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CONTROL OF PARATUBERCULOSIS IN FIVE CATTLE FARMS BY SEROLOGICAL TESTS AND FAECAL CULTURE DURING THE PERIOD 1990–1999*

OZDRAVOVÁNÍ PĚTI FAREM SKOTU OD PARATUBERKULÓZY V LETECH 1990–1999 POMOCÍ SÉROLOGICKÉHO VYŠETŘOVÁNÍ A KULTIVACE TRUSU

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ABSTRACT: In the Slovak Republic, clinical paratuberculosis was detected for the first time in two cows in the farm NT in 1989. Gradually the disease spread to another four farms: M, HJ, H, and C, where there were 1 490 cows, 1 100 calves and 1 300 heifers. The introduction of *Mycobacterium avium* subspecies *paratuberculosis* to these farms was most probably due to the importation of 60 Holstein heifers from Denmark to farm NT in the late 1970s. In the mid-1980s, because of common housing with apparently healthy heifers, the remaining four free herds also became infected with paratuberculosis. In 1990, an eradication programme against paratuberculosis was established. In the first stage (during 1990–1992) of the programme all animals older than 18 months were examined by three serological tests: AGID (agar-gel-immunodiffusion), CFT (complement fixation test) and ELISA. Animals with repeatedly positive serological results were culled from the herd including clinically suspect animals. In the second stage (from 1992) serological tests were supplemented by two parallel faecal cultures per year. In 1990, the incidence of clinical paratuberculosis among the five farms varied (M – 4.1%, HJ – 1.5%, H – 0%, C – 0% and NT – 0.5%). Cases of infection (n = 528) were divided into three groups: Ist without clinical signs and few CFU in faeces and/or gastrointestinal tract (60.8%); IIrd without clinical signs, and numerous CFU in faeces and/or gastrointestinal tract (20.1%); IIIrd clinically affected and numerous CFU in faeces and gastrointestinal tract (19.1%). Thus, faecal surveillance enabled us to cull individuals that were in the preclinical stage of the disease. The incidence of the disease as of 1999 on farm M was 0% and on farms HJ, H and C was only 2.0%. However, on farm NT the prevalence was still high (e.g. 25.8% in 1998) which may be due to the common housing of new-born calves and cows for two months between 1993–1996.

Johne's diseases; paratuberculosis; control; zootechnical procedures; cattle farms; faecal culture

ABSTRAKT: V roce 1989 byla poprvé na farmě NT na Slovensku diagnostikována klinická paratuberkulóza u dvou krav. Postupně byla nákaza rozšířena na další čtyři farmy: M, HJ, H a C, s celkovým stavem 1 490 krav, 1 100 telat a 1 300 jalovic. Infekce *Mycobacterium avium* subspecies *paratuberculosis* byla do chovu pravděpodobně zavlečena koncem 70. let importem 60 vysokobřezích jalovic holštýnského plemene z Dánska na farmu NT. Na čtyři ostatní farmy byla infekce postupně rozšířena prostřednictvím společného odchovu jalovic pocházejících ze všech pěti farem. Počátkem roku 1990 bylo zahájeno ozdravování skotu od paratuberkulózy. V první fázi do roku 1992 byla prováděna tři plošná sérologická vyšetření všech zvířat starších 18 měsíců dvěma metodami IDT (imunodifuzní test), RVK (reakce vazby komplementu) a ELISA. Opakovaně pozitivně sérologicky reagující zvířata byla vyřazována z chovu, včetně zvířat klinicky podezřelých. Ve druhé fázi od roku 1992 bylo plošné sérologické vyšetření doplněno o paralelní kultivaci trusu dvakrát ročně. Podle intenzity infekce bylo možné rozdělit infikovaná zvířata (n = 528) do tří skupin: I. klinicky zdravá a několik CFU v trusu nebo střevním traktu (60,8 %); II. klinicky zdravá s velkým množstvím CFU v trusu nebo střevním traktu (20,1 %); III. klinicky nemocná, masivně infikovaná (19,1 %). Kultivační vyšetření trusu pomohlo při ozdravování jednotlivých farem vyřadit z chovu zvířata v preklinickém stadiu onemocnění. Incidence paratuberkulózy byla v roce 1999 na farmě M 0 % a na farmách HJ, H a C do 2,0 %. Avšak na farmě

* The results were partly presented (Pavlik et al., 1999c) at the Sixth International Colloquium on Paratuberculosis (14–18th February, 1999, Melbourne, Victoria, Australia). The research was partially supported by the Ministry of Agriculture of the Czech Republic (Grant No. EP0960006087), Grant Agency of the Czech Republic (Grants No. 514/95/1594 and 524/97/0948) and Program Fair: FAIR6-CT98-4373 (EU, Brussels).

NT byla incidence stále vysoká (např. 25,8 % v roce 1998), což bylo s největší pravděpodobností způsobeno v letech 1993 až 1996 společným ustájením telat s matkami dva měsíce po narození.

Johnova choroba; paratuberkulóza; ozdravování; zootechnická opatření; chov skotu; kultivace trusu

INTRODUCTION

Paratuberculosis is a contagious disease of cattle and other ruminants, which is caused by *Mycobacterium avium* subspecies *paratuberculosis*. The infectious agent is shed in large amounts in faeces of infected animals, and infection is acquired by ingestion of contaminated feed and water. After ingestion, the bacteria multiply in the intestinal mucosa and associated lymph nodes. The disease is characterised by a long incubation period of several months to years. Thus, after the purchase of new animals, farmers are not aware of the introduction of the infection until its wide dissemination in the herd. In dairy cattle, the disease is characterised by chronic diarrhoea in only about 10% of infected cows (Chiodini et al., 1984; Pavlík et al., 1994).

The disease is spread world wide. In Victoria (Australia), in the late 1990s, about 13% of dairy cows and 0.4% of beef cattle were infected with paratuberculosis (Stephens and Aukema, 1989). In New South Wales, the disease was detected in 0.08% of cattle (out of 45 million). McNab et al. (1991), between 1986-1989, estimated a prevalence rate of 6.1% in Ontario, Canada. Paratuberculosis is also widely spread in the USA. Jones (1989b), estimated that 3% of 10.3 mil. dairy cattle and 1% of 33 mil. beef cattle were infected by *M. paratuberculosis* in the USA. It is also known in most countries of Europe (Brugére-Picoux et al., 1988).

