for the enzymes P450_{sec} and 3 β hydroxysteroid dehydrogenase are detectable as progesterone production increases (Rodgers et al., 1987).

This study reports the formation of a secondary luteal structure in the presence of a natural cyclic corpus luteum (CL) on the ovary of a cow after puncture of all follicles ≥ 3 mm and aspiration of the follicular contents. A detailed comparison between the two luteal structures is described.

*E-mail: amiridis@spark.net.gr; Fax: +0441 70906

Materials and methods

The accessory structure was formed during the course of scheduled follicular aspiration for oocyte pick up (OPU). The OPU sessions were carried out three times during the early (days 3–4), mid (days 9–12) and late (days 15–17) stages of the ovarian cycle, in eight non-pregnant dry Holstein donor cows aged 4–8 years. The day of standing heat was defined as day 0.

Follicular aspiration

Ultrasound-guided follicular aspiration was carried out as described by Scott et al. (1994). The presence of the natural CL was noted and all follicles ≥ 3 mm were aspirated. The ovaries were re-examined 6–8 days later (the day before the next OPU session) using transrectal ultrasonography. After the identification of the secondary CL the cow was sacrificed, the ovaries were removed and the corpora lutea were studied using the following techniques.

Microscopy

Five small tissue samples from each CL were prepared for light microscopy by fixation in 10% neutral buffered formalin. Samples were then dehydrated through a graded series of alcohols and embedded in poly-wax. Thin sections of 3 μ m were cut and stained with haematoxylin and eosin after fixation in 10% formalin and dehydration with alcohols. The sections were examined under light microscope (Leitz Laborlux 11, Germany) and cells were counted per field with a cell counter.

For transmission electron microscopy tissue samples from the accessory and the natural CL were prepared by pre-fixation for 24 h in Karnowsky's fixative and post-fixation in 1% osmic acid. Tissues were then dehydrated and embedded in araldite. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined in a Jeol 100CX 11 transmission electron microscope.

Radioimmunoassays

Tissue progesterone and oestradiol concentrations were determined in 5 samples from each luteal structure by double antibody RIA (Jeffcoate, 1992). Tissue samples (500 mg from each CL) were homogenized with 2 ml of assay buffer (PBS) and the homogenate was extracted with absolute ethanol.

Reverse transcription-polymerase chain reaction (RT-PCR)

Messenger RNA was extracted from five tissue samples from each luteal structure, using RNazol and complementary DNA (cDNA) synthesised by reverse transcription as described previously (O'Shaughnessy and Murphy, 1993).

This cDNA was used as a template for PCR amplification. The sequence of the oligonucleotide primers was as follows:

P450_{sc} primer 1, 5'- GCC ATC TCG TAC AAG TGC CAT TGC -3'
 primer 2, 5'- GCC GTC TAC AAG ATG ATG TTC CAC AC3'

P450_{arom} primer 1, 5'- GAG CAT GTT AGA GGT GTC CAG CAT -3'
 primer 2, 5'- GGA TTG GAA GTG CCT GCA ACT ACT -3'

P450_{17 α} primer 1, 5'- CTT GTC GGA CCA AGG AAA AGG CGT -3'
 primer 2, 5'- CAA CCA CGG GAA TAT GTC CAC CAG 3'

FSH receptor primer 1, 5'- CCT TCT GCC AGG AGA GCA AGG TAA C-3'
 primer 2, 5'- TGC CTT AAA ATA GAT TTG TTG CA -3'

Southern blotting was performed for the identification of the FSH receptor as described previously (Rajapaksha et al., 1996).

Results

Seven out of eight cows underwent follicular aspiration during the early, mid and late stages of the ovarian cycle, whereas in one cow (the one that produced the accessory CL) OPU was carried out only during the early stage. In the course of these OPU sessions 42, 70 and 65 follicles were aspirated during the early, mid and late stages of the ovarian cycle, respectively.

The secondary structure had the typical macroscopic appearance of a CL, but it was of lower weight than the natural structure (4.97 g and 6.02 g, respectively), but the natural CL was denser than the secondary one (158 ± 23 vs. 121 ± 15 cells per field, respectively). Transmission electron microscopy revealed that the secondary structure had ultrastructural features typical of luteal tissue. Large and small luteal cells contained abundant smooth endoplasmic reticulum, polymorphic mitochondria, lipid droplets and dark granules.

Mean tissue progesterone concentration in the secondary CL was 31.15 ± 3.11 compared with 58.29 ± 6.32 $\mu\text{g/g}$ tissue for the natural one, while mean tissue oestradiol concentration was significantly higher in the secondary CL (108 ± 11.6 compared with 74.2 ± 7.81 pg/g tissue).

Complementary DNA corresponding to P450_{arom} mRNA was detected by PCR only in the accessory structure, while P450_{sc} and P450_{17 α} were found in both structures.

Southern blotting for FSH receptor cDNA revealed that this was expressed in full length in the accessory structure, but not in the natural CL (Fig. 1).

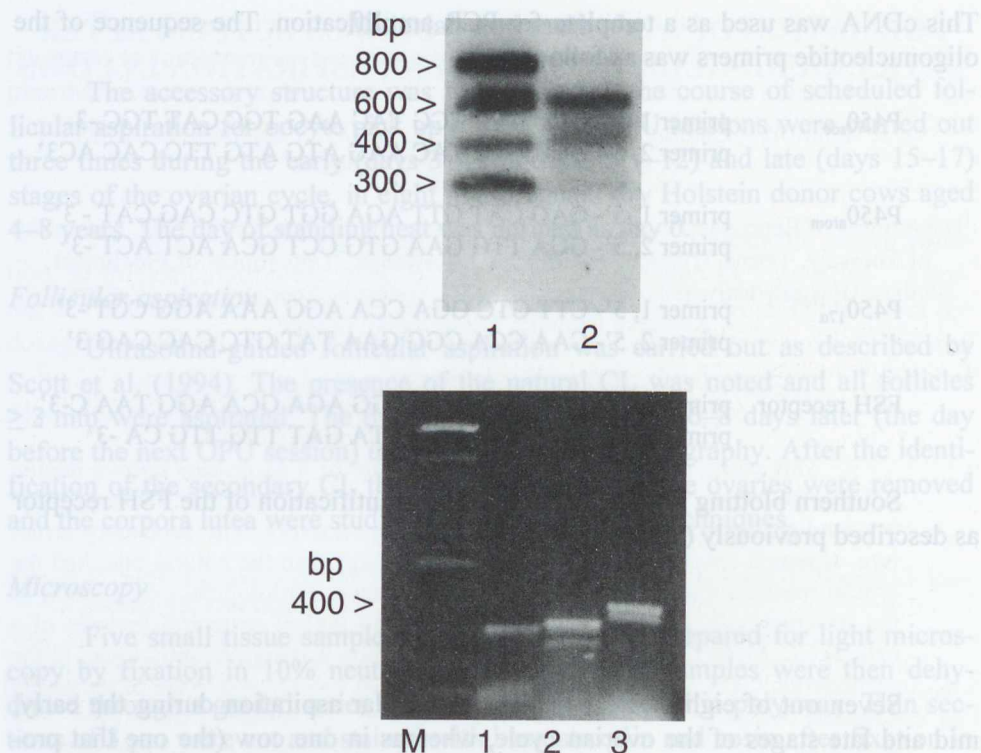


Fig. 1. Upper: Southern blot hybridisation of PCR products using FSH receptor primers. cDNA was prepared from the accessory (lane 1) and the natural (lane 2) CL. Lower: Amplification by RT-PCR, using primers for GAPDH (1), P450_{sc} (2) and P450_{arom} (3) of RNA isolated from the accessory structure. Lane M contained a 100-bp DNA ladder

Discussion

The current understanding of the mechanisms of follicular luteinisation is that corpus luteum formation is dependent on a surge of LH in a hormonal environment characterised by low plasma progesterone and high concentration of oestradiol. In the situation described here the secondary CL was produced on day 3 of the cycle, in the presence of a natural CL. One could assume that the accessory luteal structure was formed in a background of rising plasma progesterone, characterised by basal LH and oestradiol concentrations. Hormone profiles from a large group ($n = 12$) of cows treated in an identical manner indicated that the procedure of follicle ablation in the early, mid and late luteal phase did not provoke an LH surge, nor did it alter the typical profile of progesterone secretion (Amiridis, 1996).

While the role of the LH surge in ovulation and luteinisation is well documented, that of the contemporary FSH surge is not clear. It has been shown that FSH injections cause ovulation and luteinisation in hypophysectomised rats (Galway et al., 1990) and that intrafollicular administration of FSH induces ovulation in sheep (Murdoch et al., 1981). We have demonstrated that in the early, mid or late luteal phase, aspiration of the dominant follicle causes a dramatic drop in plasma oestradiol, a significant increase in plasma FSH, and only during the early luteal phase a tendency for increase in plasma LH concentration (Amiridis et al., 1999). It could be hypothesised that these changes in LH, FSH and oestradiol secretion caused by the ablation of the follicular contents, may trigger luteinisation of a punctured follicle.

Histological examination of the structure showed the normal morphological appearance of a CL. Tissue concentrations of progesterone and oestradiol were within the normal range for corpora lutea, though progesterone in the secondary structure was half that of the natural CL and oestradiol was 45% higher, perhaps indicating incomplete luteinisation.

The assumption of incomplete luteinization is supported by the results of the enzyme studies. Levels of mRNA encoding P450_{sec} and P450_{17 α} were detectable in both structures. Interestingly, P450_{arom} and FSH receptor were present only in the induced CL. These mRNA species are normally expressed only in granulosa cells and expression is down-regulated as the granulosa cells differentiate into luteal cells (Vos and Fortune, 1993). Furthermore, it has been shown that luteinised cells lose their full-length FSH receptor as early as day two after formation of the CL (Rajapaksha et al., 1996).

In conclusion, this observation suggests that the LH surge that occurs prior to ovulation is essential for rupture of the pre-ovulatory follicle and for down-regulation of some mRNA species expressed in the granulosa cells. It appears that the LH surge may not be the only factor required for differentiation of the CL. It is possible that, under certain circumstances as yet undetermined, the process of follicle rupture or the ablation of the follicular contents is sufficient to induce partial luteinisation of the granulosa cells and development of a morphologically normal CL.

References

- Amiridis, G. S. (1996): Effects of follicular aspiration on the bovine oestrus cycle. PhD Thesis, University of Glasgow.
- Amiridis, G. S., Robertson, L., Reid, S., Boyd, J. S., O'Shaughnessy, P. J. and Jeffcoate, I. A. (1999): Plasma estradiol, FSH and LH concentration after dominant follicle aspiration in the cow. *Theriogenology* **52**, 995–1003.
- Fortune, J. E. and Hansel, W. (1985): Concentration of steroids and gonadotrophins in follicular fluid from normal heifers and heifers primed for superovulation. *Biol. Reprod.* **32**, 1069–1079.

- Galway, A. B., La Polt, P. S., Tsafiri, A., Dargan, C. M., Boime, I., Hsue, A. J. W. (1990): Recombinant follicle stimulating hormone induces ovulation and tissue plasminogen activator expression in hypophysectomised rats. *Endocrinology* **127**, 3923–3031.
- Jeffcoate, I. A. (1992): Concentrations of luteinising hormone and oestradiol in plasma and response to injection of gonadotrophin-releasing hormone analogue at selected stages of anoestrous in domestic bitches. *J. Reprod. Fert.* **94**, 423–429.
- Murdoch, W. J., Dailey, R. A. and Inskeep, E. K. (1981): Preovulatory changes in prostaglandins E_2 and $F_{2\alpha}$ in ovine follicles. *J. Anim. Sci.* **53**, 192–201.
- Niswender, G. D. and Nett, T. M. (1994): The corpus luteum and its control. In: Knobil, E. and Neil, J. (eds) *The Physiology of Reproduction*. Raven Press, New York, pp. 489–525.
- O'Shaughnessy, P. J. and Murphy, L. (1993): Cytochrome P450 15- α -hydroxylase protein and mRNA in the testis of testicular feminised (tfm) mouse. *J. Mol. Endocr.* **11**, 77–82.
- Rajapaksha, W. R. A. K. J. S., Robertson, L. and O'Shaughnessy, P. J. (1996): Expression of follicle stimulating hormone receptor mRNA alternate transcripts in bovine granulosa cells during luteinisation *in vivo* and *in vitro*. *Mol. Cell. Endocr.* **120**, 25–30.
- Rodgers, R. J., Waterman, M. R. and Simpson, E. R. (1987): Levels of ribonucleic acid encoding cholesterol side chain cleavage cytochrome P450_{17 α} hydroxylase cytochrome P450 adrenodoxin and low density lipoprotein receptor in bovine follicles and corpora lutea throughout the ovarian cycle. *J. Mol. Endocr.* **3**, 274–279.
- Scott, C. A., Robertson, L., de Moura, R. T. D., Paterson, C. and Boyd, J. S. (1994): Technical aspects of transvaginal ultrasound guided follicular aspiration in the cow. *Vet. Rec.* **134**, 440–443.
- Vos, A. K. and Fortune, J. E. (1993): Levels of messenger ribonucleic acid for P450_{17 α} hydroxylase and P450 aromatase in preovulatory bovine follicles decrease after luteinising hormone surge. *Endocrinology* **132**, 2239–2245.
- Wiltbank, M. (1994): Cell types and hormonal mechanisms associated with mid cycle corpus luteum. *J. Anim. Sci.* **72**, 1873–1883.

EFFECT OF EXOGENOUS OVINE PLACENTAL LACTOGEN ON BASAL AND PROSTAGLANDIN-STIMULATED PROGESTERONE PRODUCTION BY PORCINE LUTEAL CELLS

Ewa L. GREGORASZCZUK^{1*}, A. GERTLER² and E. FUTOMA¹

¹Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Ingarden 6, 30-060 Cracow, Poland; ²Institute of Biochemistry, Food Science and Nutrition, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

(Received August 25, 1999; accepted February 1, 2000)

The ability of ovine placental lactogen (oPL) to stimulate progesterone secretion of porcine luteal cells isolated from ovaries in different stages of the oestrous cycle and to support the luteotropic action of PGE₂ or to protect the corpus luteum (CL) against the luteolytic action of PGF_{2α} was investigated. oPL in all doses used had no effect on progesterone production of cells isolated from early developing corpora lutea while in doses of 1 and 10 ng/ml it increased oestradiol secretion by this type of cells. In doses of 1, 10 and 100 ng/ml it also increased progesterone secretion of cells isolated from mature corpora lutea in a dose-dependent manner. No influence on progesterone production of cells isolated from regressing corpora lutea was observed. oPL added to the culture media had no effect on PGE₂-stimulated progesterone production by cells isolated from mature corpora lutea. However, it exerted a protective effect against the luteolytic action of PGF_{2α} observed in cultures treated with PGF_{2α} alone or in combination with PGE₂ in a ratio of 4:1. These studies provide evidence that oPL is luteotropic and supports progesterone production in swine. The fact that oPL acted directly on ovarian steroidogenesis suggests that it may also play some role under non-pregnant physiological conditions. Future studies of structural and functional proteins secreted by the porcine conceptus will help resolve this uncertainty.

Key words: Placental lactogen, porcine luteal cells, prostaglandins, steroid secretion

In the past decade, the high incidence of early embryonic losses observed in swine prompted numerous studies designed to elucidate mechanisms contributing to prenatal mortality. Embryo transfer experiments in pigs (Soares et al., 1998) showed that developmental stages of the uterus and the transferred blastocyst must be in synchrony for the successful implantation and survival of embryos. Hormonal events during the first 14 days of the oestrous cycle and pregnancy are essentially identical in swine. After that time a functional corpus luteum (CL) must be maintained for the duration of pregnancy. Loss of CL func-

*E-mail: greg@zuk.iz.uj.edu.pl; Fax: (4812) 6343716

tion at any stage of gestation leads to abortion within 24 to 36 h in the porcine species (Belt et al., 1971). In swine, 'maternal recognition of pregnancy' is mediated by production of oestrogen by the blastocyst, which rapidly stimulates the release of endometrial secretory components into the uterine lumen (Geisert et al., 1990; Soares et al., 1998).

During the early cross-species surveys, it became apparent that some mammals such as swine, horses, rabbits and dogs did not possess a classic placental lactogen (PL). Alternatively, non-classical members of the prolactin (Prl) family may be expressed in the uteroplacental tissue in these species. Sensitive and specific monoclonal antibody-based immunoassays have provided new insights into the secretion patterns of protein and glycoprotein hormones. Recent studies on human chorionic gonadotropin and its free α and β subunits indicate that they are also present in non-pregnant, healthy individuals (Madersbacher et al., 1992, 1993). Moreover, recent demonstration of the ectopic production of human placental lactogen in the ovary prompted the above authors (Madersbacher et al., 1998) to reassess its role under non-pregnant physiological conditions, suggesting auto/paracrine functions of this molecule. Placental lactogen is structurally related to growth hormone (GH) and Prl, and may act as a luteotropon in swine.

The present study was undertaken to investigate, first, whether oPL can stimulate progesterone secretion of porcine luteal cells and, second, whether oPL may exert luteoprotective effects against the luteolytic effects of prostaglandin $F_{2\alpha}$.

Materials and methods

Reagents and hormones

Ovine placental lactogen (oPL) was prepared as described previously (Sakal et al., 1997). Prostaglandins $PGF_{2\alpha}$ and PGE_2 were purchased from Sigma Chemical Co. (St. Louis, MO). Medium M199, penicillin, trypsin and calf serum were obtained from the Laboratory of Vaccines, Lublin, Poland.

Cell isolation

Ovaries of Large White sows were obtained from a local abattoir immediately after slaughter and transported to the laboratory in ice-cold PBS. The stage of the oestrous cycle was determined according to morphological criteria (Schilling, 1974; Gregoraszczuk, 1992). Ovarian tissues representing three different stages of the oestrous cycle were collected from (1) newly developed corpora lutea (CL1, 1–3 days after ovulation), (2) mature corpora lutea (CL2, 8–10 days after ovulation) and (3) regressing corpora lutea (CL3, 11–14 days after ovulation).

Luteal cells were obtained from pools of freshly excised corpora lutea of three animals in the oestrous cycle according to Gregoraszczyk (1983). Cells were suspended in medium M199 supplemented with 5% calf serum at a concentration of 3.5×10^5 cells/ml medium.

Cells were grown in multiwell plates (Nunc) at 37 °C in a humidified atmosphere with 5% CO₂ in the air for 48 h. Incubations were conducted in triplicate in 1.0 ml/well. After 48-h incubation, all cultures were terminated and the media were frozen until steroid analysis. Cell viability measured using the trypan blue exclusion test was 85%.

Experimental procedure

Experiment 1. Dispersed cells were incubated without (control) and with graded concentrations of oPL (1, 10, 50, 100, 200 and 500 ng/ml). After 48-h incubation, all cultures were terminated and the media were frozen until steroid analysis.

Experiment 2. In order to test whether the increased progesterone secretion of luteal cells was mediated by enhanced luteoprotective signals through prostaglandin E₂ (PGE₂) or by acting against the luteolytic effect of prostaglandin F_{2α} (PGF_{2α}), dispersed cells were incubated with oPL alone (100 ng/ml), or together with PGE₂ (100 ng/ml), or together with PGF_{2α} (100 ng/ml), or with PGE₂:PGF_{2α} added in a ratio of 4:1. After 48-h incubation with hormones the media were collected and frozen until steroid analysis.

Experiment 3. To test the influence of oPL on the activity of Δ^5 , 3 β -hydroxysteroid dehydrogenase (3 β -HSD), dispersed luteal cells collected from mid-luteal phase were cultured for 72 h and examined histochemically for 3 β -HSD according to Fischer and Kahn (1972).

Steroid analysis

Progesterone and oestradiol were determined by radioimmunoassay using Spectria kits (Orion Diagnostica, Finland), supplied by Polatom (Świerk, Poland).

For progesterone the sensitivity of the assay was 94 pg/ml. The coefficients of variation within and between assays were 5.8% and 2.9%, respectively. The mean recoveries were 95.1–103.7%. The cross-reaction with pregnenolone was 2.9%. All other tested steroids (5 β -dihydroprogesterone, 20 β -hydroxyprogesterone, corticosterone, testosterone, oestrone) showed less than 1% cross-reaction.

For oestradiol the detection limit of the assay was 5 pg. The coefficients of variation within and between assays were 10.28% and 2.9%, respectively. The mean recoveries were 85.6–108.9%. The cross-reaction with ethinyloestradiol was 1.4%. All other tested steroids (oestrone, oestriol, progesterone, testosterone, corticosterone) showed less than 1% cross-reaction.

Statistical analysis

All data points are expressed as means \pm SEM, from at least three separate experiments ($n = 3$) each in triplicate. The significance of differences in steroid concentration between control and experimental groups at different stages of the luteal phase was determined by analysis of variance and by Duncan's new multiple range test.

Results

Experiment 1: Effect of oPL on progesterone and oestradiol secretion

The dose-response curve of progesterone and oestradiol secretion by luteal cells isolated at different stages of the luteal phase under the influence of oPL is shown in Figs 1 and 2.

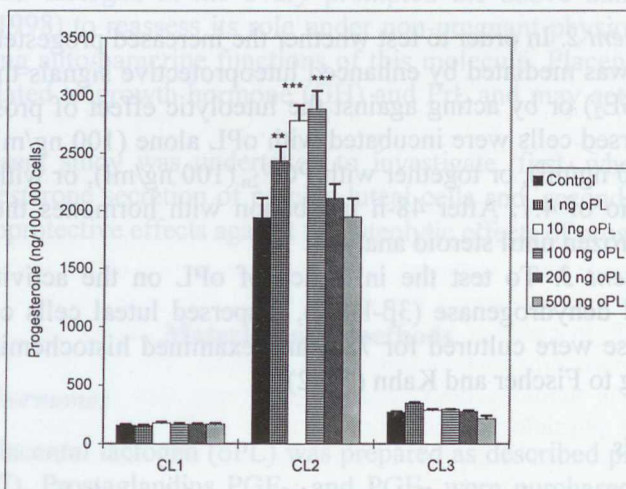


Fig. 1. Effect of graded concentrations (1, 10, 100, 200 and 500 ng/ml) of ovine placental lactogen (oPL) on progesterone secretion by porcine luteal cells isolated from corpora lutea during the early (CL1), mid- (CL2) and late luteal phase (CL3). Treatments designated with an asterisk are different from their respective controls (** $p < 0.05$; *** $p < 0.001$)

oPL added to the culture media had no effect on progesterone production of cells isolated from early developing corpora lutea (Fig. 1), whereas at doses of 1 and 10 ng/ml it increased oestradiol secretion by this type of cells (Fig. 2). A well-shaped dose-response curve was obtained and maximal increase was found at 10 ng oPL/ml. oPL also exerted a biphasic effect on progesterone production of luteal cells collected from mature corpora lutea. In doses of 1, 10 and 100 ng/ml it stimulated progesterone secretion (2065 ± 106.6 ng, 2390.0 ± 204.3 ng and 2447.5 ± 136.0 ng/ 10^5 cells, respectively vs. 1649.2 ± 143.8 ng/ 10^5 cells), while

at doses of 200 and 500 ng/ml secretion was close to control values (Fig. 1). oPL added to the culture media had no effect on progesterone and oestradiol secretion of cells isolated from regressing corpora lutea (Figs 1 and 2).