Paratuberculosis is a disease that can also lead to a tremendous economic losses. According to Merkal (1984), in the 1980s, about US$ 1.5 billion was estimated to be lost each year in the USA. Collins and Nordlund (1991) estimated that milk yield in a single infected herd was reduced by 5.4%. This means, a loss of about US$ 200 per single infected cow. The ultimate emaciation of the animals is the main reason for economic loss in individual animals. Some of the indirect consequences of the disease include low milk yield and poor quality (increased somatic cells), reproduction disorders etc. The estimated economic loss in an infected herd may be as much as 12–15% of its total production (Hole, 1958; Chiodini et al., 1984; Wilson et al., 1993).

Different approaches have been used for eradication programmes. For instance, in Northern Ireland, repeated serological examinations were used in the late 1960s. As a result, 41 out of 47 infected herds were free of the disease (Rieman and Abbas, 1983). The application of skin tests is influenced by many factors. Since the organism is an intracellular parasite, cellular immunity plays a major role in the pathogenesis of the disease (Bendixen, 1978). During the early stage of the infection, a cell-mediated response is produced, which is gradually replaced by humoral immune response (Chiodini et al., 1984). The allergenic character of individual strains of *M. paratuberculosis* used in the assay can also affect the outcome of the skin test (Chiodini et al., 1984; Wentink et al., 1984; Stephens, 1989; Pavlas, 1990). Skin tests detect 50–60% of infected animals, however, as the disease progresses, the response to this test becomes poorer (Körmendy et al., 1989). Accordingly, tests of cell-mediated immunity are rarely used because of their limited practical diagnostic value.

Some countries use faecal culture as the most reliable diagnostic method for the control of paratuberculosis (Arrigoni et al., 1988; Collins and McLaughlin, 1989; Bensch, 1993; Pohl, 1993). In Wisconsin (USA), the programme for control of paratuberculosis in cattle has been implemented since 1969. Faecal culture and vaccination of all calves younger than 4 weeks were applied in some herds. Although vaccination was used in 700 herds, until 1989 only 39 herds were free of the disease (Jones, 1989a; Collins and McLaughlin, 1989).

Benedictus and Haagsma (1986) used mesenteric lymph node culture together with skin tests, complement fixation test (CFT) and faecal culture in 233 Holstein-Friesian cattle. All animals under 9 months of age were examined. Positive reactors, including 29 confirmed cases, were culled. In the following two years, there were no reports of clinical paratuberculosis. Nevertheless, the high cost per animal, tediousness, and long incubation period hampered the use of this method.

Until the late 1980s, prevalence of paratuberculosis in the Czech Republic was sporadic, whereas in the Slovak Republic it was widely spread (Pavlas et al., 1997). In 1989, the disease was detected in 16 out of 36 districts (Hanzlíkova and Vilímek, 1989) sparking efforts to control large infected cattle herds in the early 1990s in the Slovak Republic. Our control programme required regular clinical assessments of the animals, serological examinations and faecal cultures. Moreover, additional measures were taken to prevent the spread of the disease such as pasture interruption, segregation of calves from adults, and avoiding the introduction of animals to herds from other farms. The aim of this study was to assess the efficacy of this control programme, which has been implemented since 1990.

MATERIAL AND METHODS

Characteristics of the infected herds

Paratuberculosis was first diagnosed in two clinically suspect cows (farm M) at the end of 1989. Slowly, the disease spread into all five farms (M, C, HJ, NT

and H), which comprised 1 490 cows, 1 085 calves, and 1 300 heifers (according to data from 1990). Until 1992, calves and heifers were often moved from one farm to another. Due to some operational inconveniences, it was difficult to return heifers to their farm of origin.

Intravital diagnostic methods

From 1990 to 1999, the following intravital diagnostic methods were used: regular clinical examinations, serological tests, and faecal cultures.

Clinical examination

The health status of the cattle on all farms was monitored daily by a technician and weekly by a veterinarian. Clinically suspect animals (i.e. with diarrhoea for longer than five days and emaciated) were assessed, and confirmed cases were sent to emergency slaughter. Blood and faecal samples were collected from emaciated cows suffering from diarrhoea. Serologically positive animals were immediately culled. Serologically negative animals were culled on the basis of faecal culture results.

Serum antibody detection

Antibodies to *M. paratuberculosis* were detected by the agar-gel-immunodiffusion test (AGID) described by Sherman et al. (1984). Until 1994, the antigen was produced in our laboratory by sonification; later it was produced by Bioveta, Ivanovice na Hané, Czech Republic. The ELISA method was used from 1990 to 1994 as described (Colgrove et al., 1989), and CFT from 1995 using the set produced by Bioveta, Ivanovice na Hané.

Faecal culture

Since 1992, faecal culture was conducted according to the method of Whipple et al. (1991). About 5 g of faeces were collected from the rectum using disposable gloves. Until mid-1996, the time required for preparation of faeces in HPC – Hexadecyl Pyridinium Chloride (N-cetylpyridinium chloride monohydrate, No. 102340 Merck) was one day, but later prolonged to 3 days, at room temperature (Socket, 1996, personal communication). Then 0.2 ml of the inoculum was inoculated in each of the 3 Herrold culture media with Mycobactin J (prepared according to Merkal and McCullough, 1982), and incubated at 37 °C for 14 to 16 weeks.

Postmortem diagnostic methods

To determine the extent of infection, tissue specimens were collected from clinical suspects (emaciated and/or diarrhoea), that had a positive faecal culture and positive antibody result. A total of 547 slaughtered animals were examined. Infection with *M. paratuberculosis* was detected in tissue and/or faecal samples of 306 animals and in faecal samples of 222 animals from which tissues were not collected.

Pathological examination

Immediately after slaughter, specimens were collected for pathological examination. At least four samples were cultured from each animal: two specimens from different segments of the ileal mucosa (about 30–50 cm apart); and two specimens from different adjacent mesenteric lymph nodes.