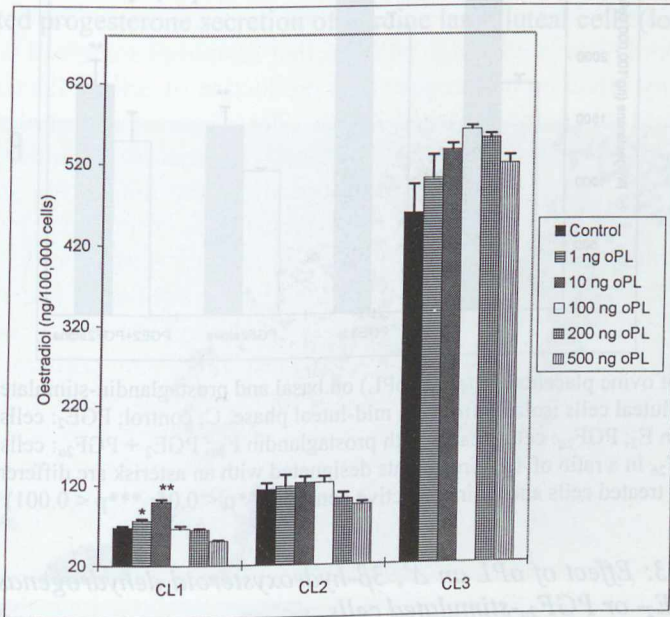


Fig. 2. Effect of graded concentrations (1, 10, 100, 200 and 500 ng/ml) of ovine placental lactogen (oPL) on oestradiol secretion by porcine luteal cells isolated from corpora lutea during the early (CL1), mid- (CL2) and late luteal phase (CL3). Treatments designated with an asterisk are different from their respective controls (* $p < 0.01$; ** $p < 0.05$)

Experiment 2: Effect of oPL, PGE₂ alone, PGF_{2α} alone or simultaneous treatment with PGE₂ and PGF_{2α} in a ratio of 4:1 on progesterone secretion

Addition of oPL to cells isolated at the mid-luteal phase and grown without PGE₂ or PGF_{2α} increased progesterone secretion from 1530 ± 26 to 2400 ± 198 ng/10⁵ cells (Fig. 3). PGE₂ alone added to the culture medium also elevated secretion to 1890 ± 20.8 ng/10⁵ cells, but the simultaneous addition of oPL had no statistically significant effect (Fig. 3). On the other hand, oPL added together with PGF_{2α} protected CL cells against the luteolytic action of PGF_{2α} (1372.5 ± 19.5 ng vs. 962.0 ± 10.5 ng/10⁵ cells in PGF_{2α}-treated cultures; $p < 0.05$). Treatment with a combination of oPL and 4:1 PGE₂/PGF_{2α} counteracted the inhibitory effect of PGF_{2α} on progesterone secretion (1716.5 ± 34.5 ng vs. 1444.75 ± 18.3 ng in PGE₂ + PGF_{2α} treated cells and vs. 962.0 ± 10.5 ng/10⁵ cells in cultures treated with PGF_{2α} alone; $p < 0.05$; Fig. 3).

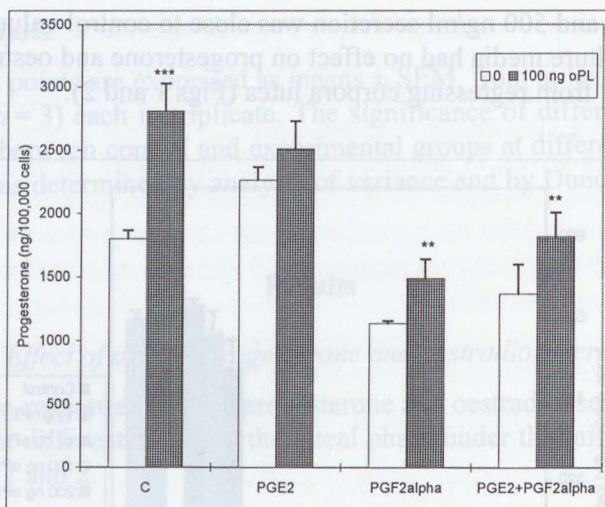


Fig. 3. Effect of ovine placental lactogen (oPL) on basal and prostaglandin-stimulated progesterone secretion by luteal cells isolated from the mid-luteal phase. C: control; PGE₂: cells treated with prostaglandin E₂; PGF_{2α}: cells treated with prostaglandin F_{2α}; PGE₂ + PGF_{2α}: cells treated with PGE₂ + PGF_{2α} in a ratio of 4:1. Treatments designated with an asterisk are different from oPL-treated cells and their respective controls (**p < 0.05; ***p < 0.001)

Experiment 3: Effect of oPL on Δ^5 , 3 β -hydroxysteroid dehydrogenase activity in control, PGE₂- or PGF_{2α}-stimulated cells

Control cultures showed a relatively weak enzyme activity (Fig. 4a) which, however, markedly increased after addition of oPL (Fig. 4b).

Discussion

In most mammals, establishment of early pregnancy requires continuous progesterone production by the CL beyond the luteal phase of the oestrous cycle. In pigs, loss of CL function at any stage of gestation leads to abortion (Belt et al., 1971). However, it has been revealed that some mammals such as the pig do not possess a classic PL (Talamantes et al., 1980; Van der Meulen et al., 1991). It complicates the issue that the notion regarding the absence of PL is based on limited experimentation. Some apparently non-expressing species may possess a classic PL with a restricted expression pattern that was not adequately tested. The presented data showed that PL added to the culture media in doses of 1–100 ng/ml increased the progesterone secretion of luteal cells collected from mature corpora lutea. A subgroup of hormones referred to as placental lactogens activates Prl and GH receptor signalling pathways and possesses a biological activity similar to that attributed to pituitary Prl and GH (McLeod et al., 1989;

Cohick et al., 1996; Yuan and Lucy, 1996b). In our earlier study a stimulatory action of Prl on the progesterone secretion of porcine luteal cells was reported (Gregoraszcuk, 1983). Prl receptor was also detected in luteal cells isolated from the porcine ovary (unpublished data). Yuan and Lucy (1996b) showed that Prl stimulated progesterone secretion of porcine large luteal cells (location of GH receptor).

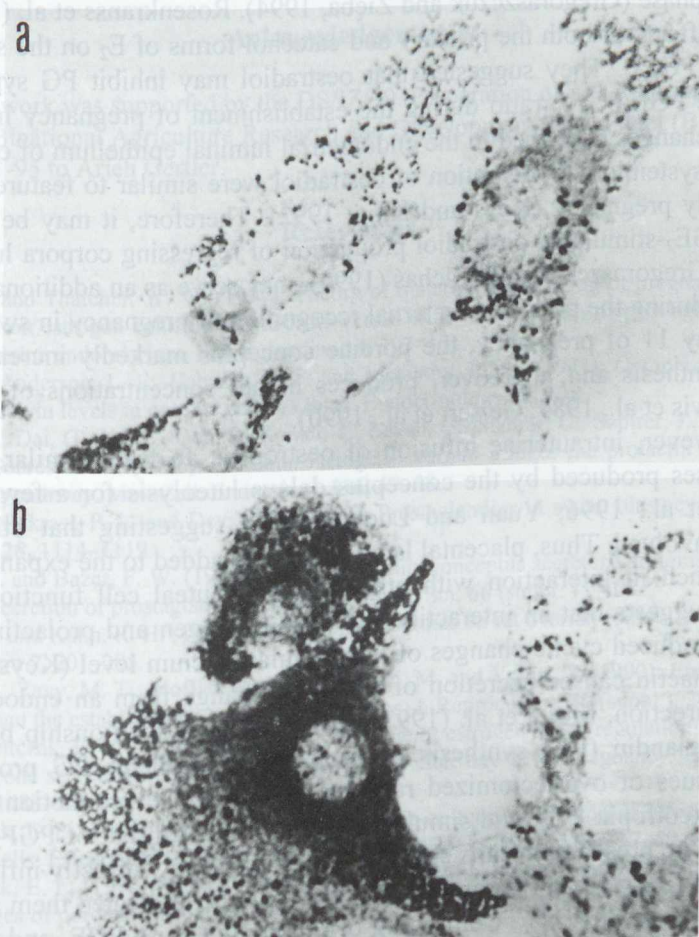


Fig. 4. The activity of Δ^5 , 3β -HSD in a 4-day monolayer of (a) control luteal cells and (b) luteal cells treated with 100 ng/ml placental lactogen

In pigs, the initial antiluteolytic signal is delivered on days 11–12 of pregnancy and is generally considered to be mediated by oestrogens released by the conceptus (Bazer and Thatcher, 1977). The presented data showed that oPL added to the culture medium increased oestradiol production by cells isolated from early-

developing and mature corpora lutea. This observation could support results obtained by Gibori et al. (1984) who observed that both prolactin and prolactin-like hormone from the placenta appear to be required for the luteotropic effect of oestrogen in the corpora lutea of pregnant rats. In a previous paper (Gregoraszczuk, 1992), we demonstrated that the concentration of oestradiol in homogenates of regressing corpora lutea is very high and also that oestradiol combined with hCG exerts stimulatory effect on the progesterone production of cells collected during the late luteal phase (Gregoraszczuk and Zięba, 1994). Rosenkranss et al. (1992) demonstrated effects of both the primary and catechol forms of E_2 on the synthesis of PGE_2 and $PGF_{2\alpha}$. They suggested that oestradiol may inhibit PG synthesis and modify the $PGE_2:PGF_{2\alpha}$ ratio during the establishment of pregnancy in pigs. The structural changes described in the endometrial luminal epithelium of cycling gilts following systemic administration of oestradiol were similar to features observed during early pregnancy (Keys and King, 1992). Therefore, it may be speculated that the PGE_2 -stimulated oestradiol production of regressing corpora lutea, as observed by Gregoraszczuk and Michaś (1999), may serve as an additional source of oestradiol during the period of maternal recognition of pregnancy in swine. Beginning on day 11 of pregnancy, the porcine conceptus markedly increases prostaglandin synthesis and, moreover, produces higher concentrations of PGE_2 than $PGF_{2\alpha}$ (Davis et al., 1983; Geisert et al., 1990).

However, intrauterine infusion of oestrogens at doses similar to physiological doses produced by the conceptus delays luteolysis for a few days only (Pusateri et al., 1996; Yuan and Lucy, 1996a), suggesting that other factors might be involved. Thus, placental lactogen can be added to the expanding list of agents, which in interaction with others regulate luteal cell functions. Recent evidence suggests that an interaction between oestrogen and prolactin enhances oestrogen-induced cyclic changes of intracellular calcium level (Keys and King, 1992). Prolactin caused secretion of $PGF_{2\alpha}$ to change from an endocrine to an exocrine direction. Motta et al. (1997) showed a direct relationship between Prl and prostaglandin (PG) synthesis. Prl selectively regulates PG production in uterine tissues of ovariectomized rats. Prl enhanced the production of uterine PGE_2 (a luteotropic PG), and simultaneously diminished that of $PGF_{2\alpha}$ (a luteolytic PG). The presented results showed that oPL not only directly influenced the progesterone secretion of porcine luteal cells, but also protected them against the luteolytic effects of $PGF_{2\alpha}$. Combining oPL treatment with PGE_2 and $PGF_{2\alpha}$ in a ratio of 4:1 counteracted the inhibitory effect of $PGF_{2\alpha}$ on progesterone secretion. The pig conceptus synthesises substantial amounts of PGE_2 and $PGF_{2\alpha}$ (Geisert et al., 1990). The developing pig conceptus also secretes a number of unique proteins which increase endometrial PGE_2 synthesis and interact with oestrogen to alter prostaglandin shift into the uterine lumen (Dubois and Bazer, 1988).

These studies offer direct evidence that oPL is luteotropic and supports progesterone production in the pig. Moreover, oPL exerted a protective effect

against the luteolytic action of $\text{PGF}_{2\alpha}$ observed in cultures treated with $\text{PGF}_{2\alpha}$ alone or in simultaneous treatment with PGE_2 . Taking into consideration the observations of Madersbacher et al. (1998) who demonstrated ectopic production of human placental lactogen in the human ovary, future specific monoclonal antibody-based immunoassays are necessary to determine whether a similar ectopic production of placental lactogen-like hormones could be demonstrated in porcine ovaries.

Acknowledgements

This work was supported by the DS/IZ/99. Preparation of oPL was supported by a grant from Binational Agriculture Research and Development USA-Israel (BARD) grant no. US-2643-95 to Arie Gertler.

References

- Bazer, F. W. and Thatcher, W. W. (1997): Theory of maternal recognizing of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $\text{F}_{2\alpha}$ by the uterine endometrium. *Prostaglandins* **14**, 397–401.
- Belt, W. D., Anderson, L. F., Cabazos, L. F. and Melampy, R. M. (1971): Cytoplasmic granules and relaxin levels in porcine corpora lutea. *Endocrinology* **89**, 1–8.
- Cohick, C. B., Dai, G., Xu, L., Deb, S., Kamei, T., Levan, G., Szpirer, C., Szpirer, J., Kwok, M. J. and Soares, M. J. (1996): Placental lactogen-I variant utilizes the prolactin receptor signaling pathway. *Mol. Cell. Endocrinol.* **116**, 49–58.
- Davis, D. L., Pakrasi, P. L. and Dey, S. K. (1983): Prostaglandins in swine blastocysts. *Biol. Reprod.* **28**, 1114–1119.
- Dubois, D. H. and Bazer, F. W. (1988): Effect of porcine conceptus secretory proteins on endometrial secretion of prostaglandins *in vitro*. *J. Anim. Sci.* **66** (suppl. 1), 404.
- Fischer, T. V. and Kahn, R. H. (1972): Histochemical studies on rat ovarian follicular cells *in vitro*. *In Vitro* **7**, 201–205.
- Geisert, R. D., Zavy, M. T., Moffatt, R. J., Blair, R. M. M. and Yellin, T. (1990): Embryonic steroids and the establishment of pregnancy in pigs. *J. Reprod. Fert.* **40** (suppl.), 293–305.
- Gibori, G., Antczak, E. and Rotchild, I. (1984): The role of estrogen in the regulation of luteal progesterone secretion in the rat during the period after day 12 of pregnancy. *Endocrinology* **100**, 1483–1495.
- Gregoraszczuk, E. L. (1983): Steroid hormone release in cultures of pig corpus luteum and granulosa cells: Effect of LH, hCG, PRL and estradiol 17β . *Endocr. Exper.* **17**, 59–63.
- Gregoraszczuk, E. L. (1992): Interrelations between steroid hormone secretion and morphological changes of porcine corpora lutea at various periods of luteal phase. *Endocrine Regulations* **26**, 189–194.
- Gregoraszczuk, E. L. and Zięba, D. (1994): Effect of estradiol- 17β on basal and hCG stimulated progesterone secretion by porcine luteal cells isolated in various stages of the luteal phase. *Endocrine Journal* **41**, 57–62.
- Gregoraszczuk, E. L. and Michas, E. (1999): Progesterone and estradiol secretion by porcine luteal cells under the influence of individual and combined treatment with prostaglandins E_2 and $\text{F}_{2\alpha}$ throughout the estrous cycle. *Prostaglandins & Other Lipid Mediators* **57**, 231–241.
- Keys, J. L. and King, G. J. (1992): Effects of topical and systemic estrogen on morphology of porcine uterine luminal epithelia. *Biol. Reprod.* **46**, 1165–1172.

- Madersbacher, S., Klieber, R., Mann, K., Marth, C., Tabarelli, M., Wick, G. and Berger, P. (1992): Free α -subunit, free β -subunit of human chorionic gonadotropin (hCG), and intact hCG in sera of healthy individuals and testicular cancer patients. *Clin. Chem.* **38**, 370–376.
- Madersbacher, S., Stulnig, T., Huber, L. A., Schonitzer, D., Dirnhofer, S., Wick, G. and Berger, P. (1993): Serum glycoprotein hormones and their free α -subunit in a healthy elderly population selected according to the SENIEUR protocol. Analyses with ultrasensitive time resolved fluoroimmunoassays. *Mech. Ageing Development* **71**, 223–233.
- Madersbacher, S., Untergasser, G., Gerth, R., Hermann, M., Schwarzler, P., Dirnhofer, S. and Berger, P. (1998): Reassessment of the role of human placental lactogen in physiological non-pregnant and pathological conditions. *Exp. Clin. Endocrinol. Diabetes* **106**, 61–67.
- McLeod, K. R., Smith, W. C., Ogren, L. and Talamantes, F. (1989): Recombinant mouse placental lactogen-I binds to lactogen receptors in mouse liver and ovary: partial characterization of the ovarian receptor. *Endocrinology* **5**, 2258–2266.
- Motta, A. B., Viggiano, J. M. and Gimeno, M. F. (1997): Prolactin regulates uterine synthesis of prostaglandins in ovariectomized rats. Effect of estradiol 17- β . *Prostaglandins, Leukotrienes and Essential Fatty Acids* **56**, 13–16.
- Pusateri, A. E., Smith, J. M., Smith, II J. W., Thomford, P. J. and Diekman, M. A. (1996): Maternal recognition of pregnancy in swine I. Minimal requirement for exogenous estradiol-17 β to induce either short or long pseudopregnancy in cycling gilts. *Biol. Reprod.* **55**, 582–589.
- Rosenkranss, C. F., Paria, B. C., Davis, D. L. and Milliken, G. (1992): Synthesis of prostaglandins by pig blastocysts cultured in medium containing estradiol or catechol estrogen. *Prostaglandins* **43**, 309–319.
- Sakal, E., Bignon, C., Grosclaude, J., Kantor, A., Shapira, R., Leibovitch, H., Helman, D., Nespoulous, C., Shamay, A., Rowlinson, S. W., Djiane, J. and Gertler, A. (1997): Large-scale preparation and characterization of recombinant ovine placental lactogen. *J. Endocrinol.* **152**, 317–327.
- Schilling, E. (1974): Stages of ovarian function in the sow. In: *Veterinary Medical Review*. G. Elwert Universitas und Verlagsbuchhandlung, Marburg-Lahn. pp 59.
- Soares, M. J., Muller, H., Orwig, K. E., Peters, T. J. and Dai, G. (1998): The uteroplacental prolactin family and pregnancy. *Biol. Reprod.* **58**, 273–284.
- Talamantes, F., Orgen, L., Markoff, E., Woodard, S. and Madrid, S. (1980): Phylogenetic distribution, regulation of secretion, and prolactin-like effects of placental lactogens. *Fed. Proc.* **39**, 2582–2587.
- Van der Meulen, J., Elsaesser, E., Oudenaarden, C. P. J. and Helmont, F. A. (1991): Effect of intrauterine estradiol-17 β administration on inter-estrus interval in the pig. *Anim. Reprod. Sci.* **24**, 305–313.
- Yuan, W. and Lucy, M. C. (1996a): Effects of growth hormone, prolactin, insulin-like growth factors, and gonadotropins on progesterone secretion by porcine luteal cells. *J. Anim. Sci.* **74**, 866–872.
- Yuan, W. and Lucy, M. C. (1996b): Messenger ribonucleic acid expression for growth hormone receptor, luteinizing hormone receptor, and steroidogenic enzymes during the estrous cycle and pregnancy in porcine and bovine corpora lutea. *Dom. Anim. Endocrinol.* **13**, 431–444.

STUDIES ON THE TOXIC INTERACTION BETWEEN MONENSIN AND TIAMULIN IN RATS: TOXICITY AND PATHOLOGY

G. SZÜCS¹*, Judit BAJNÓGEL¹, A. VARGA¹, Zsuzsa MÓRA² and P. LACZAY²

¹Department of Toxicology, EGIS Pharmaceuticals Ltd., H-1475 Budapest 10, P.O. Box 100, Hungary; ²Department of Pharmacology and Toxicology, Faculty of Veterinary Science, Szent István University, H-1400 Budapest, P.O. Box 2, Hungary

(Received October 7, 1999; accepted February 1, 2000)

The characteristics of the toxic interaction between monensin and tiamulin were investigated in rats. A three-day comparative oral repeated-dose toxicity study was performed in Phase I, when the effects of monensin and tiamulin were studied separately (monensin 10, 30, and 50 mg/kg or tiamulin 40, 120, and 200 mg/kg body weight, respectively). In Phase II, the two compounds were administered simultaneously to study the toxic interaction (monensin 10 mg/kg and tiamulin 40 mg/kg b.w., respectively). Monensin proved to be toxic to rats at doses of 30 and 50 mg/kg. Tiamulin was well tolerated up to the dose of 200 mg/kg. After combined administration, signs of toxicity were seen (including lethality in females). Monensin caused a dose-dependent cardiotoxic effect and vacuolar degeneration of the skeletal muscles in the animals given 50 mg/kg. Both compounds exerted a toxic effect on the liver in high doses. After simultaneous administration of the two compounds, there was a mild effect on the liver (females only), hydropic degeneration of the myocardium and vacuolar degeneration of the skeletal muscles. The alteration seen in the skeletal muscles was more marked than that seen after the administration of 50 mg/kg monensin alone.

Key words: Ionophore, monensin, tiamulin, comparative toxicity, toxic interaction, rats

Monovalent ionophore antibiotics are used for the prevention of coccidiosis in poultry and as growth promoters in swine and cattle. In contrast to synthetic compounds, ionophores induce the development of resistance only after a long period of use (Chapman, 1976; Hamet, 1989). From the toxicological point of view, the use of ionophores is not without risk. The therapeutic range is narrow, and ionophores can cause severe intoxication in farm animals due to overdosage, incomplete mixing into the diet (sedimentation, segregation) or use in animal species other than those specified in the indications (Matsuoka, 1976; Howell et al., 1980; Dilov et al., 1981; Halvarson et al., 1982; Bourque et al., 1986; Galitzer et al.,

*E-mail: hatastan@mail.datanet.hu; Fax: +36 (1) 404 4888

1986; Morisse et al., 1986; Pietsch and Ruffle, 1986; Rollinson et al., 1987; Sályi et al., 1988; Papp et al., 1989). The use of ionophore antibiotics also involves some risks due to the incompatibility of certain compounds.

Monensin, salinomycin and narasin are incompatible with tiamulin, erythromycin, oleandomycin and certain sulphonamides (Goff et al., 1980; Hilbrich and Trautwein, 1980; Horrox, 1980; Hamet and Bennejean, 1980; Weisman et al., 1980; Fink, 1981; Frigg et al., 1983; Mazlum et al., 1985; Laczay et al., 1987, 1989a, 1989b).

Among these compounds, the toxic interaction between monensin and tiamulin has been studied extensively due to their clinical importance. Monensin is a member of the class of compounds known as monocarboxylic acid ionophores. Tiamulin is a semi-synthetic derivative of the dipertene antibiotic pleuromutulin, and is used for the treatment and prevention of pulmonary and gastrointestinal infections in swine and poultry. *In vitro*, it is particularly active against *Mycoplasma*, *Staphylococcus*, *Streptococcus* spp., *Serpulina* (*Treponema*) *hyodysenteriae* and other species of the genus *Spirochaeta* (Högenauer, 1979).

The combined administration of monensin and tiamulin to farm animals may lead to intoxication manifested in severe clinical symptoms (ranging from growth depression to death). The clinical signs in chickens, turkeys and pigs include depression, anorexia, ataxia, leg weakness and diarrhoea, greatly resembling the symptoms seen in ionophore poisoning (Hanrahan et al., 1981; van Vleet et al., 1983). Histopathologically, the ionophore-tiamulin myopathies have been reported to show some discrepancies from the morphological changes of ionophore toxicosis in poultry (Umemura et al., 1984).