Culture of the specimen

About 1 g of the ileal mucous membrane was cut, homogenised (Lab Blender, Stomacher), prepared overnight in 0.75% HPC and cultured in the same way as the faecal culture. Isolated strains were identified by subcultivation in 3 Herrold culture media with and one culture medium without Mycobactin J (mycobactin dependency test), and PCR was used for detection of the specific insertion sequence *IS900* for *M. paratuberculosis* (Kunze et al., 1992).

Assessment of the extent of infection

Based on the results of clinical examinations, faecal and GIT cultures (ileal mucosa and adjacent lymph nodes), animals in individual farms were divided into three groups:

Group I – clinically healthy animals mostly in good body condition with positive faecal and/or GIT culture (CFU < 10 on one culture medium);

Group II – animals in fair body condition with positive faecal and/or GIT culture (CFU ≥ 10 but < 50 on one culture medium);

Group III – clinically ill animals in poor body condition (or with chronic diarrhoea), with positive faecal and/or GIT culture (CFU ≥ 50 on one culture medium).

Paratuberculosis eradication programme at individual farms

In the first stage (until 1992), all animals older than 18 months were tested three times by serological methods using AGID and ELISA. In the second stage (since 1992), faecal cultures were applied at selected farms in addition to serology. Until 1994, AGID and ELISA were the methods of choice, but between 1995 and 1999, AGID and CFT were used. Animals with positive serological reaction were serologically re-tested within 4 to 6 weeks. Animals with two positive serological results and/or positive faecal cultures, including clinically suspect animals, were culled from the herd.

Farm M (170 animals older than 18 months)

Until 1992, three serological tests were completed annually but since 1993 only two tests a year have been conducted. Between 1992 and 1996, faecal cultures were done twice a year. As the disease prevalence was dropping (1997–1999), faecal cultures were then done only once a year.

Farm HJ (400 animals older than 18 months)

At this farm three serological tests were completed annually until 1992; since 1993 two tests have been carried out each year. Since 1995, faecal culture has been conducted twice a year.

Farm H (350 animals older than 18 months)

Until 1992, serological tests were being carried out three times a year. Between 1993 and 1996, faecal cultures were conducted twice a year. During 1997–1999, since the disease incidence was declining, faecal cultures were performed only once a year.

Farm C (170 animals older than 18 months)

At this farm serological tests were carried out three times a year until 1992, but twice a year until 1994. In addition, faecal cultures were conducted twice a year from 1992 to 1994. In 1994, approximately 90 cows were sent to slaughter and 82 animals older than 18 months (mainly first-calf heifers and cows in late pregnancy) were moved to farm NT. In addition, 92 heifers older than 18 months were transferred to 3 other farms: 7 to farm M, 9 to farm HJ, and 76 to farm H. Thus, from 1995 to 1999, an assessment of epidemiological status of farm C should also include the health condition of cows shifted to farms M, HJ and H.

Farm NT (400 animals older than 18 months)

Until 1992, serological examinations were conducted three times a year, but from 1993–1999 only twice a year. Due to an apparently improved infection status between 1992 and 1994, faecal cultures were done only once a year and in the years 1996 to 1999 twice a year.

Mode of animal raising (calves, heifers and cows)

Until 1989, cows at all five farms were regularly permitted to graze on pasture from May to September. However, after the detection of paratuberculosis, pasture grazing was interrupted. Only heifers born from paratuberculosis-free dams could be introduced into the herd. Since the risk of shedding of *M. paratuberculosis* in faeces by young animals is low, bulls to the age of 18 months were left to graze without limitation.

Farms M, HJ, H and C

About 170 cows were housed at farm M. Farm HJ had four barns, each with 100 cows. Farm H had two barns, each consisting of 175 cows, and farm C with 170 managed cows. Calving of all cows took place in the barns. Calves were removed from cows within 24 hrs after birth and placed individually in boxes, where they received colostrum for 5–8 days, and later milk replacer. All heifers (2–3 months old) from the above 4 farms were moved onto another farm, where they were kept until 1992. After breeding, heifers were returned to their farm of origin and kept until 16–18 months old. Due to strict disease control, heifers have been kept at their farm of origin since 1993. Only bulls were moved onto another farm.

Farm NT

Animal rearing on this farm (calves, heifers, and cows) should be reviewed by time period:

First period (1990–1992). Until 1992, animals older than 18 months were confined in three barns. Calves were raised in the same system as in the above four farms.

Second period (1993–1996). During this period, animals were confined in pens (4 barns, each with 100 animals). About 1 month before parturition, cows were grouped in pens (10–15 cows). New-born calves were left together with cows for 4–8 weeks. Other cows not suitable for milk production (increased milk somatic cell number, chronic mastitis, poor body condition after parturition, old age, chronic arthritis, etc.) were kept in these pens with the heifers. This system increased the risk for infection and so heifers were removed to another stall of the farm, where they were kept until late pregnancy. Bulls, 2–3 months old, were moved to another farm and added to bulls from different farms of origin.

Third period (1997–1999). To improve the epidemiological situation, some changes were carried out. Only 3–5 cows were grouped in the delivery pen. Calves were removed within 3–6 hrs after birth and placed into open-air calf hutches, where they received colostrum from mother for 5–8 days, and later milk replacer.

Statistical assessment

The χ^2-test (STAT Plus) was applied for the statistical evaluation of the disease incidence in individual farms (Matoušková et al., 1992).