The pathogenic mechanisms of the interactions are not clearly understood. It has been suggested that the interactions are either caused by accumulation of the ionophores in the tissues because of an inhibition of their oxidative biotransformation by tiamulin (Meingasser et al., 1979; Anadón et al., 1989) or are generated by an increase in the formation rate of reactive metabolites of the ionophores. These changes could be related to the induction of the metabolising enzymes by the pleuromutulin derivative (Laczay et al., 1990).

The objective of these experiments was to study the toxic effects of monensin and tiamulin given separately in overdose and to investigate the characteristics of the toxic interaction after the combined administration of monensin and tiamulin to rats in doses that are therapeutically relevant in farm animals.

Materials and methods

Animals. CrI:(WI)BR rats (supplied by the SPF Animal House of EGIS Pharmaceuticals Ltd., Budapest, Hungary), 5 weeks of age upon arrival, were used in both experiments. Five rats per cage were housed from arrival until the

end of study in a conventional animal room of the Toxicological Laboratory of EGIS Pharmaceuticals Ltd. The temperature of the animal room was maintained at 22 ± 1 °C with a relative humidity of 30–70%. The animals were kept under a 12-h light/dark cycle. Except for terminal sacrifice (overnight fast), the animals were fed *ad libitum* with steam-sterilised SNIFF (SNIFF Spezialdiäten GmbH, Germany) rodent pellets. Tap water, changed daily, was freely available via drinking bottles. The cages containing wooden bedding were steam-sterilised and changed twice a week.

Compounds and dosing. After five days of acclimation, five rats per sex were randomly selected (males 152.3–183.6 g and females 130.0–154.5 g body weight at study initiation) for the test substance treated and control groups in Phase I (tolerance study). Five rats per sex were used in the control group and ten rats per sex were used for the combined administration in Phase II.

Compounds. Monensin, sodium salt (Sigma); tiamulin fumarate (Dynamitilin, Novartis).

Vehicle. Methylcellulose (Hungaropharma) and distilled water (EGIS).

A three-day comparative, oral repeated-dose toxicity study was carried out in Phase I, when the effects of monensin and tiamulin were studied separately. Approximately 6-weeks-old Wistar rats (5 animals per sex) were treated on three consecutive days with monensin at dose levels of 10, 30 and 50 mg/kg. Parallel to monensin treatment, tiamulin was administered in doses of 40, 120 and 200 mg/kg according to the above-mentioned design. A vehicle-treated (1% methylcellulose suspension, treatment volume: 10 ml/kg) control group was employed in the study.

The two compounds were administered simultaneously for the study of the toxic interaction in Phase II. The applied doses were well tolerated in Phase I (as well as therapeutic doses for farm animals). Rats of the same strain and age, obtained from the same breeder (10 animals per sex), were treated by the oral route for three consecutive days with monensin 10 mg/kg and tiamulin 40 mg/kg, respectively. A vehicle-treated (1% methylcellulose suspension, treatment volume: 10 ml/kg) control group (5 rats per sex) was included in the study.

Clinical observations. Animals were observed daily for lethality and signs of toxicity. Body weight was recorded daily and the body weight gain was calculated. Food consumption was assessed weekly.

Clinical chemistry. Blood was collected from each surviving animal before necropsy. The blood samples were taken from ether-anaesthetised animals by cutting the femoral vein. Standard methods were used for the evaluation of the serum of animals. The measurements were carried out with a Hitachi 704 automatic analyser using Boehringer and Dialab reagents at 37 °C reaction temperature.

Parameters examined (Phase I and Phase II). Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), cholesterol (CHOL), triglyceride

(TG), total bilirubin (TBIL), total protein (TP), albumin (ALB), globulin (GLOB), albumin/globulin ratio (A/G), glucose (GLUC), alkaline phosphatase (AP), creatinine (CREA), blood urea nitrogen (BUN), sodium (Na), chloride (Cl), phosphorus (PHOS), potassium (K), calcium (Ca), creatine kinase (CK), and lactate dehydrogenase (LDH).

Pathology. Complete necropsies were performed on all animals that died during the study and the organs and tissues were examined microscopically. On the rats killed by *exsanguination* according to the protocol under ether anaesthesia, a full macroscopic examination was carried out. Liver, kidney, adrenal gland, testis, spleen, brain and thymus organ weights were measured during necropsy. Organ to body weight ratios were calculated. Samples of the following organs and tissues were fixed in 8% buffered formalin, embedded in paraffin, sectioned, stained with haematoxylin and eosin, and examined microscopically with a Nikon Optiphot-2 light microscope: liver, kidneys, adrenals, testes, spleen, brain, thymus, heart, mesenteric lymph nodes, stomach, duodenum, lungs, striated muscle (*m. quadriceps femoris*), and all organs and tissues showing macroscopic alterations.

Statistical analyses. When applicable, statistical analysis was assessed by Statistica for Windows software. Barlett's test was run for homogeneity. In case of homogeneous data, analysis of variance (ANOVA) was performed. Where significance was achieved ($p < 95\%$), Tukey's HSD test was run. When data were not homogenous, Kruskal-Wallis's ANOVA by Ranks and Median non-parametric test was applied. In case of significance, the Wald-Wolfowitz Runs test was performed. The levels of statistical significance were $p < 0.05$ and $p < 0.01$, respectively.

Results

The following includes only drug-related results; if not reported, results were not different from concurrent or historical controls or were not considered to be drug-related.

Phase I

Lethality, clinical signs. All unscheduled deaths occurred in the animals given monensin at 30 (4 females out of five) and 50 mg/kg (3 males and 5 females out of five, respectively) on the third day of treatment. Females proved to be more sensitive than males. Prior to death, weakness of the hind legs and paraplegia were observed in the females treated with 50 mg/kg monensin. No clinical signs were seen in the other dose groups. Poor growth performance and decreased food consumption were seen in the animals given 30 and 50 mg/kg monensin. The body weights decreased in the females given monensin 30 (-13% , $p < 0.05$) and 50 mg/kg (-11%) on the third treatment day when compared to the control group.

The body weight gains were markedly lowered in the males (–121% and –150%) and females (–212% and –240%) given the mid- and high dose of monensin.

Clinical pathology. Moderate increases in the serum activity of ALAT (64% increase, $p < 0.01$) and ASAT (25% increase) were seen in the males given 30 mg/kg monensin (no measurements could be carried out in the females due to lethality) and females given 10 mg/kg when compared to the control group. Serum TG levels decreased (–62% versus controls, $p < 0.01$) in the females treated with monensin at 10 mg/kg. A similar trend was observed in the males treated with monensin in a dose of 30 mg/kg/day (–44% decrease versus controls); however, it did not reach statistical significance. Decreased (–9%, $p < 0.01$) serum sodium levels were recorded in the males given 30 mg/kg monensin. High inter-individual variations were found in the CK and LDH levels in all groups including the controls. The examined serum chemistry parameters were unaffected by the different dose levels of tiamulin.

Organ weight measurement. Increased liver weights were recorded in the females treated with 120 and 200 mg/kg tiamulin. Statistical significance ($p < 0.05$) was only seen in the females given the top dose of tiamulin at the evaluation of absolute liver weights.

Macroscopic pathology. Except for rapid *post-mortem* rigidity (females given 50 mg/kg monensin), there were no consistent macroscopic changes which could be attributed to treatment with monensin or tiamulin.

Microscopic examination. Diffuse vacuolation was observed in the livers of the animals given 50 mg/kg monensin. Vacuolar hydropic degeneration, showing no dose-dependent tendency, could be demonstrated in the sarcoplasm of the myocardium of all monensin-treated animals. Vacuolar degeneration was seen in the sarcoplasm of skeletal muscles of rats treated with 50 mg/kg monensin. Increased number of apoptotic hepatocytes and elevated liver cell division were found in the tiamulin-treated animals at 120 and 200 mg/kg dose levels, indicating a mild effect of treatment on the liver.

Phase II

Lethality, clinical signs. Four females died out of ten from the animals given the combined therapy with monensin and tiamulin on days 2 and 3. No remarkable clinical symptoms were observed before death (weakness of the hind legs, decreased motor activity).

Decreased body weights (–9%, $p < 0.05$ and 0.01 , respectively) were recorded in both males and females on the 3rd treatment day. The weight gains of the monensin-tiamulin-treated males and females were by –99% and –178% lower (statistical significance $p < 0.01$ and 0.05 , respectively) than those of the controls.

Clinical pathology. There was a statistically significant ($p < 0.01$) decrease in serum CHOL (–25%) and TG (–64%) levels in males and a decrease in serum TP (–10%) in females. The serum levels of Na decreased (–7% and –5%, statisti-

cal significance $p < 0.05$ and $p < 0.01$, respectively) and the levels of K increased (16% and 10%, $p < 0.01$) in both males and female rats. Moderately increased serum activity of ASAT (100%), ALAT (91%) and BUN levels (65%) were seen (statistical significance $p < 0.05$ and $p < 0.01$, respectively) in females. These parameters tended to increase in males as well, although the increase did not reach statistical significance. The CK levels increased (24% males and 37% females, respectively) versus controls; however, the difference did not reach statistical significance.

Organ weight measurement. Slightly decreased thymus and spleen weights were noted for the animals given the combined therapy. The adrenal weight relative to body weight increased slightly in females when compared to the control group. The changes in organ weights, in the absence of any histopathological alteration, could be attributed to stress and to deterioration of the condition of these animals, rather than to a significant effect of treatment on the immune system.

Microscopic examination. Slight vacuolation was revealed in the livers of female rats given monensin 10 mg/kg and tiamulin 40 mg/kg. Severe hydropic degeneration of the myocardium was seen in both sexes after the combined therapy (Fig. 1). Vacuolar degeneration could be detected in the sarcoplasm of the skeletal muscles, which alteration was more marked than that observed after 50 mg/kg monensin treatment (Fig. 2).



Fig. 1. Male rat treated with monensin 10 mg/kg and tiamulin 40 mg/kg (Phase II). Myocardium (left ventricle): severe hydropic, vacuolar degeneration. Haematoxylin and eosin (HE), $\times 864$

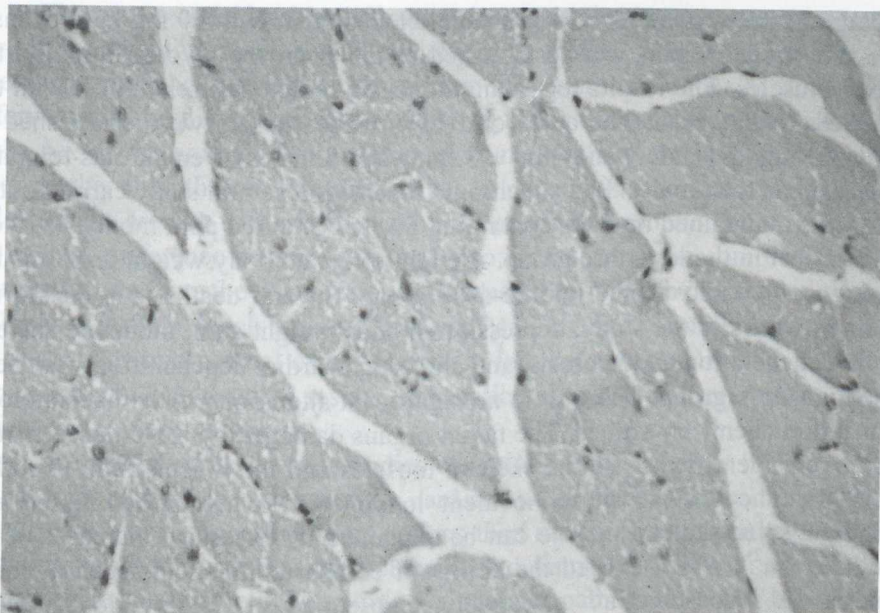


Fig. 2. Female rat treated with monensin 10 mg/kg and tiamulin 40 mg/kg (Phase II). Skeletal muscles: pronounced vacuolation in the sarcoplasm. HE, $\times 864$

Discussion

Ionophore antibiotics are known to have a narrow margin of safety and liable to overdosage. In a 30–50% overdose they usually reduce the body weight gain of chicks and, when administered in a 2- to 3-fold overdose, they may produce fatal toxicosis (Damron et al., 1977; Howell et al., 1980; Keshavarz and McDougald, 1982; Christmas and Harms, 1983; Pietsch and Rüffle, 1986; Harms and Buresh, 1987).

In the present experiment, monensin proved to be toxic to rats at doses of 30 and 50 mg/kg/day. Tiamulin was well tolerated up to the dose of 200 mg/kg/day. The maximum tolerated dose of monensin (10 mg/kg) and a non-toxic dose of tiamulin (40 mg/kg) were administered simultaneously in Phase II (therapeutic doses in farm animals). Toxic signs, including lethality (females), greatly resembling monensin intoxication appeared after three-day oral treatment. Both monensin and tiamulin exerted toxic effect on the liver in high doses. Monensin caused dose-dependent cardiotoxic effect and vacuolar degeneration of skeletal muscles at 50 mg/kg/day. After the simultaneous administration of the two compounds, there was a mild effect on the liver (females only) at microscopic examination. Hydropic degeneration of the myocardium and vacuolar degeneration of the skeletal muscles were observed in these animals. The alteration seen in

skeletal muscles was more marked than after treatment with 50 mg/kg monensin alone. The target organs were established as the liver, the myocardium and the skeletal muscles. The results regarding monensin toxicity correlate well with the published toxicity data: The acute oral LD₅₀ values in rats were established as 40.1 ± 3.0 mg/kg (males) and 28.6 ± 3.8 mg/kg (females). Female rats tolerated less monensin than males. In a subchronic repeated-dose oral study carried out in Wistar rats maintained on diets containing 50, 150 and 500 ppm monensin, there was no effect in the low-dose group. A slight effect on body weight gain was the only sign of toxicity in the middle-dose group. Several deaths (17) during the first three weeks of the study, depression in body weight gain, moderate skeletal muscle degeneration and necrosis, and slight myocardial degeneration were seen in the high-dose group. There were no significant alterations in the haematological or blood chemistry values. The target organs damaged by toxic doses of monensin were identified to be the skeletal muscles and the myocardium (Todd et al., 1984). The toxicity of monovalent ionophores to myocardial tissue and probably also to skeletal muscle can be explained by excessive increases of intracellular Ca²⁺, which exceed the ability of cellular components such as mitochondria to sequester Ca²⁺ effectively. Subsequently, Ca²⁺-overloaded cells would develop a series of degenerative alterations with subsequent membrane damage followed by swelling of the whole cell (van Vleet et al., 1983).

Based on the results of these studies, it can be concluded that tiamulin was much better tolerated in rats than monensin. Tiamulin given by itself caused no significant toxic changes. The only sign of toxicity after the high dose of tiamulin (200 mg/kg) was a mild effect on the liver. Monensin proved to be much more toxic. Even the low dose of monensin (10 mg/kg) caused slight microscopic alterations in the myocardium. The combined administration (monensin 10 mg/kg and tiamulin 40 mg/kg) resulted in toxic signs greatly resembling monensin toxicity. Vacuolar degeneration was observed in skeletal muscles. This microscopic change was only seen in the animals given 50 mg/kg monensin by itself.

Monensin-tiamulin myopathies showed two significant pathological features in chickens (Umemura et al., 1984). First, the skeletal muscles of the neck and legs were affected, whereas cardiac and pectoral muscles were consistently intact. Secondly, the histological appearance of the affected muscles was characteristic. Degenerative myopathies of avian skeletal muscles (Cheville, 1966; van Vleet and Ferrans, 1976; Hanrahan et al., 1981; Wight et al., 1981; Graziano et al., 1983, all cited by Dowling, 1992) were characterised by myocytic necrosis. In monensin-tiamulin myopathies, necrosis did not prevail in any affected muscles in poultry. The majority of fibres showed markedly enlarged nuclei and pale to basophilic sarcoplasm with partial myofibrillar lysis. Hyalinisation and floccular change appeared in a small number of muscle fibres (Dowling, 1992). In our experiment, myocytic necrosis could not develop due to the shortness of time (3-day repeated dosing).

There was considerable difference in sensitivity between sexes. Females were more sensitive to monensin toxicity than males. This gender effect is in agreement with the data published on rats: There are a number of documented differences in the disposition and toxicity of foreign compounds that are related to the sex of the animal. Sex differences in metabolism are less pronounced in species other than the rat. The differences in metabolism between males and female animals are due to the influence of hormones and genetic factors (Timbrell, 1991). This difference in sensitivity was also present after the combined administration. In this case, the sex difference in sensitivity might be explained with the differences in P450 levels (relatively lower P450 levels in females than in males, but these liver microsomal enzymes can be induced more markedly in females than in males). The detailed results of hepatic enzyme induction studies are the subject of another publication.

The results of the present studies confirm that monensin is highly toxic to rats, altering mainly the liver and the cardiac and skeletal muscles, respectively. Females are more susceptible to its actions than males. Like in poultry and pigs, the combined administration of monensin and tiamulin results in severe toxic interaction in rats as well. The clinical signs and pathological and biochemical changes caused by the simultaneous application of the two compounds greatly resemble those induced by monensin in high doses in itself, indicating the causative role of the ionophore or its metabolite(s) in the toxic interaction.

Acknowledgement

Appreciation is expressed to Attila Varga for the histological examination.

References

- Anadón, A., Martínez-Larrañaga, M. R., Díaz, M. J. and Bringas, P. (1989): Effect of tiamulin on antipyrine kinetics in chickens. *J. Vet. Pharmacol. Therap.* **12**, 94–98.
- Bourque, J. G., Smart, M. and Wobeser, G. (1986): Monensin toxicity in lambs. *Can. Vet. J.* **27**, 379–399.
- Chapman, H. D. (1976): *Eimeria tenella* in chickens: studies on resistance to the anticoccidial drugs monensin and lasalocid. *Vet. Parasitol.* **2**, 187–196.
- Christmas, R. B. and Harms, R. H. (1983): The effect of protein level and/or other selected dietary nutrients on broiler chicks receiving monensin. *Nutr. Rep. Int.* **28**, 1105–1109.
- Damron, B. L., Harms, R. H., Arafa, A. S. and Janky, D. M. (1977): The effect of dietary lasalocid or monensin in the presence of roxarsone and graded methionine levels on broiler performance and processing characteristics. *Poultry Sci.* **56**, 1487–1491.
- Dilov, P., Dimitrov, S. and Dzheurov, A. (1981): Toxicity of monensin sodium for piglets. *Vet. Med. Nauki* **18**, 55–63.
- Dowling, L. (1992): Ionophore toxicity in chickens: A review of pathology and diagnosis. *Avian Pathol.* **21**, 355–368.

- Fink, J. (1981): Arzneimittelinteraktionen: Ein Beitrag zur Frage der Verträglichkeit von Monensin und Tiamulin. *Dtsch. Tierärztl. Wschr.* **88**, 228–231.
- Frigg, M., Broz, J. and Weber, G. (1983): Compatibility studies of ionophore anticoccidials with various antibiotics and chemotherapeutics in broiler chicks. *Arch. Geflügelk.* **47**, 215–220.
- Galitzer, S., Oehme, F. W., Bartley, E. E. and Dayton, A. D. (1986): Lasalocid toxicity in cattle: acute clinicopathological changes. *J. Anim. Sci.* **62**, 1308–1316.
- Goff, S., James, T. M. and Merino, M. (1980): Tiamulin in the chicken: A summary review of compatibility studies. 29th West. Poultry Disease Conf. and 14th Calif. Health Symp. Proc. pp. 119–124.
- Halvarson, D. A., Van Dijk, C. and Brown, P. (1982): Ionophore toxicity in turkey breeders. *Avian Dis.* **26**, 634–639.
- Hamet, N. and Bennejean, G. (1980): Innocuité des associations tiamuline-monensin, tiamulin-halofuginone vis-à-vis de poulets. 6. Europ. Geflügelkonferenz Proc., Vol. II., Hamburg, pp. 356–362.
- Hamet, N. (1989): Activity of some anticoccidial drugs to turkey coccidiosis. 5th Int. Coccidiosis Conference Proc., Tours, pp. 265–266.
- Hanrahan, L. A., Corrler, D. E. and Naql, S. A. (1981): Monensin toxicosis in broiler chickens. *Vet. Pathol.* **18**, 665–671.
- Harms, R. H. and Buresh, R. E. (1987): Influence of salinomycin on the performance of broiler chicks. *Poultry Sci.* **66**, 51–54.
- Hilbrich, P. and Trautwein, G. (1980): Tiamulin-Monensin-Intoxication Beim Huhn. *Prakt. Tierarzt* **61**, 2622–2625.
- Högenauer, G. (1979): Tiamulin and Pleuromutulin. In: Hahn, F. E. (ed.) *Antibiotics: Mechanism of Action of Antibacterial Agents* **5**, Part 1. Springer Verlag, Berlin–Heidelberg. pp. 344–360.
- Horrox, N. E. (1980): Monensin-tiamulin interaction risk to poultry. *Vet. Rec.* **106**, 278.
- Howell, J., Hanson, J., Onderka, D. and Harries, W. N. (1980): Monensin toxicity in chickens. *Avian Dis.* **24**, 1050–1053.
- Keshavarz, K. and McDougald, L. (1982): Anticoccidial drugs: Growth and performance depressing effects in young chickens. *Poultry Sci.* **61**, 699–705.
- Laczay, P., Bozzay, L., Simon, F., Lehel, J., Dobos-Kovács, M., Móra, Zs. and Ribiczei, P. (1987): Study of the compatibility between monensin and other chemotherapeutics in broilers (in Hungarian, with English abstract). *Magyar Állatorvosok Lapja* **42**, 109–114.
- Laczay, P., Simon, F., Móra, Zs. and Lehel, J. (1989a): Study of the compatibility of salinomycin and narasin with other chemotherapeutics in broilers (in Hungarian, with English abstract). *Magyar Állatorvosok Lapja* **44**, 173–177.
- Laczay, P., Simon, F., Barócsai, Gy. and Vörös, G. (1989b): Study of the compatibility of XAX-M with certain ionophorous antibiotics in chickens (in Hungarian, with English abstract). *Magyar Állatorvosok Lapja* **44**, 359–363.
- Laczay, P., Simon, F. and Lehel, J. (1990): Investigations on the influence of monensin, tiamulin and of simultaneous application of both substances on the activities of the microsomal mixed function oxygenases and of the formation of peroxides in broilers. *Dtsch. Tierärztl. Wschr.* **97**, 354–357.
- Matsuoka, T. (1976): Evaluation of monensin toxicity in the horse. *J. Am. Vet. Med. Ass.* **169**, 1098–1100.
- Mazlum, Z., Pickles, R. W., Pradella, G. and Pagnani, R. (1985): Interaction between monensin, narasin or salinomycin and antibiotics erythromycin, chloramphenicol or tylosin in broiler chicks. *Clin. Vet.* **108**, 95–104.
- Meingasser, J. G., Schmook, F. P., Czok, R. and Mieth, H. (1979): Enhancement of the anticoccidial activity of polyether antibiotics in chickens by tiamulin. *Poultry Sci.* **58**, 303–313.
- Morisse, J. P., Boilletot, E. and Maurice, R. (1986): Toxicité du narasin chez le lapin: Étude de quelques cas cliniques. *Ann. Med. Vet.* **130**, 101–107.