RESULTS

During 1989, chronic diarrhoea accompanied by emaciation was observed in 16 cows in farm M (Tab. I). For the first time paratuberculosis was diagnosed in

1. Paratuberculosis detected in individual farms

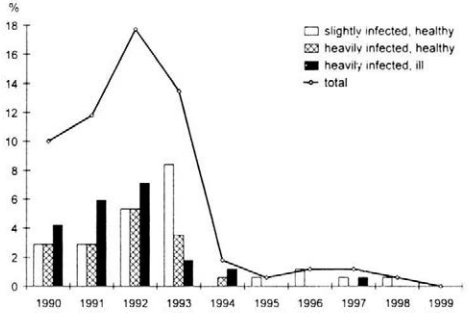
Farm number of animals	Extent of the infection	Number of infected animals										
		1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	total
M	I	5	5	9	14	0	1	2	1	1	0	38
	II	5	5	9	6	1	0	0	0	0	0	26
170	III	7(16)	10	12	3	2	0	0	1	0	0	35
Total		17	20	30	23	3	1	2	2	1	0	99
%		10.0	11.8	17.6	13.5	1.8	0.6	1.2	1.2	0.6	0	
HJ	I	5	0	5	4	0	16	8	3	5	1	47
	II	10	3	1	6	2	2	2	1	1	0	28
400	III	6(5)	3	0	5	1	5	2	1	0	0	23
Total		21	6	6	15	3	23	12	5	6	1	98
%		5.3	1.5	1.5	3.8	0.8	5.8	3.0	1.3	1.5	0.3	
H	I	0	0	0	1	5	5	4	3	2	3	23
	II	0	0	0	4	1	2	1	1	1	0	10
350	III	0(0)	0	1	3	2	0	2	1	0	0	9
Total		0	0	1	8	$8^{\&}$	$7^{\&}$	$7^{\&}$	$5^{\&\&}$	$3^{\&\&\&}$	$3^{\&\&\&}$	42
%		0.0	0.0	0.3	2.3	3.1	2.7	2.7	2.5	2.0	2.0	
C	I	0	0	7	5	2	1	0	4	3	1	23
	II	1	0	2	3	1	0	0	1	0	0	8
170	III	0(1)	2	1	0	0	0	0	0	0	0	3
Total		1	2	10	8	3	1	0^{*}	5^{**}	3^{***}	1^{****}	34
%		0.6	1.2	5.9	4.7	1.8	0.6	0	5.0	3.8	1.7	
NT	I	0	2	4	3	18	3	16	32	85	27	190
	II	3	0	0	3	1	0	2	8	13	4	34
400	III	2(2)	0	2	3	3	3	4	6	5	3	31
Total		5	2	6	9	22	6	22	46	103	34	255
%		1.3	0.5	1.5	2.3	5.5	1.5	5.5	11.5	25.8	8.5	
Total		44	30	53	63	39	38	43	63	116	39	528

Explanations:
Group I – clinically healthy animals mostly with a good body condition with positive faecal and/or GIT culture (CFU < 10 on one culture medium)
Group II – animals with a fair body condition with positive faecal and/or GIT culture (CFU ≥ 10 but < 50 on one culture medium)
Group III – clinically ill animals with poor body condition (or chronic diarrhoea), positive faecal and/or GIT culture (CFU ≥ 50 on one culture medium)
( ) – number of clinically suspected animals (chronic diarrhoea and emaciation) in 1989 before the confirmation of the disease in January 1990
Decrease in the number of animals older than 18 months in farms H and C:
$^{\&}260$, $^{\&\&}200$, $^{\&\&\&}150$, $^{*}150$, $^{**}100$, $^{***}80$, $^{****}60$

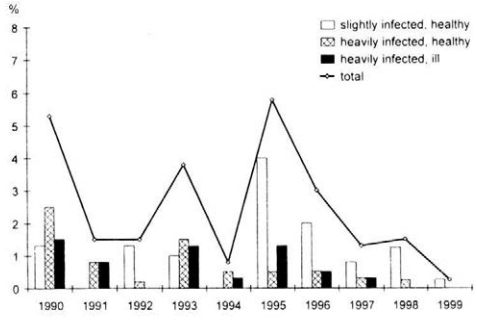
two clinically suspect cows from farm M in 1989. Since all heifers had a common farm origin (M, HJ, H, C, and NT), an eradication programme was established for all five farms. As the prevalence of the disease in each farm was different, results are given accordingly. All figures representing the percentage of the infection incidence are given based on culture and serological test results.

Farm M (Tab. I, Fig. 1)

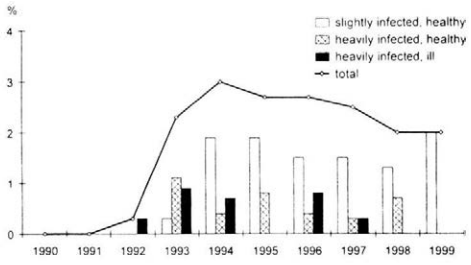
Between 1990 and 1992, this farm was found to have the highest number of clinically ill animals (4.1%, 5.9% and 7.1%). Also the number of positive reactors was increasing annually (10.0%, 11.8% and 17.6%).



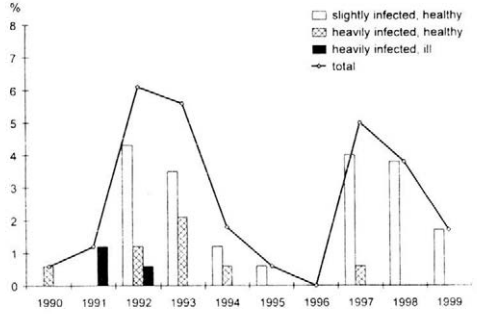
1. Incidence of paratuberculosis on farm M



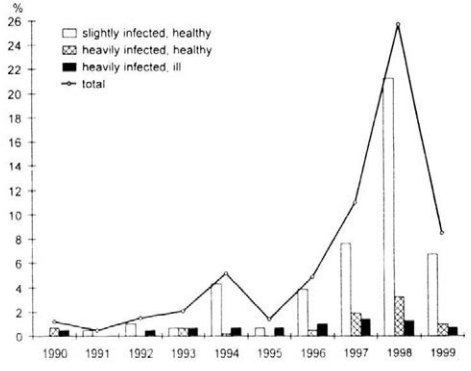
2. Incidence of paratuberculosis on farm HJ



3. Incidence of paratuberculosis on farm H



4. Incidence of paratuberculosis on farm C



5. Incidence of paratuberculosis on farm NT

Faecal culture has revealed *M. paratuberculosis* infection in 8.2% of clinically healthy animals. From 1994 to 1999 the incidence of paratuberculosis dropped from 1.8% to 0% following the culling of positive reactors. The severity of infection was also changing from time to time. From 1990–1992, there were more clinically ill animals. In the following year (1993), clinically healthy animals were observed more often. From 1995 to 1999, only one animal was clinically affected by paratuberculosis.