- Papp, L., Karsai, F., Sályi, G., Bagó, Gy., Kántás, K. and Siver, L. (1989): Narasin poisoning in dogs (in Hungarian, with English abstract). *Magyar Állatorvosok Lapja* **44**, 179–185.
- Pietsch, W. and Rüfle, E. (1986): Zur Toxizität des Monensin und zu Problemen seines Einsatzes im Broilerfutter. *Mh. Vet.-Med.* **41**, 851–854.
- Rollinson, J., Taylor, F. G. R. and Chesney, J. (1987): Salinomycin poisoning in horses. *Vet. Rec.* **124**, 126–128.
- Sályi, G., Szabó, E., Bagó, Gy., Bánhidi, Gy. and Szilágyi, M. (1988): Narasin poisoning in turkeys. *Acta Vet. Hung.* **36**, 107–114.
- Timbrell, J. A. (1991): Principles of biochemical toxicology: Factors affecting metabolism and disposition. In: Taylor & Francis Ltd., London. Second edition. pp. 144–145.
- Todd, G. C., Novilla, M. N. and Howard, L. C. (1984): Comparative toxicology of monensin sodium in laboratory animals. *J. Anim. Sci.* **8**, 1513–1517.
- Umemura, T., Nakamura, H., Goryo, M. and Itakura, C. (1984): Histopathology of monensin-tiamulin myopathy in broiler chicks. *Avian Pathol.* **13**, 459–468.
- van Vleet, J. F., Amstutz, H. E., Weirich, W. E., Rebar, A. H. and Ferrans, V. I. (1983): Clinical, clinico-pathologic, and pathologic alterations of monensin toxicosis in swine. *Am. J. Vet. Res.* **44**, 1469–1475.
- Weisman, Y., Schlosberg, A. and Egyed, M. N. (1980): Acute poisoning in turkeys caused by incompatibility of monensin and tiamulin. *Vet. Res. Commun.* **4**, 231–235.

Key words: Pesticides, acute toxicity, *in vitro*, Hen's egg test, chorioallantoic membrane, HET-CAM, Draize 1–45 test.

Pesticides must undergo extensive toxicological tests before registration. The Draize rabbit eye irritation test (Draize et al., 1944) is used for assessing ocular irritation. This test has been in use for about 40 years.

In recent years, there has been increasing pressure to find suitable alternative procedures to the Draize rabbit eye test. Most of the proposed substitute tests involve cell culture systems or isolated tissue preparations. The chorioallantoic membrane has been used extensively for many years in various fields of biological research and more recently for assessing *in vitro* eye irritation potential (Atterwill and Steele, 1987).

IN VITRO OCULAR IRRITATION TOXICITY STUDY OF SOME PESTICIDES

P. BUDAI* and L. VÁRNAGY

Department of Hygiene, Institute of Plant Protection, Georgikon Faculty of Agricultural Sciences, Veszprém University, H-8361 Keszthely, P.O. Box 71, Hungary

(Received June 28, 1999; accepted October 20, 1999)

The use of animals in toxicological screening is a controversial issue. The Draize eye irritation test receives particular criticism because of the injuries inflicted on the test animals. In recent years various *in vitro* methods have been developed to replace the heavily criticised Draize rabbit eye test for irritation testing. One of the best-studied alternative methods is the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM). In the present studies comparative screening was performed with a set of pesticides to establish parallel data on *in vitro* (HET-CAM) and *in vivo* (Draize) results. The tested pesticides included Arelon 500 FW (isoproturon), Banvel 480 (dicamba), Dikamin D (2,4 D), Karathane LC (dinocap), Ronstar (oxadiazon) and Modown 4 F (bifenox). In most cases a good correlation was found between the HET-CAM assessment and results of the Draize rabbit eye test. Although the current form of the HET-CAM test is a valuable pre-screen method for predicting the ocular irritation potential of chemicals, and can be used for reducing the number of experimental animals, a number of technical problems must still be addressed before these systems can replace whole animal tests. The HET-CAM test can be a useful component of a battery of tests needed for replacing the Draize rabbit eye test.

Key words: Pesticides, ocular irritation, *in vitro*, hen's egg test, chorioallantoic membrane, HET-CAM, Draize rabbit eye test

Pesticides must undergo numerous toxicological tests before registration. The Draize rabbit eye irritation test (Draize et al., 1944) is used for assessing ocular irritation. This test has been in use for about 40 years.

In recent years, there has been increasing pressure to find suitable alternative procedures to the Draize rabbit eye test. Most of the proposed substitute tests involve cell culture systems or isolated tissue preparations. The chorioallantoic membrane has been used extensively for many years in various fields of biological research and more recently for assessing *in vivo* eye irritation potential (Atterwill and Steele, 1987).

*E-mail: H11086bud@ella.hu; Fax: +36 (83) 315 105

The chorioallantoic membrane is a complete tissue that responds to injury with a complete inflammatory reaction. This process is similar to that induced by chemicals in the conjunctival tissue of the rabbit eye (Leighton et al., 1985).

In considering the 'reduction, refinement and replacement' of animal experimentation, it is very important to recognise that testing incubated hen's eggs is a borderline case between *in vivo* and *in vitro* systems and does not conflict with ethical and legal aspects, especially the animal welfare laws (Luepke and Kemper, 1986).

There are some tests that use the chorioallantoic membrane for predicting *in vivo* ocular irritation. In these studies, the HET-CAM test described by Luepke and Kemper (1986) was used in a comparative screening with a set of pesticides to establish parallel data on *in vitro* (HET-CAM) and *in vivo* (Draize) results.

Materials and methods

Test materials

The test preparations included Arelon 500 FW (500 g/l isoproturon), Banvel 480 S (480 g/l dicamba), Dikamin D (40% 2,4-D), Karathane LC (350 g/l dinocap), Ronstar (250 g/l oxadiazon) and Modown 4 F (40% bifentox).

Pesticides were tested at 1, 10 and 100% concentrations in the Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) test and at 100% concentration in the Draize rabbit eye irritation test.

Methods

HET-CAM test. White Leghorn Shaver Starcross 288 chicken eggs (Agricultural Combine of Bóly, Hungary) were used. The eggs were candled and all defective ones were discarded before testing. The eggs were incubated in a Ragus incubator at 37 °C temperature and 60–70% relative humidity, and were rotated for 8 days to prevent attachment of the embryo to one side of the egg. On the 9th day the eggs were candled and any non-viable eggs were discarded. Eggs were placed back into the incubator with the large end upwards but were not rotated, thus ensuring accessibility of the chorioallantoic membrane. On the 10th day the eggs were prepared for assaying. The air space was marked and the section of shell was removed with scissors. The membrane was moistened carefully with 0.9% NaCl solution and the eggs were replaced into the incubator until ready for assaying.

Standards and test solutions were prepared before each assay.

Standards: two eggs each were set up with a mixed solution of 1% sodium dodecyl sulphate and 0.1 M NaOH.

Controls: two eggs were set up with 0.9% NaCl.

Test: six eggs were set up for each concentration of the test pesticide.

Each test pesticide was run on four separate replicates. The membrane was removed carefully with tapered forceps, then 0.3 ml of test pesticide was added to the chorioallantoic membrane and the effect was observed over a period of 5 min.

The following changes can be observed on the chorioallantoic membrane: haemorrhage, vascular lysis (lysis of small blood vessels is seen as disappearance of small blood vessels accompanied by swelling of the larger blood vessels), and coagulation.

Each reaction was recorded in seconds (INVITTOX Protocol Number 47). Experimental data were evaluated with the help of a computer software using the following algorithm:

$$RI = \frac{301 - \text{secH}}{300} \times 5 + \frac{301 - \text{secL}}{300} \times 7 + \frac{301 - \text{secC}}{300} \times 9,$$

where H = haemorrhage, L = vessel lysis, C = coagulation, RI = irritation index, and sec = start second.

Draize rabbit eye irritation test. New Zealand White rabbits (Humán Ltd. Gödöllő, Hungary) were used, three animals in each assay. A separate control group was not used because the untreated other eye (left eye) served as control. Rabbits were kept in individual cages of climate-controlled animal rooms at 22–25 °C temperature and 50–70% relative humidity. Both laboratory rabbit food and drinking water (tap-water) were available *ad libitum*. Pesticides were administered in a dose of 0.1 ml or 0.1 g into the conjunctival sac of the right healthy eye. Test solutions were prepared before each assay. The treated eye was examined at 1 and 24 h and then on the 2nd, 3rd, 4th, 7th and 14th day post-instillation (OECD Guidelines for Testing of Chemicals, Number 405).

Results

Results of the HET-CAM test

The numerical data are summarised in Table 1. During the 5-min period of observation after treatment with the test pesticides the following changes were seen.

After treatment with 1% Arelon 500 FW, slight haemorrhage and vascular lysis started 100 to 137 sec after treatment. On the basis of the irritation index calculated from the observed changes with the help of a computer software, Arelon 500 FW was a weak irritant at a concentration of 1%. At 10% concentration, Arelon 500 FW induced slight haemorrhage and vascular lysis that occurred 57 to 121 sec after treatment. The irritation index showed that Arelon 500 FW was a weak irritant at a concentration of 10%. When 100% Arelon 500 FW was used, mild haemorrhage and vascular lysis occurred 10 to 43 sec after treatment.

The irritation index showed Arelon 500 FW to be a moderate irritant at a concentration of 100%.

At a concentration of 1% the pesticide Banvel 480 S caused slight haemorrhage that occurred 45 to 136 sec after treatment. At 10% concentration Banvel 480 S induced mild haemorrhage and vascular lysis that started 23 to 91 sec after treatment. After treatment with 100% Banvel 480 S, first vascular lysis occurred 8 to 18 sec after treatment, followed by mild haemorrhage that started 28 to 64 sec after treatment. On the basis of the irritation indices, Banvel 480 S was a weak irritant at 1%, a moderate irritant at 10% and a severe irritant at 100% concentration.

After treatment with 1% Dikamin D, slight haemorrhage started 78 to 268 sec and vascular lysis occurred in some eggs before the haemorrhage, at 72 to 188 sec. At 10% concentration Dikamin D caused mild haemorrhage that started 21 to 41 sec after treatment. After treatment with 100% Dikamin D, first vascular lysis started at 11 to 26 sec, followed by mild haemorrhage that occurred 25 to 59 sec after treatment. On the basis of the irritation indices, Dikamin D was a weak irritant at 1% and 10% concentration and a severe irritant at 100% concentration.

After the treatment of eggs with 1% Karathane LC, vascular lysis started at 35 to 283 sec. Treatment of eggs with 10% Karathane LC induced vascular lysis that started at 59 to 221 sec. The irritation indices showed that Karathane LC was a weak irritant at 1% and a moderate irritant at 10% concentration. Determination of the irritation index was not possible at 100% concentration as the concentrated pesticide was opaque and covered up the membrane.

At a concentration of 1% Ronstar resulted in vascular lysis that started 45 to 80 sec after treatment, followed by slight haemorrhage at 75 to 260 sec. Treatment with 10% Ronstar caused slight haemorrhage that occurred at 47 to 105 sec. When applied in 100% concentration, Ronstar induced vascular lysis that started at 25 to 38 sec and then mild haemorrhage that started 57 to 80 sec after treatment in some eggs. Most eggs showed mild haemorrhage that started 20 to 70 sec after treatment. On the basis of the irritation indices, Ronstar was a weak irritant at 1% and 10% concentrations and a moderate irritant at 100% concentration.

After the treatment of eggs with 1% Modown 4 F, vascular lysis started at 148 to 262 sec. At 10% concentration Modown 4 F induced vascular lysis that started 160 to 286 sec after treatment and slight haemorrhage that started at 169 to 300 sec. According to the irritation indices, Modown 4 F was a weak irritant at 1% and 10% concentrations. Determination of irritation index was not possible at 100% concentration, because the concentrated pesticide was opaque and covered up the membrane.

Treatment with 0.1 M NaOH and 1% SDS first resulted in vascular lysis that started at 7 to 15 sec, followed by mild haemorrhage that occurred 15 to 23 sec after treatment. On the basis of the irritation indices 0.1 M NaOH and 1% SDS were severe irritants.

Table 1

Irritation indices determined by the HET-CAM test

Pesticide concentration	Irritation index		
	1%	10%	100%
Arelon 500 FW	3.42	4.22	5.71
Banvel 480 S	3.12	5.75	11.05
Dikamin D	3.59	4.62	9.14
Karathane LC	4.64	5.06	—
Ronstar	4.91	3.94	5.28
Modown 4 F	2.15	1.94	—
NaOH 0.1 M + SDS 1%	11.53	—	—

The obtained irritation indices can be evaluated using the classification categories shown in Table 2.

Table 2

Classification of results of the HET-CAM test

Irritation index	Irritation category
0–0.9	no irritation
1–4.9	weak irritation
5–8.9	moderate irritation
9–21	severe irritation

Results of the Draize rabbit eye irritation test

The numerical data obtained by this test are summarised in Table 3 and the grading system used is presented in Table 4.

After instillation of Arelon 500 FW there was no reaction on the cornea. Iritis was not observed at any observation times after instillation of Arelon 500 FW. Positive conjunctival responses with slight redness, slight chemosis and slight discharge were noted until the first day after instillation of Arelon 500 FW. All the treated eyes returned to normal on the 2nd day after the instillation of Arelon 500 FW. On the basis of the irritation index Arelon 500 FW had a slight irritant effect on the rabbit eye.

Moderate and slight corneal opacity was observed in one and two rabbits, respectively, after instillation of Banvel 480 S. Opacity was permanent during the observation period. Iritis was noted after instillation of Banvel 480 S and was permanent throughout the observation period. Positive conjunctival responses with severe redness, severe chemosis and strong discharge were noted up to the 7th day post-instillation. Severe conjunctival responses turned slight on post-instillation day 14. On the basis of the irritation index Banvel 480 S had a severe irritant effect on the rabbit eye.

Table 3

Draize irritation indices of the test pesticides with the corresponding irritation categories

Pesticides	Irritation index	Category
Arelon 500 FW	8	slight irritation
Banvel 480 S	65	severe irritation
Dikamin D	55	severe irritation
Karathane LC	52	severe irritation
Ronstar	49	moderate irritation
Modown 4 F	4	no irritation

The instillation of Dikamin D was followed by expressed corneal responses with opacity that eventually led to the development of cataract in all cases. Iritis was not found during the observation period. Positive conjunctival responses with severe redness, severe chemosis and strong discharge were observed up to day 14 after treatment. According to the irritation index Dikamin D caused severe eye irritation in rabbits.

After instillation of Karathane LC moderate corneal opacity was a consistent finding. Opacity persisted throughout the period of observation, and cataract developed in one case. Iritis was not seen. Redness, chemosis and discharge of moderate degree were noted up to post-instillation day 7. These responses turned slight by day 14 after instillation of Karathane LC. On the basis of the irritation index Karathane LC was a severe eye irritant in rabbits.

The instillation of Ronstar was followed by slight corneal opacity that persisted up to day 4 after treatment. Iritis was not seen at any of the observation times. Redness, chemosis and discharge of moderate degree occurred up to post-instillation day 2. Responses turned slight from day 3 after instillation. All the treated eyes returned to normal by day 7. On the basis of the irritation index Ronstar had a moderate irritant effect on the rabbit eye.

After the instillation of Modown 4 F there was no corneal reaction. Iritis did not develop. Positive conjunctival responses with slight redness were noted up to the first day after instillation of Modown 4 F. All the treated eyes returned to normal on the 2nd day after treatment. On the basis of the irritation index Modown 4 F caused no eye irritation in rabbits.

Table 4

Grading system used in the Draize eye irritation test

0-19	no irritation or slight irritation
20-49	moderate irritation
50-79	severe irritation
80-110	super irritation

Discussion

The HET-CAM test is a rapid, inexpensive, sensitive and simple toxicity test that provides information about the membrane irritation potency of chemicals. A disadvantage of the HET-CAM test is that the evaluation of results is subjective. The test cannot be used for opaque chemicals that tend to cover up the membrane, preventing evaluation. The rinsing variety of the HET-CAM test can be used for powder or granule formulations. This technique is more complicated than the simple method because of the risk of injury caused by rinsing.

Several substances and formulations have been tested by HET-CAM and a good correlation has been found between the HET-CAM results and reported data based on the Draize eye test (Luepke and Wallat, 1987). In 1988 through 1994 a validation study was carried out in Germany to evaluate the potential of two *in vitro* tests to replace the Draize eye test for severe irritants. These two tests were the HET-CAM test and the NRU test using 3T3 mouse fibroblast cells (3T3 NRU). The validation study was co-ordinated by the Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the Bundesgesundheitsamt (BGA), and was supported financially by the German Department of Research and Technology (BMBF). The authors of the BGA/BMBF study concluded that chemicals could be classified as severe irritants (R41) with sufficient reliability by the combined use of the HET-CAM test and the 3T3 NRU test, both of which are well-validated test, as required by the OECD Guidelines No. 405 (1981). Since 1992, the German authorities have accepted the use of HET-CAM data for the classification of R41 chemicals in the notification of new industrial chemicals (Balls et al., 1999).

The present study showed good correlation between results obtained by the HET-CAM test and those of the Draize rabbit eye test in most cases (Table 5).

Table 5

Irritation categories determined by the HET-CAM test and the Draize rabbit eye test

Pesticides	Categories by the HET-CAM test	Categories by the Draize rabbit eye test
Arelon 500 FW	moderate irritation	slight irritation
Banvel 480 S	severe irritation	severe irritation
Dikamin D	severe irritation	severe irritation
Karathane LC	—	severe irritation
Ronstar	moderate irritation	moderate irritation
Modown 4 F	—	no irritation

The actual form of the HET-CAM test is a valuable pre-screen for determining the ocular irritation potential of chemicals, and can be used for substantially reducing the use of animals. However, at the present time it cannot replace the Draize rabbit eye irritation test. More perfect or complete *in vitro* tests have to

be developed to replace the whole animal test. The HET-CAM test can be a useful component of a battery of tests needed for replacing the Draize rabbit eye irritation test.

References

- Atterwill, C. K. and Steele, C. E. (eds) (1987): *In vitro* Methods in Toxicology. Cambridge University Press, Cambridge.
- Balls, M., Berg, N., Bruner, L., Curren, R. D., deSilva, O., Earl, L. K., Esdaile, D. J., Fentem, J. H., Liebsch, M., Ohno, Y., Prinsen, M. K., Spielmann, H. and Worth, A. P. (1999): Eye irritation testing: The way forward. *ATLA* **27**, 53–77.
- Draize, J. H., Woodard, G. and Calvery, H. O. (1944): Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J. Pharmacol. Exp. Ther.* **82**, 377–390.
- Invitox Protocol Number 47. HET-CAM test 1990.
- Leighton, J., Nassauer, J. and Tchao, R. (1985): The chick embryo in toxicology: an alternative to the rabbit eye. *Fd. Chem. Toxic.* **23**, 293–298.
- Luepke, N. P. and Kemper, F. H. (1986): The HET-CAM test: an alternative to the Draize eye test. *Fd. Chem. Toxic.* **24**, 495–496.
- Luepke, N. P. and Wallat, S. (1987): HET-CAM reproducibility studies. *Altern. Meth. Toxicol.* **5**, 353–363.
- OECD (1981): OECD Guidelines for Testing of Chemicals, Number 405. Paris, 1981.

GENE IMMUNIZATION OF MICE WITH PLASMID DNA EXPRESSING RABIES VIRUS GLYCOPROTEIN

I. FODOR^{1,2*}, L. KUCSERA³, Nadja FODOR¹, V. PÁLFI⁴ and V. I. GRABKO^{5**}

¹Agricultural Biotechnology Center, H-2101 Gödöllő, P.O. Box 411, Hungary;

²Center for Molecular Biology and Gene Therapy, Loma Linda University School of Medicine, Loma Linda, USA; ³State Control Institute for Veterinary Biologicals, Drugs and Feeds, Budapest, Hungary; ⁴Central Veterinary Institute, Budapest, Hungary;

⁵Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia

(Received December 10, 1998; accepted October 20, 1999)

Gene immunization can be an effective vaccine strategy eliciting both humoral and cell-mediated immune responses. We constructed plasmid vectors expressing the full-length Vnukovo-32 rabies virus glycoprotein G under the control of CMV IE promoter and enhancer, adenovirus tripartite leader sequences and poly A signal of SV40. The gene vaccines were evaluated for the ability to elicit neutralizing antibodies and to protect BALB/c mice against lethal rabies virus challenge. First, mice were injected intramuscularly (i.m.) into the left hind leg and by the intradermoplar (i.d.p.) route with equal amounts of plasmid DNA (0.25–0.1 mg). Two weeks later, immunization was boosted with an additional dose of the DNA. The immunized mice were challenged by intracerebral (i.c.) inoculation of CVS-27 (10–50 LD₅₀) rabies virus. All mice produced anti-rabies virus neutralizing antibodies with a titre of $\geq 1:45$ after immunization with 0.1–0.4 mg of DNA. In challenge experiments, 83 to 91.6% protection was observed. These results confirm that a DNA vaccine could be a simple and effective solution for preventing the spread of rabies.

Key words: Antigen, recombinant DNA, rabies, protection, DNA vaccine

Rabies virus, a member of the family *Rhabdoviridae*, contains a negative-stranded RNA genome encoding five structural proteins (Wunner et al., 1988). Glycoprotein G (gpG) forms 10 nm long peplomers on the external surface of the virus membrane. It is responsible for the induction and binding of virus-neutralizing antibodies, for conferring immunity against lethal infection with rabies virus (Wunner et al., 1985), and for the determination of virulence (Coulon et al., 1982, 1983; Dietzschold et al., 1983).

* Corresponding author: I. Fodor, Center for Molecular Biology and Gene Therapy, Loma Linda University School of Medicine, 11085 Campus St., Loma Linda, CA 92354, USA; E-mail: ifodor@som.llu.edu; Fax: 909-478-4177

** Present address: Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

The nucleotide sequences of the gpG of several laboratory and street strains were determined (Anilionis et al., 1981; Yelverton et al., 1983; Tordo et al., 1986; Morimoto et al., 1989; Conzelmann et al., 1990; Benmansour et al., 1992; Xianhe et al., 1993; Fodor et al., 1994). In each strain, the precursor of gpG is 524 amino acids (aa) long. It was shown that virus-neutralizing antibodies, a major constituent of protective immunity against rabies, are solely directed against the gpG of the virus (Wiktor et al., 1984). Various gene expression systems, including recombinant vaccinia virus (Ruprecht and Kieny, 1988), adenovirus (Prevec et al., 1990), and baculovirus (Prehaud et al., 1989) have been used for developing recombinant rabies vaccines. A novel approach for induction of immune responses has been recently described which opens new powerful methods for vaccine research and to curb emerging disease threats (Whalen, 1996; Chattergoon et al., 1997). These methods involve the introduction of DNA plasmids carrying and expressing an antigen-encoding gene into the cells *in vivo*, causing an immune response. DNA vaccines have been used against various viruses including influenza A (Montgomery et al., 1993), HIV (Wang et al., 1993), bovine herpesvirus 1 (Cox et al., 1993), HSV-1 (Kuklin et al., 1997), hepatitis B (Davis et al., 1996) and C viruses (Major et al., 1995). The plasmid could be inoculated by the use of synthetic materials (Ledley, 1995) or a 'gene gun' (Fynan et al., 1993). Recently, DNA-based immunization against rabies virus has also been reported (Xiang et al., 1995; Ray et al., 1997).