Farm HJ (Tab. I, Fig. 2)

In 1990, the incidence of paratuberculosis reached 5.3%. In the following years (1991–1994), it dropped to < 4%. Clinical cases were reported in about 1.3% of animals. In 1995, the two faecal culture screens revealed an increase in incidence of 5.8% due to latent infection, but from this time to 1999 decreased to 0.3% (no clinical case was reported in 1998 and 1999).

Farm H (Tab. I, Fig. 3)

At this farm, the disease was for the first time detected in a single clinically ill cow in 1992. The incidence of clinical paratuberculosis was under 1%. Between 1995–1996, incidence of the infection reached 2.7%, while between 1997–1999 it decreased to 2.0%.

Farm C (Tab. I, Fig. 4)

The follow-up of farm C can be divided into two periods. In the first period, animals were kept in farm C, and in the second period they were moved to other farms.

First period (1990–1994)

In 1990 and 1991, paratuberculosis was diagnosed in one subclinically ill and two clinically ill animals, respectively. In 1992 and 1993, infection incidence increased to 5.9% and 4.7%, respectively. This was believed to be because of the detection of 4.1% and 2.9% of latently infected animals. However, in 1994, since the incidence decreased to 1.8%, the movement of some young animals to other farms was permitted.

Second period (1995–1999)

During this period the disease incidence was low. However, in the years 1997–1999, it started to rise as

paratuberculosis was detected in 8 animals shifted to farm NT, and in one animal, moved to farm HJ.

Farm NT (Tab. I, Fig. 5)

Similarly, the follow-up of this farm was divided into three periods according to the mode of calf raising.

First period (1990–1992)

During this time, animals were confined in three barns. Calves were placed in calf boxes after birth. The disease incidence was ≤ 1.5%. The infection was detected only in 4 clinically ill cows, 3 subclinically ill cows, and 6 clinically healthy cows.

Second period (1993–1996)

Cows were kept in three free-stall barns (each with 100 cows). Calves were placed together with cows for 4–8 weeks. In the years 1994–1996, large numbers of animals were discovered to be shedding *M. paratuberculosis* in faeces. In each of these three years, clinical paratuberculosis was detected in only 4 animals (1% of the herd).

Third period (1997–1999)

Some modifications were executed: new-born calves were transferred to open-air calf boxes. Nevertheless, there was a dramatic increase in incidence to 25.8% in 1998. However, incidence of clinical cases did not exceed 1.5%.

Statistical assessment of the disease incidence at individual farms

Farms with the highest disease incidence during the period 1990–1999

In the years 1990–1993, the highest incidence based on the isolation of *M. paratuberculosis*, was found at farm M (10.0%, 11.8%, 17.6% and 13.5%). In 1995, farm HJ had the highest incidence (5.8%), and in 1994 and 1996–1999, farm NT had the highest incidence (5.5%, 5.5%, 11.5%, 25.8% and 8.5%).

Difference in the system of calf-rearing

A comparison, based on the technique of calf raising, was conducted among farms M, HJ, H and NT. At farm NT, where for two months between 1993 and 1996 new-born calves and cows were housed together, the incidence started to increase in 1993 (except 1995) to 25.8% in 1998. During the years 1997–1998 the statistical incidence was significantly higher ($p = 0.01$) than the incidence of each year during the previous years of 1990–1996. During the period 1996–1998 the annual incidence was significantly higher ($p = 0.01$) than the previous year. During the period 1997–1999 (calves have been kept with dams during the first 4–8 weeks after birth since 1993) the incidence was significantly higher at farm NT ($p = 0.01$) than at farms M, HJ and H. There, calves were removed from cows after birth and placed into open-air boxes.

The effect of faecal cultures on the decrease of incidence during the control program

The introduction of faecal culture promoted the decrease of the incidence on farm M (during 1992–1995), farm HJ (1995–1999) and farm C (1992–1996). This decrease was statistically highly significant ($p = 0.01$) at two farms. Farm M from 1992 to 1994 and farm HJ from 1995 to 1997. At farm C the decrease was statistically significant ($p = 0.05$) only from 1992 to 1994. However at farm NT the decrease of incidence was not observed after the introduction of faecal culture examination during the years 1995–1998.

DISCUSSION

The exact source of the infection at the farms has yet to be confirmed. The most probable source of infection is the introduction of 60 late pregnant Holstein heifers from Denmark to farm NT during the late 1970s. In our study, we discovered that it is most likely that the imported ruminants were the reservoir of the organism (Pavlík et al., 1995). This farm was infected with *M. paratuberculosis* that was identified by RFLP (Restriction Fragment Length Polymorphism) as type B-C1 (Pavlík et al., 1999b). This RFLP type was often identified in ruminants imported from Denmark (Pavlík et al., 1996, 1999a). In the mid 1990s, heifers from this farm were raised in one central farm together with heifers from the above mentioned four farms. Transfer of heifers from this farm to others contributed to the spread of paratuberculosis as well.

Farm M (Tab. I, Fig. 1)

Between 1990 and 1993 the incidence of clinical and subclinical paratuberculosis was different among the different farms. This was attributed to various factors such as mode of calf rearing, level of animal husbandry, etc. The reason why the highest incidence of paratuberculosis was detected at farm M remains unclear. However, from Tab. I, it was evident that the infection incidence had gradually decreased from 17.6% in 1992 to 0% in 1999. From an epidemiological point of view, the main issue was the reduction in clinically ill animals since 1993, which obviously minimises intrauterine infection, thus providing uninfected young replacements for the farm.

This result was achieved by culling animals with positive results in serological assays and faecal cultures. Other important factors include avoiding the introduction of new animals from other herds and practising measures to block the spread of infection (pasture restriction, removal of calves from dams). According to most authors, calves are highly prone to the disease. Obasanjo et al. (1997), demonstrated in the New York State Paratuberculosis Control Program that the exposure of a 6 week old calf to contaminated faeces of adult animals provides one of the highest risks for the spread of paratuberculosis in a herd. The same conclusion was reached by Dutch and other authors (Reinders, 1986; Benedictus, 1985a,b).

Farm HJ (Tab. I, Fig. 2)

From Tab. I and Fig. 2, it was evident that the occurrence of clinical paratuberculosis at this farm was somehow sporadic (≤ 1.5%) between 1990 and 1994. This was due to pasture restriction in 1990 and improvement of calf raising in open-air individual boxes. However in 1995, faecal culture revealed latently infected animals (negative in serology), thus increasing the disease incidence. The pattern changed between 1995 and 1999 when the number of clinical and subclinical cases declined consistently.

Farm H (Tab. I, Fig. 3)

Paratuberculosis was detected for the first time in 1992 only in one clinically ill cow. The reason for the low incidence might have been due to the closed herd status and effective infection control methods (such as temporary pasture restriction, removal of calves from cows immediately after birth, etc.). During 1993–1999 the constant incidence of paratuberculosis between 2.0–3.1% remains unclear.

Farm C (Tab. I, Fig. 4)

Between 1990 and 1996 a few clinical cases were reported. However, between 1990–1991, the incidence at this farm was very low and the disease was diagnosed only in 3 animals. Results from faecal culture indicated that the incidence suddenly increased to 5.9% in 1992. Then, as a result of regular faecal examination performed twice a year, the incidence has decreased from 5.9% to 0% in 1996. However, after the transfer of some cows to farms NT and HJ, the epidemiological situation changed. Between 1997 and 1999, one cow in farm HJ and 8 cows at farm NT were found to be infected with *M. paratuberculosis*. It was difficult to verify whether these animals acquired the infection prior to their arrival to farm C. Since the infection pressure at farm NT was very high between 1997 and 1999 (Fig. 5) it may be assumed that they were infected in farm NT. This assumption could be supported by the microbiological and immunological examinations of these cows in another study (Toman et al., 1999).

Farm NT (Tab. I, Fig. 5)

At the beginning of the eradication programme, the incidence of the infection was low. However, due to some alterations in the system of animal raising, calf rearing and stress factors, the epidemiological situation worsened over time. In 1994, faecal culture detected 5.5% of latently infected cows. Although increasing incidence appeared during the period 1996–1998 when new-born calves were kept with cows to the age of 4–8 weeks. This condition perpetuated a higher risk of infection in the future. As a result, a lot of adult cows born after 1992, tested positive in faecal culture during the following period 1996–1999.

CONCLUSION

It can be concluded that even though clinical examinations, serological tests, and especially faecal cultures are the best choices in detecting infections and are necessary for the eradication of the disease, efforts should also be directed towards sanitation. Removing calves from cows immediately after birth is particularly important since paratuberculosis is acquired early in life.

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MORPHOLOGY OF APOPTOSIS OF POLYMORPHONUCLEAR LEUKOCYTES ISOLATED FROM JUVENILE BOVINE MAMMARY GLANDS*

MORFOLOGIE APOPTÓZY POLYMORFONUKLEÁRNÍCH LEUKOCYTŮ JUVENILNÍ MLÉČNÉ ŽLÁZY SKOTU

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ABSTRACT: The paper describes the occurrence and morphological pattern of apoptosis of polymorphonuclear leukocytes (PMN) of juvenile bovine mammary glands as imaged by light, fluorescence, confocal, and transmission electron microscopy. The cells were obtained by lavage of 40 juvenile bovine mammary glands stimulated with muramyl dipeptide. Twenty-four hours after the stimulation, the percentages of necrotic and apoptotic PMN were 7.9 and 4.2, respectively. Light, fluorescence and confocal microscopy of the apoptotic PMN showed morphological alterations including condensation of chromatin (karyopyknosis), cell shrinking, loss of pseudopodia, and spherical shape, while transmission electron microscopy revealed aggregation of chromatin, intact organelles and cell membrane, and vacuolisation of cytoplasm. The apoptotic PMN were eventually phagocytosed by macrophages as demonstrated by electron microscopy, and light microscopy in preparations stained for myeloperixidase, as well as by confocal microscopy. The finding of degenerating and necrotic PMN indicates that there exist two forms of death of this cell type and is suggestive of the existence of factors alterating the apoptotic programme and shortening the life span of PMN.

polymorphonuclear leukocyte; apoptosis; juvenile mammary gland

ABSTRAKT: Předmětem této práce bylo dokumentovat výskyt a popsat morfologii procesu apoptózy polymorfonukleárních leukocytů (PMN) juvenilní mléčné žlázy skotu s využitím světelné, fluorescenční, konfokální a transmisní elektronové mikroskopie. Morfologie apoptózy PMN byla studována na preparátech pocházejících z laváží 40 stimulovaných juvenilních mléčných žláz skotu. 24 hodin po stimulaci syntetickým derivátem muramyldipeptidu bylo zaznamenáno 7,9 % nekrotických a 4,2 % apoptotických PMN. Apoptotické PMN vykazovaly morfologické změny, které zahrnovaly kondenzaci chromatinu (karyopyknosis), svraštění buněk, ztrátu pseudopodií a sféricitu buněk ve světelné, fluorescenční a konfokální mikroskopii. A dále pak agregaci chromatinu, intaktní organely a cytoplazmatickou membránu a vakuolizaci cytoplazmy patrné v transmisním elektronovém mikroskopu. Osudem apoptotických PMN byla jejich fagocytóza makrofágy, což bylo potvrzeno elektronovou mikroskopií, dále barvením na myeloperoxidázu, kterým bylo stanoveno zastoupení makrofágů fagocytujících apoptotické PMN, a rovněž konfokální mikroskopií. Přítomnost degenerativních a nekrotických PMN svědčí o existenci faktorů působících alteraci apoptotického programu PMN a zkracování životnosti těchto buněk.

polymorfonukleární leukocyty; apoptóza; juvenilní mléčná žláza

INTRODUCTION

Active polymorphonuclear leukocytes (PMN) are an important component of the natural defence system of the bovine mammary gland. Chemotactic substances induce the migration of PMN from blood vessels through tissues to their site of action which is the cavitary system of the mammary gland (Jain, 1976; Nickerson, 1985; Paape et al., 1991; Sordillo et al., 1997).