In our previous study, we cloned and characterized the gpG gene isolated from the vaccine strain Vnukovo-32 (Fodor et al., 1994; Grabko et al., 1994). This strain is widely used for prophylactic or post-exposure immunization in Central and Eastern Europe and in Asia. Furthermore, preliminary data indicated that the recombinant gpG expressed by vaccinia virus and plasmid DNA induced a protective immune response in various animals (Kucsera et al., 1997). This report describes efficacious immunization and protection of mice against rabies using DNA plasmids expressing gpG.

Materials and methods

Plasmid construction

The vector pWS4 was kindly provided by Dr. R. Dornburg (University of Medicine and Dentistry, New Jersey, USA). The gpG gene of rabies virus vaccine strain Vnukovo-32 (Selimov, 1978) is derived from the plasmid pVG19 (Fodor et al., 1994). To construct expression plasmid pWR-21, pVG19 was digested with *Bam*HI and *Pst*II restriction enzymes, the obtained fragment separated by agarose gel electrophoresis, the band containing the gpG gene excised and the gene inserted into expression vector pWS4 (Sheay et al., 1993) cut with *Bam*HI-*Pst*II. To construct the expression plasmid pWR-24, pWR-21 was treated

with *KpnI* and *PstI*, blunt-ended with T4 DNA polymerase and ligated with T4 ligase, resulting in the deletion of restriction sites at the 3'-end of the gpG gene. The structure of each recombinant plasmid was verified by DNA sequencing.

Plasmid pWGP2 is another derivative of pWS4 containing the VP2 gene of infectious bursal disease virus (Fodor et al., 1999). Plasmid DNAs were purified from the *E. coli* XL1-Blue in large scale using a method described previously (Fodor et al., 1999).

Mouse immunization and challenge

Four-week-old BALB/c female mice were used for the immunization studies. Groups ($n = 12$) of mice were injected intramuscularly (i.m.) into the left hind leg and by the intradermoplar route (i.d.p.) with plasmids diluted in 100 μ l of PBS. Mice were boosted two weeks later with an additional dose of plasmid and challenged on day 28 post-immunization by intracerebral (i.c.) inoculation of CVS-27 virus (10–50 LD₅₀). Animals were observed for the following three weeks for symptoms indicative of rabies virus infection. Mice that developed the disease were euthanized.

Neutralization assay for rabies virus specific antibodies

Blood samples were obtained by retroorbital puncture. Sera were prepared and inactivated for 30 min at 56 °C and stored at –20 °C. Virus-neutralizing antibody (VNA) titres were determined on baby hamster kidney (BHK-21) cells using infectious CVS virus at 1 plaque forming unit per cell and series of serum dilutions. BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 5% fetal bovine serum (FBS). Sera and virus were incubated at 37 °C for 90 min and cell suspension was added. After 72 h of incubation, cells were fixed with acetone and stained using fluorescein isothiocyanate (FITC) conjugated with anti-rabies antibodies, and were analyzed in an immunofluorescence microscope. Data are expressed as neutralization titres that are the reciprocals of the serum dilution resulting in a 50% reduction in the number of infected cells. Standard deviations were within 10%.

Results

Constructed plasmids were purified using a modified method developed in our laboratory (Fodor et al., 1999). Extensive fractionations of DNA from the crude extracts allowed us to use only one step of CsCl gradient centrifugation in the final stage of purification. Yield of the purified plasmids varied from 0.6 to 12 mg/litre depending on the volume and the construct. The final DNA preparations contained very low, if any, levels of contaminant cytotoxic lipopolysaccharides (LPS).

In this study, two recombinant plasmids, pWR-21 and pWR-24, were constructed using the expression vector pWS4. The two plasmids (Fig. 1) differ at the junction of the 3' end of the gpG gene and poly A signal: construct pWR-21 had a DNA segment with 9 restriction sites, which was deleted in pWR-24.

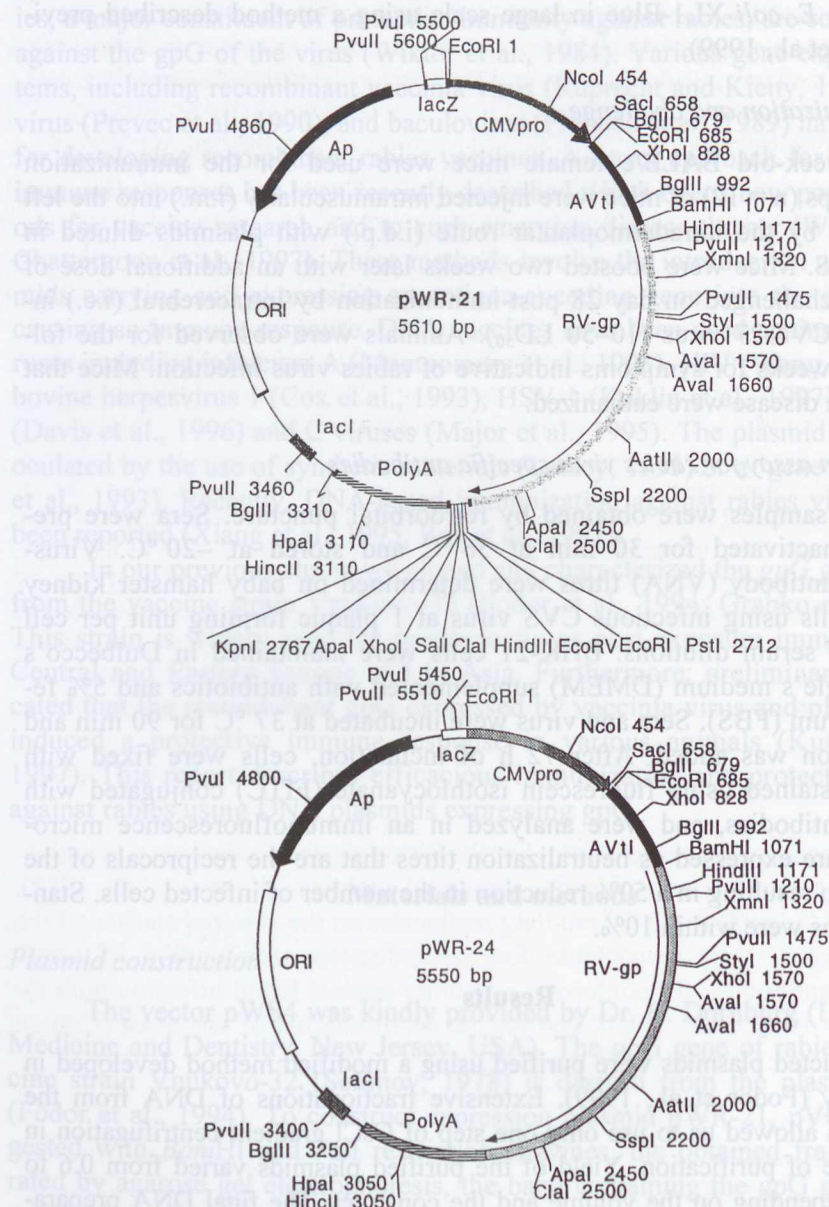


Fig. 1. Map of the expression plasmids. Both plasmids expressing rabies gpG are derived from pWS4 and contain regulatory elements of cytomegalovirus (CMV), adenovirus and SV40

For animal vaccination, various routes of DNA administration were tested by protection and seroconversion analyses. In preliminary experiments with pWR-21 subcutaneous (s.c.) inoculation resulted in 25% protection (VNA test: 1:60). In i.m. injection experiments, we obtained 37.5% protection and the presence of neutralizing antibodies of 1:240 (data not shown). However, more effective (80%) protection was observed using pWR-24 when mice were inoculated both i.m. and i.d.p. (first with $2 \times 100 \mu\text{g}$ of DNA, two weeks later boosted with $2 \times 33 \mu\text{g}$ of DNA). Since this design of vaccination seemed to be more promising, in the following experiment animals were immunized with pWR-24 using the same design. The data of this experiment presented in Table 1 confirmed the high efficacy of two consecutive inoculations of mice with a total of 400 (A), 200 (B) and 100 (C) μg of DNA. VNA assays performed prior to challenge determined the titre of neutralizing antibodies to be not less than 1:45 in all animals. These titres were not less than 0.5 IU using the WHO standard for rabies immunoglobulin and indicated a significant seroconversion. In contrast, mice of control groups inoculated with pWGP2 or PBS (Table 1, D and E, respectively) did not develop anti-rabies virus antibodies and all developed the disease. Challenge of immunized animals resulted in 83–91.6% protection.

Table 1

Gene immunization by pWR-24 and protection of mice against pathogenic rabies virus strain CVS-27

Mouse groups	Injection (day 1)	Boost (day 14)	Dead	Survived	Protected (%)	VN assay		IU
						day 1	day 28	
A (n = 12)	0.1 mg i.m. + 0.1 mg i.d.p	0.1 mg i.m. + 0.1 mg i.d.p.	1	11	91.6	< 1:4	$\geq 1:45$	≥ 0.5
B (n = 12)	0.05 mg i.m. + 0.05 mg i.d.p	0.05 mg i.m. + 0.05 mg i.d.p.	1	11	91.6	< 1:4	$\geq 1:45$	≥ 0.5
C (n = 12)	0.025 mg i.m. + 0.025 mg i.d.p.	0.025 mg i.m. + 0.025 mg i.d.p.	2	10	83.3	< 1:4	$\geq 1:45$	≥ 0.5
D (n = 19)	0.1–0.2 mg i.m. + i.d.p	0.05–0.1 mg i.m. + i.d.p.	19	0	0	< 1:4	< 1:4	–
E (n = 5)	0	0	5	0	0	< 1:4	< 1:4	–

Discussion

We investigated the protection elicited against rabies in mice by DNA vaccines expressing the gpG of the rabies virus. Using neutralizing monoclonal antibodies directed against gpG, several antigenic epitopes were localized on the gly-

coprotein (Wunner et al., 1985). Most Mabs recognized conformational epitopes (Bunschoten et al., 1989; Grassi et al., 1989). In addition, linear epitope III was closely associated with the pathogenicity of the virus (Dietzschold et al., 1983; Seif et al., 1985). Thus, gpG is an immunodominant antigen of the virus and anti-rabies vaccines should contain this component. We used the recombinant gpG of the Vnukovo-32 rabies virus strain.

For vaccination experiments, highly purified DNA is needed since contaminant PLS may cause serious side effects in immunized animals. Our DNA purification procedure allowed us to purify 1–10 mg DNA suitable for vaccination from 0.5–2 litres of bacterial culture. Delivery systems, routes of administration and certain features of the expression vector are likely to play a role in the efficacy of the DNA vaccine. One of these factors is the promoter driving the expression of the foreign antigen. The bacterial expression plasmid pWS4 containing the cytomegalovirus (CMV) immediate early promoter and enhancer, the tripartite leader sequence of an adenovirus downstream of the CMV sequences, and an SV40 polyadenylation sequence were used for the expression of the glycoprotein gene. It was reported that this combination of viral regulatory elements, compared to standard CMV-based vectors, significantly enhanced the foreign gene expression in mammalian cells (Sheay et al., 1993).

This study showed that vaccination with highly purified DNA encoding the gpG of Vnukovo-32 rabies virus under control of potent regulatory elements and delivered by a combination of i.m. and i.d.p. inoculations elicited neutralizing antibodies and effectively protected mice against a lethal dose of a pathogenic rabies virus. Our data are in agreement with earlier published data on DNA vaccination of laboratory animals against rabies (Xiang et al., 1995; Ray et al., 1997). It was previously demonstrated that anti-rabies virus neutralizing antibodies elicited by vaccination with recombinant antigen cross-neutralized a global spectrum of rabies virus variants (Ray et al., 1997). Thus, gene vaccination against rabies could be a solution for the developing countries. To achieve fully protective immunity, we plan to inoculate animals using cationic liposomes as a carrier for delivering DNA. DNA vaccines have distinct advantages: they can be manufactured far more easily than traditional live or inactivated vaccines, or subunit vaccines composed of subcellular or recombinant protein. In addition, gene vaccines are very stable and resist temperature extremes.

Acknowledgements

We thank E. Erdélyi and M. Katona for technical assistance and Dr. R. Dornburg for providing the pWS4 plasmid. This work was supported by ICGEB, Trieste, Italy.

References

- Anilionis, A., Wunner, W. H. and Curtis, P. J. (1981): Structure of the glycoprotein gene in rabies virus. *Nature* **294**, 275–278.
- Benmansour, A., Brahimi, M., Tuffereau, C., Coulon, P., Lafay, M. and Flamand, A. (1992): Rapid sequence evolution of street rabies glycoprotein is related to the highly heterogeneous nature of the viral population. *Virology* **187**, 33–45.
- Bunschoten, H., Gore, M., Claasen, I. J., Uytdehaag, F. G., Dietzschold, B., Wunner, W. H. and Osterhaus, A. D. (1989): Characterization of a new virus-neutralizing epitope that denotes a sequential determinant on the rabies virus glycoprotein. *J. Gen. Virol.* **70**, 291–298.
- Chattergoon, M., Boyer, J. and Weiner, D. B. (1997): Genetic immunization: A new era in vaccines and immune therapeutics. *FASEB J.* **10**, 753–763.
- Conzelmann, K. K., Cox, J. H., Schneider, L. G. and Thiel, H. J. (1990): Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. *Virology* **175**, 485–489.
- Coulon, P., Rollin, P. E. and Flamand, A. (1982): Molecular basis of rabies virulence. I. Selection of avirulent mutants of the CVS strain with anti-G monoclonal antibodies. *J. Gen. Virol.* **61**, 97–100.
- Coulon, P., Rollin, P. E. and Flamand, A. (1983): Molecular basis of rabies virulence. I. Identification of a site on the CVS glycoprotein associated with virulence. *J. Gen. Virol.* **64**, 693–696.
- Cox, G. J. M., Zamb, T. J. and Babiuk, L. A. (1993): Bovine herpesvirus 1 immune responses in mice and cattle injected with plasmid DNA. *J. Virol.* **67**, 5664–5667.
- Davis, H. L., McCluskie, M. J., Gerin, J. L. and Purcell, R. H. (1996): DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines. *Proc. Natl. Acad. Sci. USA* **93**, 7213–7218.
- Dietzschold, B., Wunner, W. H., Wiktor, T. J., Lopez, A. D., Lafon, M., Smith, M. and Koprowski, H. (1983): Characterization of an antigen determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proc. Natl. Acad. Sci. USA* **80**, 70–74.
- Fodor, I., Grabko, V. I., Khozinski, V. V. and Selimov, M. A. (1994): Nucleotide and deduced amino sequences of the glycoprotein gene of rabies virus vaccine strain Vnukovo-32. *Arch. Virol.* **135**, 451–459.
- Fodor, I., Horváth, E., Fodor, N., Nagy, E., Rencendorsh, A., Vakharia, V. N. and Dube, S. K. (1999): Induction of protective immunity in chickens immunized with plasmid DNA encoding infectious bursal disease virus. *Acta Vet. Hung.* **47**, 481–492.
- Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C. and Robinson, H. L. (1993): DNA vaccination: protective immunization by parental, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **90**, 11478–11482.
- Grabko, V. I., Fodor, I., Selimov, M. A., Khozinski, V. V. and Aksenova, T. A. (1994): Synthesis of functionally active gene of the glycoprotein-G of rabies virus strain Vnukovo-32 by polymerase chain reaction method (PCR). *Dokl. Akad. Nauk (Moscow)* **337**, 117–121.
- Grassi, M., Wandeler, A. I. and Peterhans, E. (1989): Enzyme-linked immunosorbent assay for determination of antibodies to the envelope glycoprotein of rabies virus. *J. Clin. Microbiol.* **27**, 899–902.
- Kucsera, L., Timiryasova, T., Grabko, V. I., Fodor, N., Khozinski, V., Pálfi, V., Selimov, M. A. and Fodor, I. (1997): Immunological and protection studies of vaccinia- and plasmid-vectored recombinant glycoprotein of rabies virus. In: *Proceedings of the Meeting: Molecular Biology and Biotechnology for Development*. ICGEB, Trieste, Italy, pp. 44–46.
- Kuklin, N., Daheshia, M., Karem, K., Manickan, E. and Rouse, B. T. (1997): Induction of mucosal immunity against herpes simplex virus by plasmid DNA immunization. *J. Virol.* **71**, 3138–3145.
- Ledley, F. D. (1995): Nonviral gene therapy: the promise of genes as pharmaceutical products. *Human Gene Therapy* **6**, 1129–1144.

- Major, M. E., Vitvitski, L., Mink, M. A., Schleef, M., Whalen, R. G., Treppe, C. and Inchauspe, G. (1995): DNA-based immunization with chimeric vectors for the induction of immune responses against the Hepatitis C virus nucleocapsid. *J. Virol.* **69**, 5798–5805.
- Montgomery, D. L., Shiver, J. W., Leander, K. R., Perry, H. C., Friedman, A., Martinez, D., Ulmer, J. B., Donnelly, J. J. and Liu, M. A. (1993): Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol.* **12**, 777–783.
- Morimoto, K., Ohkubo, A. and Kawai, A. (1989): Structure and transcription of the glycoprotein gene of attenuated HEP-Flury strain of rabies virus. *Virology* **173**, 465–477.
- Prehaud, C., Takehara, K., Flamand, A. and Bishop, D. H. L. (1989): Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors. *Virology* **173**, 390–399.
- Prevec, L., Campbell, J. B., Christie, B. S., Belbeck, L. and Graham, F. L. (1990): A recombinant human adenovirus vaccine against rabies. *J. Infect. Dis.* **161**, 27–30.
- Ray, N. B., Ewalt, L. C. and Lodmell, D. L. (1997): Nanogram quantities of plasmid DNA encoding the rabies virus glycoprotein protect mice against lethal rabies virus infection. *Vaccine* **15**, 892–895.
- Kuprecht, C. E. and Kieny, M.-P. (1988): Development of a vaccinia-rabies glycoprotein recombinant virus vaccine. In: Campbell, J. B. and Charlton, K. M. (eds) *Rabies*. Kluwer Academic, Boston, pp. 335–380.
- Seif, I., Coulon, P., Rollin, P. E. and Flamand, A. (1985): Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. *J. Virol.* **53**, 926–934.
- Selimov, M. A. (1978): *Rabies* (in Russian). Nauka, Moscow.
- Sheay, W., Nelson, S., Martinez, I., Chu, T.-H. T., Bhatia, S. and Dornburg, R. (1993): Downstream insertion of the adenovirus tripartite leader sequence enhances expression in universal eukaryotic vectors. *Biotechniques* **15**, 856–862.
- Tordo, N., Poch, O., Ermine, A., Keith, G. and Rougeon, F. (1986): Walking along the rabies genome: is the large G-L intergenic region a remnant gene? *Proc. Natl. Acad. Sci. USA* **83**, 3914–3918.
- Wang, B., Ugen, K. E., Srikantan, V., Agadjanyan, M. G., Dang, K., Refaeli, Y., Sato, A. I., Boyer, J., Williams, W. V. and Weiner, D. B. (1993): Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90**, 4156–4160.
- Whalen, R. G. (1996): DNA vaccines for emerging infectious diseases: what if? *Emer. Infect. Dis.* **2**, 168–175.
- Wiktor, T. J., MacFarlan, R. I., Reagan, K. J., Dietzschold, B., Curtis, P. J., Wunner, W. H., Kieny, M.-P., Lathe, R., Lecocq, J.-P., Macket, M., Moss, B. and Koprowski, H. (1984): Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. *Proc. Natl. Acad. Sci. USA* **81**, 7194–7198.
- Wunner, W. H., Dietzschold, B., MacFarlan, R. L., Smith, C. L., Golub, E. and Wiktor, T. J. (1985): Localization of immunogenic domains on the rabies virus glycoprotein. *Ann. Inst. Pasteur Virology* **136 E**, 353–362.
- Wunner, W. H., Larson, J. K., Dietzschold, B. and Smith, C. L. (1988): The molecular biology of rabies virus. *Rev. Infect. Dis.* **10**, S771–S784.
- Xiang, Z. Q., Spitalnik, S. L., Cheng, J., Erikson, J., Wojczyk, B. and Ertl, C. J. (1995): Immune responses to nucleic acid vaccines to rabies virus. *Virology* **209**, 569–579.
- Xianhe, B., Warner, C. K. and Fekadu, M. (1993): Comparisons of nucleotide and deduced amino acid sequences of the glycoprotein genes of a Chinese street strain (CGX89-1) and a Chinese vaccine strain (3aG) of rabies virus. *Virus Res.* **27**, 101–112.
- Yelverton, E., Norton, S., Obijeski, J. F. and Goeddel, D. V. (1983): Rabies virus glycoprotein analogs: biosynthesis in *Escherichia coli*. *Science* **219**, 614–620.

IMMUNOSTIMULATORY EFFECTS OF THE MURAMYL DIPEPTIDE ANALOGUE LK415 IN CHICKENS IMMUNIZED WITH A VACCINE STRAIN OF INFECTIOUS BURSAL DISEASE VIRUS

Olga ZORMAN ROJS^{1*}, Manica ČERNE¹, I. MRZEL¹, U. URLEB² and Shizuko MURAOKA³

¹Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenija,

²Pharmaceutical Faculty, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenija, ³Fujimoto Pharmaceutical Corporation, Osaka 580-0011, Japan

(Received October 25, 1999; accepted February 1, 2000)

The effects of muramyl dipeptide (MDP) synthetic analogue LK415 on the immune response of chickens immunized with a live vaccine against infectious bursal disease (IBD) were studied in two independent trials, using levamisole hydrochloride as comparative immunostimulant. Groups of five-week-old commercial chickens (Isa Brown) were immunized orally with 10 doses of the vaccine strain of IBV (Winterfield strain). The chickens were then given four injections of the MDP analogue LK415 in a dosage of either 0.25 mg/kg body weight (b.w.) or 2.5 mg/kg b.w. or levamisole at a daily dose of 15 mg/kg b.w. for four consecutive days, starting from the day of immunization. Histological examinations of bursal tissue collected on days 2, 4 and 7 postimmunization (p.i.) showed a lower degree of destruction of bursal follicles and earlier renewal of bursal tissue in LK415-treated chickens compared to levamisole-treated and untreated immunized groups. Compared to the other groups, the LK415-treated chickens showed a significantly higher antibody response to IBV on days 14 and 28 p.i. ($P < 0.01$) as measured by commercial ELISA. The present study indicates some potent immunostimulatory effects of the MDP analogue LK415 on the chicken immune system.

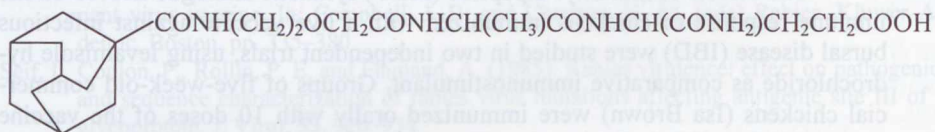
Key words: Muramyl dipeptide, infectious bursal disease, vaccination, immune response, chickens

N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) has been known since 1974 as the smallest immunologically active fragment of the cell wall of bacteria (Ellouz et al., 1974). A large number of MDP derivatives have been prepared in order to study structure-activity relationships as well as to avoid toxic side-effects of native MDP. Consequently, the immunostimulatory effect of MDP and its derivatives is very well documented in different animal species (e.g. LeClerc et al., 1979) as well as in humans (e.g. Lopez-Bernstein et al., 1983; Ozaki et al., 1989;

*E-mail: rojsol@mail.vf.uni-lj.si; Fax: +386 1 332 308

Furuse and Sakuma, 1989). However, very limited information is available about the effects of these compounds on the immune response of chicken. Only poor effects on selected chicken immune functions have been documented in the literature (Neumann et al., 1982).

In U.S. Patent No. 5,514,654 (Pečar et al., 1996) a new group of desmuremyl analogues was described, which includes an adamantyl derivative, LK415. It was found that LK415 could influence the level of individual cytokines (Ochi et al., 1999). Some other congeners from this family of analogues were found to be capable of increasing the survival of infected immunocompromised mice, enhancing the maturation of B lymphocytes to plasma cells and increasing the activity of T lymphocytes as well as that of macrophages (Sollner et al., 1993, 1995, 1996). The chemical structure of LK415 [N-(2-(2-(1-adamantanecarboxamido)-ethoxy)-acetyl)-L-alanyl-D-isoglutamine] is as follows:



The main physicochemical properties of LK415 are described elsewhere (Pečar et al., 1996).

In the present study the influence of LK415 on the chicken immune response has been studied. Infectious bursal disease (IBD) is an acute viral disease of young chickens caused by a double-stranded and bi-segmented RNA virus (IBDV) of the *Birnaviridae* family. Chickens are clinically most susceptible to IBDV between the ages of 3 and 7 weeks, although susceptibility has also been reported at earlier and later ages. IBDV is known to have a tropism for B lymphocytes and causes severe necrosis in lymphoid follicles of the bursa of Fabricius. Other lymphoid organs may also be affected, but to a lesser degree (Lukert and Saif, 1997). Predilection of the virus for B cells and its replication in the bursal tissue is a mechanism responsible for the immunosuppressive effect of IBDV infection (Rodenberg et al., 1994). Vaccination is the principal method used for the control of IBD. To achieve good protection in broiler flocks and to protect future layers and breeders, the use of live or live and inactivated vaccines is recommended.

The present experiment was designed to evaluate the efficacy of the MDP synthetic analogue LK415 as an immunopotentiating agent to enhance the immune response of chickens immunized with the vaccine strain (Winterfield) of IBDV. Several approaches to introducing adjuvant to viral vaccines have been tried in order to obtain higher vaccine efficacy. Levamisole, which has also been studied extensively in chickens (Maheswaran et al., 1979; Singh and Dhawedkar, 1993), was used as comparative immunostimulant.

Materials and methods

Chickens

Chickens (Isa Brown) used in the experiment were obtained from a commercial hatchery. They were kept in an isolation house on deep litter before and during the experiment. The chickens were fed an ordinary chicken feed and provided drinking water *ad libitum*.

Immunomodulators

Two immunomodulators were used in the study: levamisole hydrochloride, the commercially available preparation of pharmaceutical grade, in a dosage of 15 mg/kg body weight (b.w.), and LK415 – a synthetic analogue of MDP – in a dosage either of 0.25 mg/kg b.w. or 2.5 mg/kg b.w.

Immunization

The live attenuated IBD vaccine containing the Winterfield strain (Gumbokal SPF, Pliva Zagreb, Croatia) was used for immunization. On the first day of the trials, each bird received 10 standard oral doses of the vaccine strain suspended in 0.1 ml PBS.

Experimental design

The experiment was performed in two independent trials.

Trial 1

At the age of 35 days, 115 chickens were weighed and then divided into 5 groups (23 chicks per group). The doses of immunomodulators were determined according to the body weight (mean weight about 300 g). Levamisole was dissolved in sterile phosphate buffer saline (PBS) to give a final concentration of 4.5 mg per 0.1 ml; LK415 was suspended in sterile PBS to give a final concentration of 0.075 mg per 0.1 ml or 0.75 mg per 0.1 ml.

Birds from Groups 1, 2, 3 and 4 were immunized with the IBDV vaccine. Treatment with immunostimulants was also started on the day of vaccination. Each bird was treated subcutaneously (s.c.) in the neck for four consecutive days as follows:

Group 1: Each bird received an injection of 0.1 ml PBS.

Group 2: Birds were given levamisole at a daily dose of 4.5 mg in 0.1 ml PBS.

Group 3: Birds were given LK415 at a daily dose of 0.075 mg in 0.1 ml PBS.

Group 4: Birds were treated with LK415 at a daily dose of 0.75 mg per 0.1 ml PBS.

Group 5: Birds served as an untreated control group.

Before the immunization and then on days 2, 4 and 7 day post immunization (p.i.), the bursae of two euthanized birds per group were taken for histological examinations. In order to examine the humoral immune response, blood samples were collected before the immunization and then on days 7, 14, 21 and 28 p.i. Serum was obtained from these samples and stored at -20°C until tested.

Trial 2

The second trial was performed on 69 birds aged 32 days. As immunomodulator, LK415 was used in a dose of 0.25 mg/kg b.w. The birds were weighed, divided into three groups of 23 birds each and treated as follows:

Group 1: Each bird was immunized as in Trial 1 and treated with 0.1 ml PBS s.c. in the neck for four consecutive days.

Group 2: Birds were immunized with the vaccine strain of IBDV as above and given four injections of LK415 at a daily dose of 0.075 mg per 0.1 ml s.c. in the neck, starting on the day of immunization.

Group 3: Birds were neither immunized nor treated.

For histological examinations bursal tissues were taken from two euthanized and necropsied chickens per group at the same time as in the first trial: before the immunization and then on days 2, 4 and 7 p.i.. Blood samples were taken before immunization and on day 14 p.i., and then at weekly intervals over a period of 5 weeks. Serum samples were stored at -20°C until tested.

Histology

Samples of bursa were fixed in a 4% neutral buffered formaldehyde solution, dehydrated in graded alcohol, cleared with histolemon and infiltrated and embedded in paraplast. The embedded tissues were sectioned at $6\text{ }\mu\text{m}$ and stained with haematoxylin and eosin using conventional procedures.

The destruction and recovery of bursal tissue were observed and compared at the same intervals for all groups. Virus-induced lesions were determined according to Nunoya et al. (1992). The recovery of bursal tissue was determined by the refilling of the cortex or cortex and medulla of affected follicles and by the presence of newly formed follicles.

Measurement of antibody titres to IBDV

Serum samples were assayed in a single 1:500 dilution using a commercial total antibodies ELISA (Idexx Corporation, Westbrook, Maine, USA) according to the manufacturer's instruction. Mean titres and standard deviation were calculated using Idexx software.

Statistical analysis

All data were statistically processed using the ANOVA statistical program. The differences of $P < 0.05$ were considered significant.

Results

Effect of immunomodulators on antibody response to IBDV

At the beginning of the trial the chickens from all groups were free of antibodies to IBDV. An increase in the ELISA antibody titre to IBDV was shown in all the immunized groups.

The presence of antibodies to IBDV in the sera of chickens immunized and treated with different immunomodulators in Trial 1 are shown in Table 1.

The administration of LK415 resulted in a significantly higher serological response on days 14 and 28 p.i. as compared with the other groups. The highest ELISA mean titre to IBDV was observed on day 28 p.i. in the group treated with LK415 of 2.5 mg/kg. A slightly lower ELISA mean titre was observed on the same day in chickens treated with a 10 times lower dose of LK415, but the difference was not statistically significant. Antibodies to IBDV were not detected in the negative control group (data not shown).

Very similar serological results were obtained in Trial 2 (Table 2). Like in Trial 1, both immunized groups of chickens showed an increase in antibodies against IBDV. Positive results were confirmed throughout the examination period for up to 7 weeks after the immunization. The chickens treated with LK415 exhibited significantly higher ELISA titre to IBDV on days 14 and 28 p.i. as compared to Group 1. The maximum ELISA titre was observed 4 weeks after immunization. Although higher ELISA titres were observed also on days 35 and 49 p.i., the differences were not statistically significant.

Histology

Specific changes of bursal tissue induced by the vaccine strain were present on days 2 (Fig. 1a), 4 and 7 p.i. in chickens immunized with the vaccine strain of IBDV (Group 1, both trials). On days 2 and 4 p.i. interstitial oedema, cortical and medullar lymphoid depletion and focal lymphocytolysis were seen. In some cases medullar follicular cysts and surface epithelial cysts were also detected. On day 7 p.i. 25% of the bursal follicles were still affected, but on the same day the recovery started in half of the affected follicles (Fig. 1b).

Table 1

Results of ELISA test for IBDV in immunized chickens treated with different immunomodulators – Trial 1

Days p.i.	ELISA IBDV											
	Group 1: PBS			Group 2: Levamisole 15 mg/kg b.w.			Group 3: LK415 0.25 mg/kg b.w.			Group 4: LK415 2.5 mg/kg b.w.		
	No. of samples	MT ^a	SD ^b	No. of samples	MT	SD	No. of samples	MT	SD	No. of samples	MT	SD
0	15	59	197.9	15	61	225.7	15	28	48.8	15	26	52.4
7	15	2681	1060.5	15	2469	983.6	15	2022	596.6	15	2588	713.9
14	15	2815	1023.3	14	2159	751.3	15	4007 ^c	827.4	15	3930 ^c	1103.2
21	15	4238	1016.8	13	4230	1820.7	15	3557	894.8	15	3920	824.0
28	14	3754	974.6	13	3361	920.2	19	4883 ^c	1556.6	17	5572 ^c	1145.3

^aMean titre; ^bStandard deviation; ^cSignificant difference $P < 0.01$

Table 2

Results of ELISA test for IBDV in chickens treated with 0.025 mg/kg LK415 – Trial 2

Days p.i.	ELISA IBDV								
	Group 1: PBS			Group 2: LK415 0.25 mg/kg b.w.			Group 3: Negative control		
	No. of samples	MT ^a	SD ^b	No. of samples	MT ¹	SD	No. of samples	MT ¹	SD
0	19	3	0.0	19	6	0.0	19	2	0.0
14	16	1493	703.4	16	2154 ^d	675.3	16	0	0.0
21	16	2212	1020.2	16	2649	990.8	ND ^c		
28	16	3308	817.8	16	3922 ^c	802.1	16	0	0.0
35	16	3508	673.2	16	3725	873.9	ND		
42	11	3604	724.1	10	3590	498.2	ND		
49	16	2700	1056.2	16	3220	806.7	16	0	0.0

^aMean titre; ^bStandard deviation; ^cNot done; ^dSignificant difference $P < 0.01$; ^eSignificant difference $P < 0.05$

In the levamisole-treated group (Trial 1, Group 2) vasal hyperaemia without interstitial oedema and very uniform follicular lymphopenia without cyst formation were observed. On day 2 p.i. focal lymphocytolysis was seen in 45% of the follicles. On day 7 p.i. the follicular cortex was restored in almost all follicles. Renewal of the medulla was slower than that of the cortex.

In the groups of chickens treated with LK415 at a dose of 0.25 mg/kg b.w. (Trial 1, Group 3 and Trial 2, Group 2) on day 2 p.i. marked medullar lymphopenia with medullar cyst formation and cortical focal lymphocytolysis were seen in the follicles under the plical epithelia (Fig. 2a). Follicles situated deeper in the tissue were less affected. Focal lymphocytolysis was observed in 25% of the follicles. Renewal in the medulla and cortex of the affected follicles started on day 4 p.i. Newly formed follicles were becoming visible near the affected area. On day 7 p.i. almost all the follicles were restored (Fig. 2b).

In the group treated with LK415 at a dose of 2.5 mg/kg b.w. (Trial 1, Group 4) only 25% of the follicles were affected. Predominant follicular lymphopenia without cyst formation, intestinal oedema and vasal hyperaemia were expressed on day 2 p.i. Corticomedullar epithelia in the affected follicles were evident till day 4 p.i. Compared with the other groups, cortical renewal was faster with more newly formed follicles. On day 7 p.i. the picture of bursal tissue was comparable with that of the control groups (Trial 1, Group 5; Trial 2, Group 3), where no histological changes were detected.

Discussion

Many investigators have demonstrated that MDP and its derivatives exhibit adjuvant activities to virus vaccines in different species, reflecting the potentiation of the humoral and cellular immune response (Yoo et al., 1992; Tamura et al., 1995; Yoo et al., 1998). Contrary to these findings, a lack of effects of MDP and its derivatives on the cellular as well as on the humoral immune response was reported in chickens (Neumann et al., 1982).

LK415 is a distinctive inducer of interferon gamma (Ochi et al., 1999), and some researchers are looking at interferon gamma as a potential adjuvant for vaccines (Schijns et al., 1995; Lowenthal et al., 1998). Therefore, the present experiment was designed to evaluate the efficacy of the MDP structural analogue LK415 as an immunopotentiating agent to enhance the immune response of chickens immunized with a vaccine strain of IBDV. Levamisole was used as comparative immunostimulant. The criteria for testing were based on the production of specific antibodies to IBDV immunization and on histological examinations of the bursa of Fabricius as the central lymphoid organ which provides B lymphocytes.

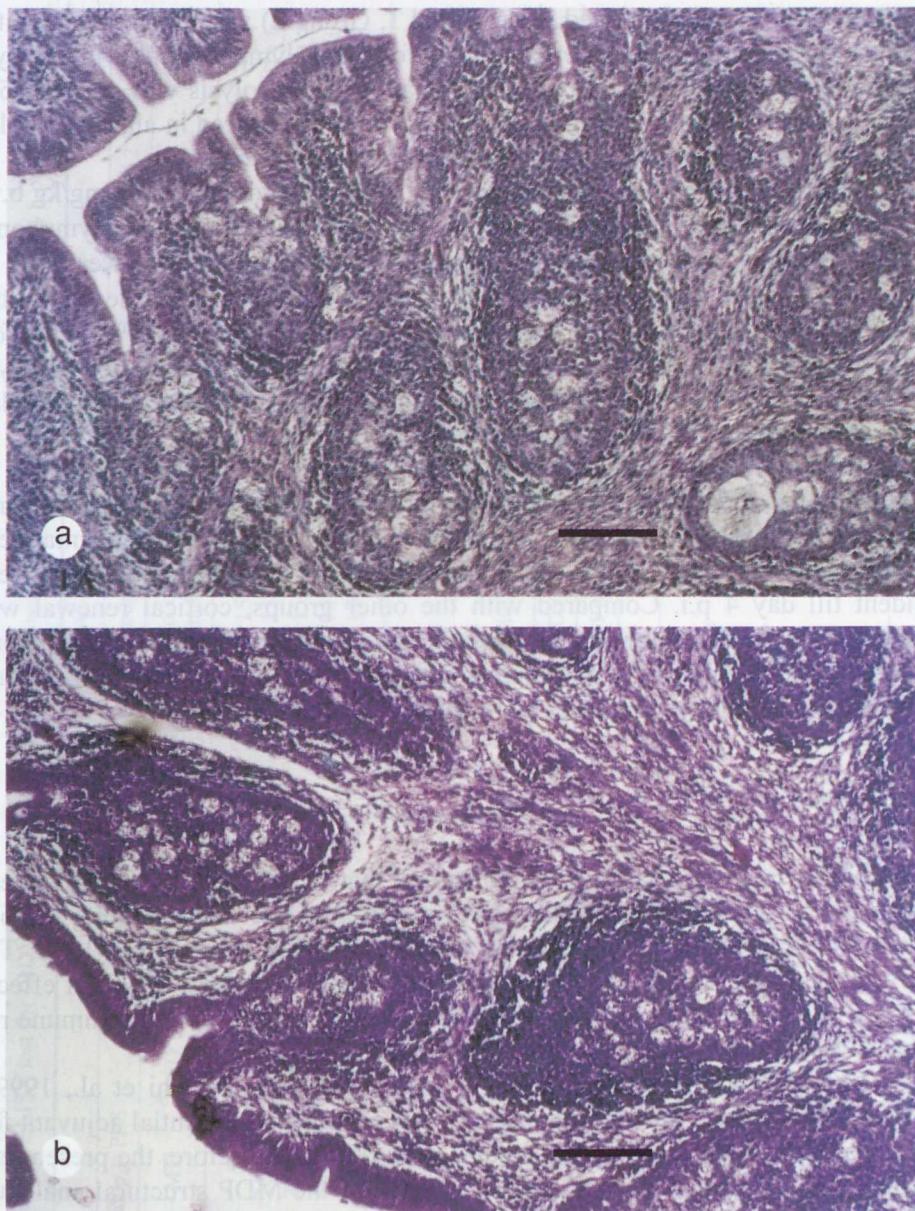


Fig. 1. Bursa of chickens immunized with the vaccine strain of IBDV. (a) Day 2 p.i.: focal lymphocytolysis, cortical and medullary lymphoid depletion with medullary cyst formations. Haematoxylin and eosin (HE). Bar = 100 μ m. (b) Day 7 p.i.: renewal of follicular cortex has commenced but some focal lymphocytolysis is still present. HE. Bar = 100 μ m

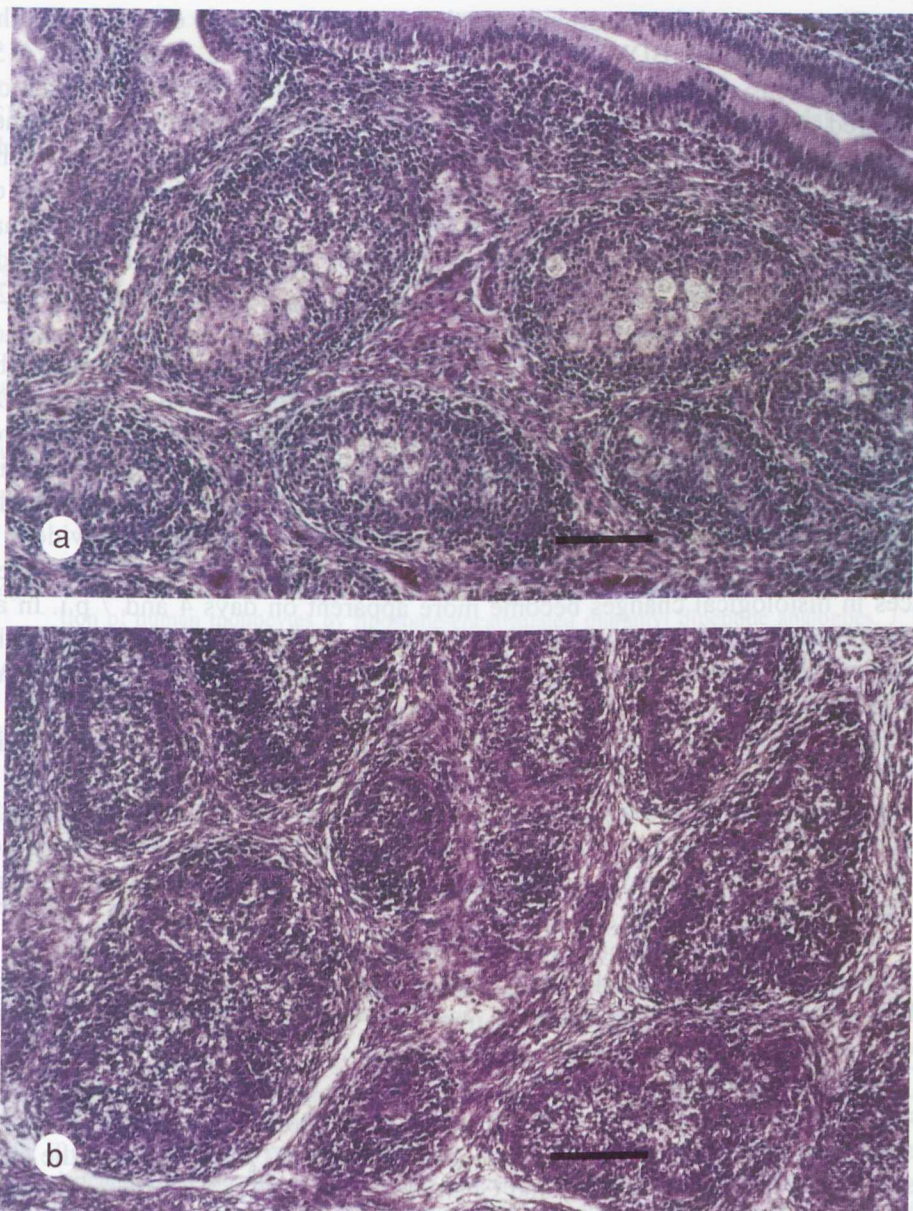


Fig. 2. Bursa of chickens immunized with the vaccine strain of IBDV and treated with LK415.

(a) Day 2 p.i.: lymphopenia and focal lymphocytolysis without cyst formation. HE.

Bar = 100 µm. (b) Day 7 p.i.: almost all bursal follicles are restored. HE. Bar = 100 µm

The results of this study indicate that LK415 is capable of enhancing the antibody response to IBDV. From Tables 1 and 2 it can be seen that LK415-treated chickens developed a higher antibody response to IBDV immunization compared to levamisole-treated and untreated groups. Significantly higher ELISA antibody titres to IBDV ($P < 0.01$) were detected in both trials on the days 14 and 28 p.i. Furthermore, the discrepancy observed in the third week of the first trial prompted the second trial. Since the above discrepancy is permanent, it is suggested that the influence of LK415 on the potentiation of antibody production is biphasic. The cause of the lower immune response seen on day 21 is not clear and needs further investigation.

The higher immune response observed on days 14 and 28 p.i. might be due to earlier renewal of the bursal tissue seen in chickens treated with the MDP analogue LK415. Histological examinations indicate that LK415 somehow protects bursal follicles against destruction and induces faster renewal of bursal tissue. The vaccine strain induced specific destruction of bursal follicles in all the immunized groups. On day 2 p.i. the destruction of follicles was less evident in the LK415-treated groups compared to the other immunized groups. The differences in histological changes become more apparent on days 4 and 7 p.i. In all groups treated with LK415 the renewal of bursal tissue started earlier than in the groups serving for comparison (Trial 1, Groups 1 and 2; Trial 2, Group 1). Although the negative changes of bursal tissue were expressed in a lower degree in the chickens treated with the higher dose of LK415 (Trial 1), there was no statistically significant difference in antibody response between the two LK415-treated groups. LK415 may induce the production of antibody that is not cytophilic or not cytotoxic to bursal follicle cells modified by the viral antigen, since interferon gamma is known to facilitate or prevent a specific antibody class switch (Paludan, 1998).

The fact that the immune response of IBDV-infected chickens is normal or even enhanced (Skeeles et al., 1979; Da Silva Martins et al., 1992) suggests that other lymphoid organs may have an important role in the development of immune response. This was not studied in this work.

Previous studies on the action of levamisole indicated unpredictability of its immunomodulatory activity, which can be stimulatory, inhibitory or not manifested (Payne and Howes, 1980; Singh and Dhawedkar, 1993). Like in the studies of Payne and Howes (1980) and Chenchev et al. (1981), no immunostimulatory effect of levamisole was observed in these experiments.

Commercial flocks receive a number of vaccines to protect them from environmental exposure to pathogens. Immunomodulation has several potential benefits: it could improve vaccine-induced immunity and reduce the negative impact of immunosuppression caused by extraneous agents or certain vaccine viruses. The present study indicates some potent immunostimulatory effects of the MDP analogue LK415 on the chicken immune system.

Acknowledgement

The authors thank the Ministry of Science and Technology of the Republic of Slovenia and Lek d.d. Pharmaceutical and Chemical Company for financial support of this work.