Morphological characteristics of PMN became the object of studies of somatic cell differential counts in bovine milk. The morphology of PMN was investigated using light and electron microscopy in samples collected from juvenile mammary glands of heifers (Wardley et al., 1976; Sládek and Ryšánek, 1999a, b), adult glands of lactating dairy cows (Schalm et al., 1971; Paape and Wergin, 1977; Lee et al., 1980; McDonald and Anderson, 1981), and involuting mammary glands of dairy cows (Paape and Wergin, 1977; Lee et al., 1980; McDonald and Anderson, 1981). Already these morphological and the subsequent functional studies demonstrated among the normal also degenerating PMN charac-

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terised by fusion and swelling of nuclear segments, loss of pseudopodia, vacuolisation of cytoplasm and swelling of the cells resulting in spherical shape. The terminal stages of this process were karyolysis and cytolysis (Nickerson et al., 1985, 1986; Lintner and Eberhart, 1990; Paape et al., 1990; Sládek and Ryšánek, 1999a, b). The structural abnormalities were associated with a loss of the phagocytic activity (Nickerson et al., 1985; Lintner and Eberhart, 1990) and release of the histotoxic content of granules into the surrounding tissue (Weiss, 1989).

The degenerative processes and the subsequent necrosis are one of the forms of death of PMN in tissues (Hurley, 1983). However, PMN are predisposed also to another form which is apoptosis (Raff, 1992).

Apoptosis or programmed cell death, is an active process characterised by specific structural and biochemical alterations including chromatin condensation (karyopyknosis), shrinking of cells, zeiosis of the cytoplasmatic membrane, splitting of DNA and finely fragmentation of the cells into apoptotic bodies (Kerr et al., 1972; Wyllie et al., 1980; Wyllie, 1981). A prompt phagocytosis of the apoptotic cells by macrophages prevents the release of the histotoxic content of granules into the surrounding tissues (Savill et al., 1989; Cooter et al., 1994; Haanen and Vermes, 1995).

In vivo apoptosis of PMN was first described by Savill et al. (1989) in a human patient suffering from acute arthritis. Subsequently, this form of PMN death was described in human clinical samples (Grigg et al., 1991; Savill et al., 1992) and in experimental materials collected from laboratory animals (Yamamoto et al., 1993; Cox et al., 1995; Hughes et al., 1997; Hussain et al., 1998; Ishii et al., 1998; Meszaros et al., 1999).

Unlike degeneration and necrosis, the apoptosis of PMN of the cavitary system of the bovine mammary gland has not yet been described.

In addition to degenerating and necrotic PMN, apoptosis of this cell type was also observed within our experimental studies of the juvenile bovine mammary gland (Sládek and Ryšánek, 1999a). It is therefore apparent that apoptotic PMN should be considered in the differentiation of milk somatic cells.

This fact encouraged our morphological studies of apoptosis of PMN isolated from juvenile bovine mammary glands using light, fluorescence, confocal, and transmission electron microscopy. Simple methods for the detection of apoptosis of PMN of the bovine mammary gland avoiding the use of molecular biological techniques (Gavrieli et al., 1992; Koopman et al., 1994; Ben-Sasson et al., 1995; Martin et al., 1995; Van Oostveldt et al., 1999) are also described.

MATERIAL AND METHODS

Animals

The morphology of apoptosis was described using selected (n = 40) preparations obtained within several experiments. The whole set included 220 mammary glands of 55 unbred heifers, crosses of the Holstein x Bohemian Red Pied breeds, aged 16 to 18 months.

Stimulation of mammary glands

A lavage of mammary glands of the experimental animals (Ryšánek et al., 1999) was followed by the administration of 10 ml the synthetic derivative of muramyl dipeptide nor MurNAc-L-Abu-D-IsoGln (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague) diluted with PBS (1 μg/1 ml). Mammary gland's lavage (MLG) with 20 ml of PBS heated to 37 °C for the isolation of PMN was done 24 h thereafter.

Processing of cell suspension – cell separation

Samples of MGL were tested bacteriologically by inoculation onto blood agar plates containing 5% of washed ram erythrocytes and aerobic incubation at 37 °C for 24 h (Quinn et al., 1994). Only samples collected from healthy, noninfected glands were selected for further processing. Absolute cell counts in each MGL was determined using the conventional haemocytometer (Bürker cell). Cell viability, assessed by staining with Trypan Blue (Jain and Jasper, 1967), exceeded 97% in all the tested MGL. The cell suspensions were centrifuged at 200 x g and 4 °C for 10 min. One millilitre of the uppermost layer of the supernatant was withdrawn to be used, after the removal of the rest of the supernatant, for resuspension of the cell pellet.

The following techniques were used for morphological studies of apoptosis

Light microscopy (LM)

Cell suspension was prepared by the conventional haematological procedure and stained panoptically by the Papanicolau method (Bessis, 1973).

Transmission electron microscopy (TEM)

For TEM the MGL's cells were processed according with procedure published (Sládek and Ryšánek, 1999b).

Fluorescent microscopy (FM)

Smears from pellet were prepared using the standard haematological technique. Apoptosis of PMN was determined using a dye mixture of 10μM acridine orange and 10μM ethidium bromide (Sigma Chemical Co., Prague, Czech Republic) prepared in PBS (Duke and Cohen, 1992). Acridine orange (fluorescent DNA-binding dye) intercalates into DNA, making it appear green.

Ethidium bromide is only taken up by nonviable cells, overwhelming the fluorescence of acridine orange, making the chromatin of necrotic cells appear orange.

MGL histochemistry

The interaction of macrophages (MAC) with apoptotic PMN was assessed by staining for myeloperoxidase (MPO) using MGL of ten randomly selected mammary glands. Smears prepared by the conventional haematological method were dried, fixed in 2% glutaraldehyde in PBS for 5 min and stained with o-dianisidine in hydrochloric acid with hydrogen peroxide (Sigma Chemical Co., Prague, Czech Republic) as described by Henson et al. (1978). MAC with MPO-positive PMN got stained light orange to brown.