References

- Chenchev, I., Kostov, G., Cholakova, R. and Bonovska, M. (1981): Trials with levamisole to stimulate immunity against Newcastle disease in poultry. *Vet. Med. Nauki* **18**, 104–108.
- Da Silva Martins, N. R., Mockett, A. P. A. and Cook, J. K. A. (1992): The immunoglobulin M response in chicken serum to infectious bursal disease virus. *Avian Pathol.* **21**, 517–521.
- Ellouz, F., Adam, A., Ciobaru, R. and Lederer, E. (1974): Minimal requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem. Biophys. Res. Comm.* **59**, 1317–1325.
- Furuse, K. and Sakuma, A. (1989): Activation of the cytokine network by murectasin as a remedy for leukopenia and thrombopenia. *Arzneimittelforsch.* **39**, 915–917.
- LeClerc, C., Juy, D. Y., Bourgeois, E. and Chedid, L. (1979): *In vivo* regulation of humoral and cellular immune responses of mice by a synthetic adjuvant, N-acetyl-muramyl-L-alanyl-D-isoglutamine, muramyl dipeptide for MDP. *Cell. Immunol.* **45**, 199–206.
- Lopez-Bernstein, G. L., Mehta, K., Mehta, R., Juliana, R. L. and Hersh, E. M. (1983): The activation of human monocytes by liposome-encapsulated muramyl dipeptide analogues. *J. Immunol.* **130**, 1500–1502.
- Lowenthal, J. W., York, J. J., O'Neil, T. E., Steven, R. A., Strom, D. G. and Digby, M. R. (1998): Potential use of cytokine therapy in poultry. *Vet. Immunol. Immunopathol.* **63**, 191–198.
- Lukert, P. D. and Saif, Y. M. (1997): Infectious bursal disease. In: Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M. (eds) *Diseases of Poultry*. 10th edn, Mosby-Wolfe, London. pp. 648–663.
- Maheswaran, S. K., Dua, S. K. and Thies, E. S. (1979): Studies on *Pasteurella multocida* IX. Levamisole-induced augmentation of immune responses to a live fowl cholera vaccine. *Avian Dis.* **24**, 71–81.
- Neumann, U., Wischner, H. and Siegmann, O. (1982): Lack of immunostimulatory effect of N-acetyl-L-alanyl-D-isoglutamine on selected chicken immune functions. *Comp. Imm. Microbiol. Infect. Dis.* **5**, 413–421.
- Nunoya, T., Otaki, Y., Tajima, M., Hirage, M. and Saito, T. (1992): Occurrence of acute infectious bursal disease with high mortality in Japan and pathogenicity of field isolates in specific pathogen free chickens. *Avian Dis.* **36**, 597–609.
- Ochi, C., Norisada, N., Moriguchi, M., Štalc, A., Urleb, U. and Muraoka, S. (1999): Interleukin-10 inducing activity of a phtalimido desmuramyl dipeptide compound. *Arzneimittelforsch.* **49**, 72–79.
- Ozaki, T., Maeda, M. and Hayashi, H. (1989): Role of alveolar macrophages in neutrophil-dependent defense system against *Pseudomonas aeruginosa* infection in the lower respiratory tract. Amplifying effect of muramyl dipeptide analog. *Am. Rev. Respir. Dis.* **140**, 1595–1601.
- Payne, L. N. and Howes, K. (1980): Lack of beneficial effect of levamisole on Marek's disease. *Avian Pathol.* **9**, 525–529.
- Paludan, S. P. (1998): Interleukin-4 and interferon gamma: the quintessence of a mutual antagonistic relationship. *Scand. J. Immunol.* **48**, 459–468.

BOOK REVIEWS

Eberhard Grunert and Aart de Kruif (editors): **Fertilitätsstörungen beim weiblichen Rind (Fertility Disorders in Female Cattle)**. 3rd, revised and enlarged edition, Parey Buchverlag, Berlin, 1999. 430 pages with 446 figures (130 colour), 49 tables. ISBN 3-8263-3150-8.

This book, the first edition of which was published in 1982, is a comprehensive manual for people working in the field of reproduction. Many outstanding authors, mostly veterinarians, contributed to the new edition, edited by two internationally renowned professors of buiatrics. Aart de Kruif is the head of the Department of Reproduction, Faculty of Veterinary Medicine, Gent, and the president of the European Society for Domestic Animal Reproduction, the late Eberhard Grunert was full professor of the Veterinary High School in Hanover, and Honoris Causa professor of several other veterinary faculties.

This fully revised edition, with its 430 pages, 446 figures (of which 130 are colour pictures) and 49 tables, deals with the basic knowledge of female reproduction in cattle, including the newest scientific findings. The book has 21 chapters.

Chapter 1 is about the physiology of reproduction, sexual and breeding maturity, definition and course of the oestrous cycle in each part of the genital tract. A clear overview of the central and peripheral regulation of reproductive processes is also added.

Chapter 2 shows the state-of-the-art gynaecological examination beginning with the case history and including the general clinical investigation, the rectal and vaginal examination and additional diagnostic methods such as ultrasonography, microbiology, serology, vaginal impedance, histology and endoscopy, as well as their correct interpretation. Special focus is devoted to the clinicochemical background of fertility problems, heat detection and early pregnancy diagnosis.

Chapter 3 shows the development of the sexual organs and their disturbances such as intersexuality, and the causes of developmental abnormalities accompanied by dysfunction. This part of the book deals with failures like aplasia and hypoplasia of the ovaries and inappropriate pituitary control. Delayed ovulation, follicular atresia, follicular and lutein cysts as well as luteal dysfunction are also mentioned from the casuistic, diagnostic and therapeutic point of view. Inappropriate luteal activity (insufficiency) and neoplasia of the ovary are frequently found in cases of reproductive failure, especially persistent corpus luteum, the successful treatment of which is absolutely necessary for recovery from chronic endometritis. In humans, periovarial adhesions play an outstanding role in female infertility; from this book we get acquainted with analogous diseases of the bovine species (oophoritis, adhesions and phlegmones).

Chapter 5 deals with irregularities of the oestrous cycle and the disturbances of heat. Lack of cycling and silent heat are known to be the most important causes of extended calving interval. This part of the book deals with them along with irregular (long or short) cycle length or abnormal duration of heat. The following chapter is devoted to

the general physiological and pharmacological background of hormone therapy followed by a detailed list of relevant hormones ranging from pituitary FSH and LH (and their pharmacological substituents, PMSG and hCG) to ovarian steroids such as oestrogens and gestagens. In addition to these hormones the key reproductive regulator prostaglandins are also discussed, including their chemical composition, mechanism of action, indication, side effects and metabolism.

Chapter 7 contains a list of oviductal abnormalities, periovarial adhesions, salpingitis, pyo- and hydrosalpinx, their symptoms, diagnosis and therapy. It is followed by a chapter providing detailed information on the pathogenesis, differential diagnosis and treatment of endometritis. Chronic endometritis is classified according to Richter, and endometritis cases of Grades I–II and Grades III–IV are discussed separately, based on their different treatment and prognosis. Peri- and parametritis, mucometra, haemometra and pneumometra are also mentioned here briefly, along with tumours and leukosis. The presentation of diseases of the vestibulum and vulva starts with the description of vaginitis and vaginal prolapse because their incidence is higher than that of urovagina, vestibulitis, vaginal neoplasms, cysts and injuries.

Chapter 11 deals with contagious diseases of the genital tract such as IBR-IPV, vibriosis (campylobacteriosis), trichomonosis and genital catarrh, their pathogenesis, symptoms, diagnosis and treatment, while sporadic diseases like tuberculosis, brucellosis, mycoplasmosis, chlamydiosis, *Haemophilus somnus* and *Coxiella burnetii* are discussed later.

The next part introduces readers to the pathology of pregnancy including embryonic mortality, developmental failures of spermatozoa or ova, mentioning briefly oviductal, uterine and luteal dysfunctions and climatic, housing and nutritional factors as well. This chapter is followed by a short description of non-contagious abortion and a detailed presentation of contagious abortion (brucellosis, salmonellosis, leptospirosis, listeriosis, IBR-IPV, trichomonosis, BVD-MD, etc.). The veterinary measures associated with abortion and the induction of abortion are also discussed here.

Chapter 14 is short but discusses very important factors such as improper timing and technical failures of artificial insemination as well as quality problems of processed semen or failures of the dam. This part leads us to the next two chapters about hereditary causes of infertility and alimentary, seasonal and housing influences, with special regard to oestrus detection.

In the developed countries much attention will be paid to herd health and reproductive management. Methods for organisation and supervision of reproduction and for the control of the oestrous cycle, along with their cost/benefit calculations, are discussed in Chapters 17 and 18.

The next part of the book introduces readers to the theoretical and technical background and application of bovine embryo transfer, which has undergone rapid development in the last two decades and greatly contributes to the genetic progress seen in the cattle industry. Second-generation assisted reproductive technologies like cryopreservation of embryos, *in vitro* fertilisation and culture, as well as cloning and transgenesis are also discussed here.

The last two chapters of the book deal with gynaecological operations like perineal rupture, reconstructive surgery of the vulva and vagina, unilateral ovariectomy, as

well as forensic veterinary aspects of reproduction, with special attention to the consequences of misdiagnosis and treatment failures.

In conclusion, the book is clearly structured, well designed and offers a good aid for those who dedicated themselves to animal reproduction, an interesting but rather complicated field of the veterinary medicine. Containing more information than undergraduates would need, this handbook can be recommended not only for students but also for veterinarians having an advanced knowledge of reproduction.

László Solti

Jean-Pierre Vaillancourt and Bernard Toma (editors): Dictionary of Veterinary Epidemiology. Iowa State University Press, Ames, 1999. 300 pages, illus., paperback. \$69.95 (£51). ISBN 0-8138-2639-X.

This dictionary, prepared with the support of the Office International des Epizooties (Paris), comprises the first English-language source of definitions of terminology used worldwide in the study and practice of veterinary epidemiology. Based on the translation of the *Glossaire d'épidémiologie animal* (1991) and expanded to include North American terminology, *Dictionary of Veterinary Epidemiology* provides valuable information regarding transport, wellness, and medical treatment of animals as well as control of the spread of diseases within and among animal populations. It includes a vast terminology and is liberally illustrated with figures and tables. It provides a comprehensive A-to-Z listing of terms used in reference to veterinary epidemiology.

Dictionary of Veterinary Epidemiology contains:

- Formal, commonly accepted definitions of words and expressions used in reference to epidemiologic principles and methods by veterinarians and other animal health professionals in North America and Europe.
- Nomenclature of economics and biostatistics, two disciplines highly relevant to epidemiology and animal health.
- Comments and/or examples, synonyms, and cross-reference(s) to related terms in the book that accompany each definition.
- A list of principle sources used to compile the dictionary, which provides a source of recommended reading for additional information about specific topics.

In addition to the translators, the text has nine editors, two associate editors, and 55 contributors, veterinary medicine experts, from the United States, Canada, Belgium, and the United Kingdom.

Veterinary medicine students and practitioners, animal scientists, government employees involved in animal regulation worldwide, and veterinary epidemiologists will find this book to be a valuable resource.

NOTES FOR CONTRIBUTORS

Acta Veterinaria Hungarica publishes original research papers, review articles and short communications on veterinary science and medicine. Submissions are accepted on the understanding that they have not been published or submitted for publication elsewhere and that their use is subject to peer review. The covering letter of submitted manuscripts should be signed by all co-authors, or a declaration of transfer from the co-authors should be enclosed. Papers accepted for publication by the Editorial Board are subject to editorial revision. A copy of the Publishing Agreement will be sent to the authors of papers accepted for publication. Manuscripts will be processed only after receiving the signed copy of the agreement.

Format. Manuscripts must be in English and clearly and concisely written. They should be typed double spaced with a 40-mm left-hand margin. Three copies of the manuscript should be submitted with two sets of any illustrations. For general papers, the whole length should not exceed 100 pages (that is, about 10–11 printed pages of *Acta Veterinaria Hungarica*). Short communications should not exceed 1000 words and should include only one or two figures or tables. Concise papers will be given priority during the production process.

Manuscripts on disk. After acceptance, manuscripts should be submitted in electronic version (Word for Windows), on a 3.5" disk or as an e-mail attachment, together with electronic versions of the figures.

Title. The title should be a clear and concise statement of the subject, no less than 24 words (about 100 characters). A short running title of not more than 30 characters should also be provided. This is followed by the authors' initials (full first name of veteran) and surname, and the name of the institution where the work was done. The mailing address, e-mail address and fax number of the corresponding author must also be given in a footnote to the first page.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Key words (if any) should also be supplied.

Introduction. This part should state briefly the nature and purpose of the study and acknowledge any work by others.

Materials and methods. Describe materials, methods, apparatus, experimental conditions and technical methods in sufficient detail to allow other authors to reproduce the results. The methods used must be stated. The experimental methods and treatments applied shall comply with the requirements of the European Union Directive on the treatment and care of animals.

Results. Experimental data should be presented clearly and concisely, in a straightforward way.

Discussion should be focused on the interpretation of experimental findings.

Acknowledgement of grants and technical help.

References. In the text references should be given as follows: (Hagen 1988), (Hagen et al. 1988), (Hagen 1988a), (Hagen et al. 1988b), (Hagen 1988c), (Hagen 1988d), (Hagen 1988e), (Hagen 1988f), (Hagen 1988g), (Hagen 1988h), (Hagen 1988i), (Hagen 1988j), (Hagen 1988k), (Hagen 1988l), (Hagen 1988m), (Hagen 1988n), (Hagen 1988o), (Hagen 1988p), (Hagen 1988q), (Hagen 1988r), (Hagen 1988s), (Hagen 1988t), (Hagen 1988u), (Hagen 1988v), (Hagen 1988w), (Hagen 1988x), (Hagen 1988y), (Hagen 1988z), (Hagen 1989a), (Hagen 1989b), (Hagen 1989c), (Hagen 1989d), (Hagen 1989e), (Hagen 1989f), (Hagen 1989g), (Hagen 1989h), (Hagen 1989i), (Hagen 1989j), (Hagen 1989k), (Hagen 1989l), (Hagen 1989m), (Hagen 1989n), (Hagen 1989o), (Hagen 1989p), (Hagen 1989q), (Hagen 1989r), (Hagen 1989s), (Hagen 1989t), (Hagen 1989u), (Hagen 1989v), (Hagen 1989w), (Hagen 1989x), (Hagen 1989y), (Hagen 1989z), (Hagen 1990a), (Hagen 1990b), (Hagen 1990c), (Hagen 1990d), (Hagen 1990e), (Hagen 1990f), (Hagen 1990g), (Hagen 1990h), (Hagen 1990i), (Hagen 1990j), (Hagen 1990k), (Hagen 1990l), (Hagen 1990m), (Hagen 1990n), (Hagen 1990o), (Hagen 1990p), (Hagen 1990q), (Hagen 1990r), (Hagen 1990s), (Hagen 1990t), (Hagen 1990u), (Hagen 1990v), (Hagen 1990w), (Hagen 1990x), (Hagen 1990y), (Hagen 1990z), (Hagen 1991a), (Hagen 1991b), (Hagen 1991c), (Hagen 1991d), (Hagen 1991e), (Hagen 1991f), (Hagen 1991g), (Hagen 1991h), (Hagen 1991i), (Hagen 1991j), (Hagen 1991k), (Hagen 1991l), (Hagen 1991m), (Hagen 1991n), (Hagen 1991o), (Hagen 1991p), (Hagen 1991q), (Hagen 1991r), (Hagen 1991s), (Hagen 1991t), (Hagen 1991u), (Hagen 1991v), (Hagen 1991w), (Hagen 1991x), (Hagen 1991y), (Hagen 1991z), (Hagen 1992a), (Hagen 1992b), (Hagen 1992c), (Hagen 1992d), (Hagen 1992e), (Hagen 1992f), (Hagen 1992g), (Hagen 1992h), (Hagen 1992i), (Hagen 1992j), (Hagen 1992k), (Hagen 1992l), (Hagen 1992m), (Hagen 1992n), (Hagen 1992o), (Hagen 1992p), (Hagen 1992q), (Hagen 1992r), (Hagen 1992s), (Hagen 1992t), (Hagen 1992u), (Hagen 1992v), (Hagen 1992w), (Hagen 1992x), (Hagen 1992y), (Hagen 1992z), (Hagen 1993a), (Hagen 1993b), (Hagen 1993c), (Hagen 1993d), (Hagen 1993e), (Hagen 1993f), (Hagen 1993g), (Hagen 1993h), (Hagen 1993i), (Hagen 1993j), (Hagen 1993k), (Hagen 1993l), (Hagen 1993m), (Hagen 1993n), (Hagen 1993o), (Hagen 1993p), (Hagen 1993q), (Hagen 1993r), (Hagen 1993s), (Hagen 1993t), (Hagen 1993u), (Hagen 1993v), (Hagen 1993w), (Hagen 1993x), (Hagen 1993y), (Hagen 1993z), (Hagen 1994a), (Hagen 1994b), (Hagen 1994c), (Hagen 1994d), (Hagen 1994e), (Hagen 1994f), (Hagen 1994g), (Hagen 1994h), (Hagen 1994i), (Hagen 1994j), (Hagen 1994k), (Hagen 1994l), (Hagen 1994m), (Hagen 1994n), (Hagen 1994o), (Hagen 1994p), (Hagen 1994q), (Hagen 1994r), (Hagen 1994s), (Hagen 1994t), (Hagen 1994u), (Hagen 1994v), (Hagen 1994w), (Hagen 1994x), (Hagen 1994y), (Hagen 1994z), (Hagen 1995a), (Hagen 1995b), (Hagen 1995c), (Hagen 1995d), (Hagen 1995e), (Hagen 1995f), (Hagen 1995g), (Hagen 1995h), (Hagen 1995i), (Hagen 1995j), (Hagen 1995k), (Hagen 1995l), (Hagen 1995m), (Hagen 1995n), (Hagen 1995o), (Hagen 1995p), (Hagen 1995q), (Hagen 1995r), (Hagen 1995s), (Hagen 1995t), (Hagen 1995u), (Hagen 1995v), (Hagen 1995w), (Hagen 1995x), (Hagen 1995y), (Hagen 1995z), (Hagen 1996a), (Hagen 1996b), (Hagen 1996c), (Hagen 1996d), (Hagen 1996e), (Hagen 1996f), (Hagen 1996g), (Hagen 1996h), (Hagen 1996i), (Hagen 1996j), (Hagen 1996k), (Hagen 1996l), (Hagen 1996m), (Hagen 1996n), (Hagen 1996o), (Hagen 1996p), (Hagen 1996q), (Hagen 1996r), (Hagen 1996s), (Hagen 1996t), (Hagen 1996u), (Hagen 1996v), (Hagen 1996w), (Hagen 1996x), (Hagen 1996y), (Hagen 1996z), (Hagen 1997a), (Hagen 1997b), (Hagen 1997c), (Hagen 1997d), (Hagen 1997e), (Hagen 1997f), (Hagen 1997g), (Hagen 1997h), (Hagen 1997i), (Hagen 1997j), (Hagen 1997k), (Hagen 1997l), (Hagen 1997m), (Hagen 1997n), (Hagen 1997o), (Hagen 1997p), (Hagen 1997q), (Hagen 1997r), (Hagen 1997s), (Hagen 1997t), (Hagen 1997u), (Hagen 1997v), (Hagen 1997w), (Hagen 1997x), (Hagen 1997y), (Hagen 1997z), (Hagen 1998a), (Hagen 1998b), (Hagen 1998c), (Hagen 1998d), (Hagen 1998e), (Hagen 1998f), (Hagen 1998g), (Hagen 1998h), (Hagen 1998i), (Hagen 1998j), (Hagen 1998k), (Hagen 1998l), (Hagen 1998m), (Hagen 1998n), (Hagen 1998o), (Hagen 1998p), (Hagen 1998q), (Hagen 1998r), (Hagen 1998s), (Hagen 1998t), (Hagen 1998u), (Hagen 1998v), (Hagen 1998w), (Hagen 1998x), (Hagen 1998y), (Hagen 1998z), (Hagen 1999a), (Hagen 1999b), (Hagen 1999c), (Hagen 1999d), (Hagen 1999e), (Hagen 1999f), (Hagen 1999g), (Hagen 1999h), (Hagen 1999i), (Hagen 1999j), (Hagen 1999k), (Hagen 1999l), (Hagen 1999m), (Hagen 1999n), (Hagen 1999o), (Hagen 1999p), (Hagen 1999q), (Hagen 1999r), (Hagen 1999s), (Hagen 1999t), (Hagen 1999u), (Hagen 1999v), (Hagen 1999w), (Hagen 1999x), (Hagen 1999y), (Hagen 1999z), (Hagen 2000a), (Hagen 2000b), (Hagen 2000c), (Hagen 2000d), (Hagen 2000e), (Hagen 2000f), (Hagen 2000g), (Hagen 2000h), (Hagen 2000i), (Hagen 2000j), (Hagen 2000k), (Hagen 2000l), (Hagen 2000m), (Hagen 2000n), (Hagen 2000o), (Hagen 2000p), (Hagen 2000q), (Hagen 2000r), (Hagen 2000s), (Hagen 2000t), (Hagen 2000u), (Hagen 2000v), (Hagen 2000w), (Hagen 2000x), (Hagen 2000y), (Hagen 2000z), (Hagen 2001a), (Hagen 2001b), (Hagen 2001c), (Hagen 2001d), (Hagen 2001e), (Hagen 2001f), (Hagen 2001g), (Hagen 2001h), (Hagen 2001i), (Hagen 2001j), (Hagen 2001k), (Hagen 2001l), (Hagen 2001m), (Hagen 2001n), (Hagen 2001o), (Hagen 2001p), (Hagen 2001q), (Hagen 2001r), (Hagen 2001s), (Hagen 2001t), (Hagen 2001u), (Hagen 2001v), (Hagen 2001w), (Hagen 2001x), (Hagen 2001y), (Hagen 2001z), (Hagen 2002a), (Hagen 2002b), (Hagen 2002c), (Hagen 2002d), (Hagen 2002e), (Hagen 2002f), (Hagen 2002g), (Hagen 2002h), (Hagen 2002i), (Hagen 2002j), (Hagen 2002k), (Hagen 2002l), (Hagen 2002m), (Hagen 2002n), (Hagen 2002o), (Hagen 2002p), (Hagen 2002q), (Hagen 2002r), (Hagen 2002s), (Hagen 2002t), (Hagen 2002u), (Hagen 2002v), (Hagen 2002w), (Hagen 2002x), (Hagen 2002y), (Hagen 2002z), (Hagen 2003a), (Hagen 2003b), (Hagen 2003c), (Hagen 2003d), (Hagen 2003e), (Hagen 2003f), (Hagen 2003g), (Hagen 2003h), (Hagen 2003i), (Hagen 2003j), (Hagen 2003k), (Hagen 2003l), (Hagen 2003m), (Hagen 2003n), (Hagen 2003o), (Hagen 2003p), (Hagen 2003q), (Hagen 2003r), (Hagen 2003s), (Hagen 2003t), (Hagen 2003u), (Hagen 2003v), (Hagen 2003w), (Hagen 2003x), (Hagen 2003y), (Hagen 2003z), (Hagen 2004a), (Hagen 2004b), (Hagen 2004c), (Hagen 2004d), (Hagen 2004e), (Hagen 2004f), (Hagen 2004g), (Hagen 2004h), (Hagen 2004i), (Hagen 2004j), (Hagen 2004k), (Hagen 2004l), (Hagen 2004m), (Hagen 2004n), (Hagen 2004o), (Hagen 2004p), (Hagen 2004q), (Hagen 2004r), (Hagen 2004s), (Hagen 2004t), (Hagen 2004u), (Hagen 2004v), (Hagen 2004w), (Hagen 2004x), (Hagen 2004y), (Hagen 2004z), (Hagen 2005a), (Hagen 2005b), (Hagen 2005c), (Hagen 2005d), (Hagen 2005e), (Hagen 2005f), (Hagen 2005g), (Hagen 2005h), (Hagen 2005i), (Hagen 2005j), (Hagen 2005k), (Hagen 2005l), (Hagen 2005m), (Hagen 2005n), (Hagen 2005o), (Hagen 2005p), (Hagen 2005q), (Hagen 2005r), (Hagen 2005s), (Hagen 2005t), (Hagen 2005u), (Hagen 2005v), (Hagen 2005w), (Hagen 2005x), (Hagen 2005y), (Hagen 2005z), (Hagen 2006a), (Hagen 2006b), (Hagen 2006c), (Hagen 2006d), (Hagen 2006e), (Hagen 2006f), (Hagen 2006g), (Hagen 2006h), (Hagen 2006i), (Hagen 2006j), (Hagen 2006k), (Hagen 2006l), (Hagen 2006m), (Hagen 2006n), (Hagen 2006o), (Hagen 2006p), (Hagen 2006q), (Hagen 2006r), (Hagen 2006s), (Hagen 2006t), (Hagen 2006u), (Hagen 2006v), (Hagen 2006w), (Hagen 2006x), (Hagen 2006y), (Hagen 2006z), (Hagen 2007a), (Hagen 2007b), (Hagen 2007c), (Hagen 2007d), (Hagen 2007e), (Hagen 2007f), (Hagen 2007g), (Hagen 2007h), (Hagen 2007i), (Hagen 2007j), (Hagen 2007k), (Hagen 2007l), (Hagen 2007m), (Hagen 2007n), (Hagen 2007o), (Hagen 2007p), (Hagen 2007q), (Hagen 2007r), (Hagen 2007s), (Hagen 2007t), (Hagen 2007u), (Hagen 2007v), (Hagen 2007w), (Hagen 2007x), (Hagen 2007y), (Hagen 2007z), (Hagen 2008a), (Hagen 2008b), (Hagen 2008c), (Hagen 2008d), (Hagen 2008e), (Hagen 2008f), (Hagen 2008g), (Hagen 2008h), (Hagen 2008i), (Hagen 2008j), (Hagen 2008k), (Hagen 2008l), (Hagen 2008m), (Hagen 2008n), (Hagen 2008o), (Hagen 2008p), (Hagen 2008q), (Hagen 2008r), (Hagen 2008s), (Hagen 2008t), (Hagen 2008u), (Hagen 2008v), (Hagen 2008w), (Hagen 2008x), (Hagen 2008y), (Hagen 2008z), (Hagen 2009a), (Hagen 2009b), (Hagen 2009c), (Hagen 2009d), (Hagen 2009e), (Hagen 2009f), (Hagen 2009g), (Hagen 2009h), (Hagen 2009i), (Hagen 2009j), (Hagen 2009k), (Hagen 2009l), (Hagen 2009m), (Hagen 2009n), (Hagen 2009o), (Hagen 2009p), (Hagen 2009q), (Hagen 2009r), (Hagen 2009s), (Hagen 2009t), (Hagen 2009u), (Hagen 2009v), (Hagen 2009w), (Hagen 2009x), (Hagen 2009y), (Hagen 2009z), (Hagen 2010a), (Hagen 2010b), (Hagen 2010c), (Hagen 2010d), (Hagen 2010e), (Hagen 2010f), (Hagen 2010g), (Hagen 2010h), (Hagen 2010i), (Hagen 2010j), (Hagen 2010k), (Hagen 2010l), (Hagen 2010m), (Hagen 2010n), (Hagen 2010o), (Hagen 2010p), (Hagen 2010q), (Hagen 2010r), (Hagen 2010s), (Hagen 2010t), (Hagen 2010u), (Hagen 2010v), (Hagen 2010w), (Hagen 2010x), (Hagen 2010y), (Hagen 2010z), (Hagen 2011a), (Hagen 2011b), (Hagen 2011c), (Hagen 2011d), (Hagen 2011e), (Hagen 2011f), (Hagen 2011g), (Hagen 2011h), (Hagen 2011i), (Hagen 2011j), (Hagen 2011k), (Hagen 2011l), (Hagen 2011m), (Hagen 2011n), (Hagen 2011o), (Hagen 2011p), (Hagen 2011q), (Hagen 2011r), (Hagen 2011s), (Hagen 2011t), (Hagen 2011u), (Hagen 2011v), (Hagen 2011w), (Hagen 2011x), (Hagen 2011y), (Hagen 2011z), (Hagen 2012a), (Hagen 2012b), (Hagen 2012c), (Hagen 2012d), (Hagen 2012e), (Hagen 2012f), (Hagen 2012g), (Hagen 2012h), (Hagen 2012i), (Hagen 2012j), (Hagen 2012k), (Hagen 2012l), (Hagen 2012m), (Hagen 2012n), (Hagen 2012o), (Hagen 2012p), (Hagen 2012q), (Hagen 2012r), (Hagen 2012s), (Hagen 2012t), (Hagen 2012u), (Hagen 2012v), (Hagen 2012w), (Hagen 2012x), (Hagen 2012y), (Hagen 2012z), (Hagen 2013a), (Hagen 2013b), (Hagen 2013c), (Hagen 2013d), (Hagen 2013e), (Hagen 2013f), (Hagen 2013g), (Hagen 2013h), (Hagen 2013i), (Hagen 2013j), (Hagen 2013k), (Hagen 2013l), (Hagen 2013m), (Hagen 2013n), (Hagen 2013o), (Hagen 2013p), (Hagen 2013q), (Hagen 2013r), (Hagen 2013s), (Hagen 2013t), (Hagen 2013u), (Hagen 2013v), (Hagen 2013w), (Hagen 2013x), (Hagen 2013y), (Hagen 2013z), (Hagen 2014a), (Hagen 2014b), (Hagen 2014c), (Hagen 2014d), (Hagen 2014e), (Hagen 2014f), (Hagen 2014g), (Hagen 2014h), (Hagen 2014i), (Hagen 2014j), (Hagen 2014k), (Hagen 2014l), (Hagen 2014m), (Hagen 2014n), (Hagen 2014o), (Hagen 2014p), (Hagen 2014q), (Hagen 2014r), (Hagen 2014s), (Hagen 2014t), (Hagen 2014u), (Hagen 2014v), (Hagen 2014w), (Hagen 2014x), (Hagen 2014y), (Hagen 2014z), (Hagen 2015a), (Hagen 2015b), (Hagen 2015c), (Hagen 2015d), (Hagen 2015e), (Hagen 2015f), (Hagen 2015g), (Hagen 2015h), (Hagen 2015i), (Hagen 2015j), (Hagen 2015k), (Hagen 2015l), (Hagen 2015m), (Hagen 2015n), (Hagen 2015o), (Hagen 2015p), (Hagen 2015q), (Hagen 2015r), (Hagen 2015s), (Hagen 2015t), (Hagen 2015u), (Hagen 2015v), (Hagen 2015w), (Hagen 2015x), (Hagen 2015y), (Hagen 2015z), (Hagen 2016a), (Hagen 2016b), (Hagen 2016c), (Hagen 2016d), (Hagen 2016e), (Hagen 2016f), (Hagen 2016g), (Hagen 2016h), (Hagen 2016i), (Hagen 2016j), (Hagen 2016k), (Hagen 2016l), (Hagen 2016m), (Hagen 2016n), (Hagen 2016o), (Hagen 2016p), (Hagen 2016q), (Hagen 2016r), (Hagen 2016s), (Hagen 2016t), (Hagen 2016u), (Hagen 2016v), (Hagen 2016w), (Hagen 2016x), (Hagen 2016y), (Hagen 2016z), (Hagen 2017a), (Hagen 2017b), (Hagen 2017c), (Hagen 2017d), (Hagen 2017e), (Hagen 2017f), (Hagen 2017g), (Hagen 2017h), (Hagen 2017i), (Hagen 2017j), (Hagen 2017k), (Hagen 2017l), (Hagen 2017m), (Hagen 2017n), (Hagen 2017o), (Hagen 2017p), (Hagen 2017q), (Hagen 2017r), (Hagen 2017s), (Hagen 2017t), (Hagen 2017u), (Hagen 2017v), (Hagen 2017w), (Hagen 2017x), (Hagen 2017y), (Hagen 2017z), (Hagen 2018a), (Hagen 2018b), (Hagen 2018c), (Hagen 2018d), (Hagen 2018e), (Hagen 2018f), (Hagen 2018g), (Hagen 2018h), (Hagen 2018i), (Hagen 2018j), (Hagen 2018k), (Hagen 2018l), (Hagen 2018m), (Hagen 2018n), (Hagen 2018o), (Hagen 2018p), (Hagen 2018q), (Hagen 2018r), (Hagen 2018s), (Hagen 2018t), (Hagen 2018u), (Hagen 2018v), (Hagen 2018w), (Hagen 2018x), (Hagen 2018y), (Hagen 2018z), (Hagen 2019a), (Hagen 2019b), (Hagen 2019c), (Hagen 2019d), (Hagen 2019e), (Hagen 2019f), (Hagen 2019g), (Hagen 2019h), (Hagen 2019i), (Hagen 2019j), (Hagen 2019k), (Hagen 2019l), (Hagen 2019m), (Hagen 2019n), (Hagen 2019o), (Hagen 2019p), (Hagen 2019q), (Hagen 2019r), (Hagen 2019s), (Hagen 2019t), (Hagen 2019u), (Hagen 2019v), (Hagen 2019w), (Hagen 2019x), (Hagen 2019y), (Hagen 2019z), (Hagen 2020a), (Hagen 2020b), (Hagen 2020c), (Hagen 2020d), (Hagen 2020e), (Hagen 2020f), (Hagen 2020g), (Hagen 2020h), (Hagen 2020i), (Hagen 2020j), (Hagen 2020k), (Hagen 2020l), (Hagen 2020m), (Hagen 2020n), (Hagen 2020o), (Hagen 2020p), (Hagen 2020q), (Hagen 2020r), (Hagen 2020s), (Hagen 2020t), (Hagen 2020u), (Hagen 2020v), (Hagen 2020w), (Hagen 2020x), (Hagen 2020y), (Hagen 2020z), (Hagen 2021a), (Hagen 2021b), (Hagen 2021c), (Hagen 2021d), (Hagen 2021e), (Hagen 2021f), (Hagen 2021g), (Hagen 2021h), (Hagen 2021i), (Hagen 2021j), (Hagen 2021k), (Hagen 2021l), (Hagen 2021m), (Hagen 2021n), (Hagen 2021o), (Hagen 2021p), (Hagen 2021q), (Hagen 2021r), (Hagen 2021s), (Hagen 2021t), (Hagen 2021u), (Hagen 2021v), (Hagen 2021w), (Hagen 2021x), (Hagen 2021y), (Hagen 2021z), (Hagen 2022a), (Hagen 2022b), (Hagen 2022c), (Hagen 2022d), (Hagen 2022e), (Hagen 2022f), (Hagen 2022g), (Hagen 2022h), (Hagen 2022i), (Hagen 2022j), (Hagen 2022k), (Hagen 2022l), (Hagen 2022m), (Hagen 2022n), (Hagen 2022o), (Hagen 2022p), (Hagen 2022q), (Hagen 2022r), (Hagen 2022s), (Hagen 2022t), (Hagen 2022u), (Hagen 2022v), (Hagen 2022w), (Hagen 2022x), (Hagen 2022y), (Hagen 2022z), (Hagen 2023a), (Hagen 2023b), (Hagen 2023c), (Hagen 2023d), (Hagen 2023e), (Hagen 2023f), (Hagen 2023g), (Hagen 2023h), (Hagen 2023i), (Hagen 2023j), (Hagen 2023k), (Hagen 2023l), (Hagen 2023m), (Hagen 2023n), (Hagen 2023o), (Hagen 2023p), (Hagen 2023q), (Hagen 2023r), (Hagen 2023s), (Hagen 2023t), (Hagen 2023u), (Hagen 2023v), (Hagen 2023w), (Hagen 2023x), (Hagen 2023y), (Hagen 2023z), (Hagen 2024a), (Hagen 2024b), (Hagen 2024c), (Hagen 2024d), (Hagen 2024e), (Hagen 2024f), (Hagen 2024g), (Hagen 2024h), (Hagen 2024i), (Hagen 2024j), (Hagen 2024k), (Hagen 2024l), (Hagen 2024m), (Hagen 2024n), (Hagen 2024o), (Hagen 2024p), (Hagen 2024q), (Hagen 2024r), (Hagen 2024s), (Hagen 2024t), (Hagen 2024u), (Hagen 2024v), (Hagen 2024w), (Hagen 2024x), (Hagen 2024y), (Hagen 2024z), (Hagen 2025a), (Hagen 2025b), (Hagen 2025c), (Hagen 2025d), (Hagen 2025e), (Hagen 2025f), (Hagen 2025g), (Hagen 2025h), (Hagen 2025i), (Hagen 2025j), (Hagen 2025k), (Hagen 2025l), (Hagen 2025m), (Hagen 2025n), (Hagen 2025o), (Hagen 2025p), (Hagen 2025q), (Hagen 2025r), (Hagen 2025s), (Hagen 2025t), (Hagen 2025u), (Hagen 2025v), (Hagen 2025w), (Hagen 2025x), (Hagen 2025y), (Hagen 2025z), (Hagen 2026a), (Hagen 2026b), (Hagen 2026c), (Hagen 2026d), (Hagen 2026e), (Hagen 2026f), (Hagen 2026g), (Hagen 2026h), (Hagen 2026i), (Hagen 2026j), (Hagen 2026k), (Hagen 2026l), (Hagen 2026m), (Hagen 2026n), (Hagen 2026o), (Hagen 2026p), (Hagen 2026q), (Hagen 2026r), (Hagen 2026s), (Hagen 2026t), (Hagen 2026u), (Hagen 2026v), (Hagen 2026w), (Hagen 2026x), (Hagen 2026y), (Hagen 2026z), (Hagen 2027a), (Hagen 2027b), (Hagen 2027c), (Hagen 2027d), (Hagen 2027e), (Hagen 2027f), (Hagen 2027g), (Hagen 2027h), (Hagen 2027i), (Hagen 2027j), (Hagen 2027k), (Hagen 2027l), (Hagen 2027m), (Hagen 2027n), (Hagen 2027o), (Hagen 2027p), (Hagen 2027q), (Hagen 2027r), (Hagen 2027s), (Hagen 2027t), (Hagen 2027u), (Hagen 2027v), (Hagen 2027w), (Hagen 2027x), (Hagen 2027y), (Hagen 2027z), (Hagen 2028a), (Hagen 2028b), (Hagen 2028c), (Hagen 2028d), (Hagen 2028e), (Hagen 2028f), (Hagen 2028g), (Hagen 2028h), (Hagen 2028i), (Hagen 2028j), (Hagen 2028k), (Hagen 2028l), (Hagen 2028m), (Hagen 2028n), (Hagen 2028o), (Hagen 2028p), (Hagen 2028q), (Hagen 2028r), (Hagen 2028s), (Hagen 2028t), (Hagen 2028u), (Hagen 2028v), (Hagen 2028w), (Hagen 2028x), (Hagen 2028y), (Hagen 2028z), (Hagen 2029a), (Hagen 2029b), (Hagen 2029c), (Hagen 2029d), (Hagen 2029e), (Hagen 2029f), (Hagen 2029g), (Hagen 2029h), (Hagen 2029i), (Hagen 2029j), (Hagen 2029k), (Hagen 2029l), (Hagen 2029m), (Hagen 2029n), (Hagen 2029o), (Hagen 2029p), (Hagen 2029q), (Hagen 2029r), (Hagen 2029s), (Hagen 2029t), (Hagen 2029u), (Hagen 2029v), (Hagen 2029w), (Hagen 2029x), (Hagen 2029y), (Hagen 2029z), (Hagen 2030a), (Hagen 2030b), (Hagen 2030c), (Hagen 2030d), (Hagen 2030e), (Hagen 2030f), (Hagen 2030g), (Hagen 2030h), (Hagen 2030i), (Hagen 2030j), (Hagen 2030k), (Hagen 2030l), (Hagen 2030m), (Hagen 2030n), (Hagen 2030o), (Hagen 2030p), (Hagen 2030q), (Hagen 2030r), (Hagen 2030s), (Hagen 2030t), (Hagen 2030u), (Hagen 2030v), (Hagen 2030w), (Hagen 2030x), (Hagen 2030y), (Hagen 2030z), (Hagen 2031a), (Hagen 2031b), (Hagen 2031c), (Hagen 2031d), (Hagen 2031e), (Hagen 2031f), (Hagen 2031g), (Hagen 2031h), (Hagen 2031i), (Hagen 2031j), (Hagen 2031k), (Hagen 2031l), (Hagen 2031m), (Hagen 2031n),

NOTES FOR CONTRIBUTORS

Acta Veterinaria Hungarica publishes original research papers, review articles and short communications on veterinary science and medicine. Submissions are accepted on the understanding that they have not been published or submitted for publication elsewhere and that they are subject to peer review. The covering letter of submitted manuscripts should be signed by all co-authors, or a declaration of consent from the co-authors should be enclosed. Papers accepted for publication by the Editorial Board are subject to editorial revision. A copy of the Publishing Agreement will be sent to the authors of papers accepted for publication. Manuscripts will be processed only after receiving the signed copy of the agreement.

Format. Manuscripts must be in English and clearly and concisely written. They should be typed double spaced with a 40-mm left-hand margin. Three copies of the manuscript should be submitted with two sets of any illustrations. For *general papers*, the whole length should not exceed 4000 words (that is, about 10–11 printed pages of *Acta Veterinaria Hungarica*). *Short communications* should not exceed 1500 words and should include only one or two figures or tables. Concise papers will be given priority during the publication process.

Manuscripts on disk. After acceptance, manuscripts should be submitted in electronic version (Word for Windows), on a 3.5" disk or as an e-mail attachment, together with electronic versions of the figures.

Title. The title should be a clear and concise statement of the contents in not more than 15 words (about 100 characters). A short running title of not more than 50 characters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done. *The mailing address, e-mail address and fax number of the corresponding author must also be given in a footnote to the first page.*

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. **Key words** (4–6) should also be supplied.

Introduction. This part should state briefly the nature and purpose of the work and cite recent important work by others.

Materials and methods. Describe materials, methods, apparatus, experimental procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings. The experimental methods and treatments applied shall conform to the most recent guidelines on the humane treatment and care of animals.

Results. Experimental data should be presented clearly and concisely, in a non-repetitive way.

Discussion should be focused on the interpretation of experimental findings.

Acknowledgement of grants and technical help.

References. In the text references should be given as follows: Hinton (1995) described.... / ... recorded earlier (Starr et al., 1978; Manson and Starr, 1979). List of references should be given in date order in the text but alphabetically in the reference list. Different publications having the same author(s) and year will be distinguished by, e.g., 1992a, 1992b. The reference list at the end of the paper, arranged in alphabetical order of the authors' surnames, without serial numbers, should be drawn up according to the following models:

- For journals: names and initials of all authors, year of publication (in parentheses), colon, English title of the paper (if the original title is not English, indicate in parentheses, e.g. [in French]), abbreviated journal title, volume number, issue number in parentheses, first and last pages.
- For books: names and initials of authors/editors, year (in parentheses), title, publisher, place of publication, page number.
- For book chapters: names and initials of authors, year (in parentheses), title of chapter, In., names and initials of editor(s), title of book, publisher, place of publication, first and last page numbers of chapter.

Tables. They should be typed on separate sheets and have a concise heading each. Tables are to be numbered consecutively using Arabic numerals.

Illustrations. These should be numbered consecutively using Arabic numerals. Figures prepared by computer shall also be sent in electronic version. The author's name, title of the paper and number of the figure should be pencilled lightly on the back of each illustration. Good-quality computer graphs produced on a laser printer are acceptable. High quality half tones (photographs) should be prepared on glossy paper at their expected final size. Statements on magnification are not acceptable, and bars should be used instead. Histograms should be kept simple, be two-dimensional, have no background grid, and tones and colours should be avoided. A limited number of colour photographs will be accepted but the extra cost of reproduction in colour must be borne by the authors. Figure captions should be provided on a separate sheet. All figures should be referred to in the text and their approximate place indicated on the margin.

Abbreviations and symbols. Measurements should be expressed in the metric system or in SI units. All abbreviations should be spelt out in full the first time they are used in the text. Please identify unusual symbols on the margin.

Proofs, page charge and reprints. One set of proofs will be provided, which is requested to be returned within 48 hours of receipt to the Editor. A copy of the bank certificate documenting the transfer of a *page charge of USD 10 per printed page* shall be enclosed to the proofs. Twenty-five reprints of each paper are supplied free of charge. Additional reprints can be ordered at cost price at the time the page proof is returned.

Food hygiene

- Determination of residues of pyrethroid and organophosphorous ectoparasiticides in foods of animal origin. *Mária Szerletics Túri, Katalin Soós and Emőke Végh* 139

Mycotoxin research

- Natural deoxynivalenol (DON) contamination of wheat samples grown in 1998 as determined by high-performance liquid chromatography. *B. Fazekas, E. T. Hajdu, A. K. Tar and J. Tanyi* 151

Nutrition

- Effects of dietary protein and carbohydrate source on rumen fermentation and nutrient flow in sheep. *Hedvig Fébel, Szilvia Huszár and Ildikó Zsolnai Harczy* 161

Parasitology

- Potential diagnostic test for experimental and natural ovine *Taenia hydatigena* cysticercosis. *M. R. Panda, S. Ghosh and T. K. Varma* 173
A survey of chickens for viable toxoplasms in Croatia. *Viktorija Kutičić and Th. Wikerhauser* 183

Pharmacology

- Pharmacokinetics, urinary excretion and dosage regimen of diminazene in crossbred calves. *Gurmeet Kaur, R. K. Chaudhary and A. K. Srivastava* 187

Reproduction

- Formation of a secondary corpus luteum after ultrasound-guided follicular aspiration in cows. *G. S. Amiridis, Lindsay Robertson, I. A. Jeffcoate, Sophia Belibasaki, J. S. Boyd and P. J. O'Shaughnessy* 193
Effect of exogenous ovine placental lactogen on basal and prostaglandin-stimulated progesterone production by porcine luteal cells. *Ewa L. Gregoraszczuk, A. Gertler and E. Futoma* 199

Toxicology

- Studies on the toxic interaction between monensin and tiamulin in rats: Toxicity and pathology. *G. Szűcs, Judit Bajnógel, A. Varga, Zsuzsa Móra and P. Laczay* 209
In vitro ocular irritation toxicity study of some pesticides. *P. Budai and L. Várnagy* 221

Virology

- Gene immunization of mice with plasmid DNA expressing rabies virus glycoprotein. *I. Fodor, L. Kucsera, Nadja Fodor, V. Pálfi and V. I. Grabko* 229
Immunostimulatory effects of the muramyl dipeptide analogue LK415 in chickens immunized with a vaccine strain of infectious bursal disease virus. *Olga Zorman Rojs, Manica Černe, I. Mrzel, U. Urleb and Shizuko Muraoka* 237
Book reviews 249