Thereafter, the same smears were stained using a modification of the Giemsa-Romanowski method. The MPO-positive areas in the cytoplasm of MAC acquired a granular structure and a contrastive brownish-grey hue. Moreover, pyknotic and strongly basophilic nuclei of apoptotic PMN became visible in the suspension or in the cytoplasm of the MPO-positive MAC.

After enumeration of 200 MAC, the interaction of MAC with apoptotic PMN was quantified by calculation of the percentage of MPO-positive from the total MAC count. The percentage of MAC with recently phagocytosed apoptotic PMN from the population of the MPO-positive MAC was also calculated.

Confocal microscopy (CM)

The smears stained for MPO as given above were also evaluated using the confocal microscope Olympus BX60 (Olympus, Hamburg, Germany) with the immersion Nomarsky lens.

RESULTS

Absolute and differential cell counts in MGL

The stimulation of the mammary glands with synthetic muramyl dipeptide resulted in a massive influx of cells into the cavitary system of the juvenile mammary glands. Already within 24 h, the absolute cell count in MGL rose to 37.8 x 10^6 per 1 ml (corrected for the standard 20 ml volume of PBS).

I. Characteristics of the cell population of the juvenile bovine mammary gland 24 h after stimulation with a synthetic derivative of muramyl dipeptide

Cell category	%
Normal PMN	81.8
Necrotic PMN	7.9
Apoptotic PMN	4.2
MAC	3.9
LYM	2.2

The cell population of MGL included highly predominating (94%) polymorphonuclear leukocytes (PMN), macrophages (MAC), and lymphocytes (LYM). Differential cell counts in MGL of the juvenile bovine mammary glands 24 h after the stimulation are shown in Tab. I. The structural abnormalities found in a part of the PMN population were interpreted as results of degeneration or necrosis, and apoptosis.

Structure of apoptotic PMN

The apoptotic PMN showed typical morphological characteristics. The whole process of programmed cell death could be divided into three morphologically distinct stages. Condensation of chromatin of nuclear segments (karyopyknosis) and their subsequent fusion into a single spherical structure were observed during early apoptosis (Figs. 1 and 2) which was further characterised by cytoplasm condensation and cell shrinking resulting in a reduction to almost one half of the original volume. Pseudopodia disappeared from the surface and the cells acquired a spherical shape (Figs. 1 and 2).

The second stage – zeiosis – was characterised by progressive blebbing of the cytoplasmatic membrane (Fig. 2) and disruption of the pyknotic nucleus into several small spheric fargments (Figs. 1, 2, and 3).

In the third stage, the disruption resulted in the formation of apoptotic bodies (Fig. 1) which can be described as relatively small (approx. 3 μm in diameter) structures with fragmented nuclei and condensed chromatin (Fig. 1).

The three stages of apoptosis were observed both in the free PMN in the suspension and in PMN already phagocytosed by MAC. Fig. 4 shows a MAC with vacuolised cytoplasm containing a recently phagocytosed, relatively intact apoptotic PMN with a distinct pyknotic nucleus and eosinophilic cytoplasm.

Ultrastructure of apoptotic PMN

Like in LM, three ultrastructurally different stages of apoptosis of PMN could be distinguished by transmission electron microcopy.

In the first stage nuclear chromatin condensed and aggregated to form an electron-dense semilunar or sickle-shaped mass surrounded by intact karyolemma. The nuclear segments merged into a single spherical structure with a central region of a less electron-dense mass and nucleolar prominence (Fig. 5). Like the nucleus, also the cytoplasm condensed. Azurophilic and specific granules and mitochondria were intact (Fig. 5). Glycogen particles disappeared from the cytoplasm, the number of granules was reduced, and small vacuoles were observed sporadically in some apoptotic PMN. By loss of pseudopodia, the cells acquired spherical shape.

Surface protuberances and bubbles as a sign of the forthcoming zeiosis with the subsequent nuclear and

whole cell fragmentation into apoptotic bodies (Fig. 6) were seen in apoptotic PMN.

The spherical or ovoid apoptotic bodies contained a nuclear fragment with condensed electron-dense chromatin and several granules (Fig. 7) Cytoplasmatic membranes of the apoptotic PMN and apoptotic bodies were intact.

TEM also confirmed the presence in MAC cytoplasm of phagocytosed PMN. Phagocytosed PMN at various stages of degradation of their nuclear and cytoplasmatic structures and apoptosis were found in phagosomes and phagolysosomes of the phagocytising MAC. The MAC phagosomes contained PMN both in the stage of early apoptosis and in the stage of apoptotic bodies.

Fig. 8 shows two phagocytosed PMN at different stages of cell structure degradation. While distinguishable morphological characteristics of apoptosis, such as condensation of nuclear chromatin, intact organelles, or vacuolisation of cytoplasm can be seen in the upper PMN, the lower PMN shows signs of advanced degradation of cell structures indicative of the active processing by MAC. Most of the phagocytosed apoptotic PMN were seen at this stage of degeneration.

Fluorescence microscopy of apoptotic PMN

Combined staining with acridine orange and ethidium bromide allowed us to distinguish between apoptotic and necrotic PMN.

FM highlighted particularly changes in the nuclear structure of PMN undergoing apoptosis. Unlike normal PMN with regularly segmented nuclei, the apoptotic PMN contained spherical, highly condensed, bright yellowish-green nuclei.

On the other hand, necrotic PMN were characterised by the presence of irregularly shaped, red-staining and mostly segmented nuclei (Fig. 9).

Both free and phagocytosed apoptotic PMN were clearly distinguishable. The cytoplasm of the MAC at the right of Fig. 9 is saturated with bright yellowish-green nuclei of the phagocytosed apoptotic PMN.

Histochemistry of apoptotic PMN

The staining for MPO allowed us to identify MAC containing in their cytoplasm myeloperoxidase originating from granules of phagocytosed apoptotic PMN. The distribution pattern of MPO depended on the time and stage of the degradation of the phagocytosed apoptotic PMN. An MPO-negative, i.e. non-phagocytising, MAC and an MPO-positive MAC containing a recently phagocytosed PMN with globular aggregation of the MPO-positive material can be seen at the extreme left and the extreme right of Fig. 10, respectively. The degradation of the phagocytosed apoptotic PMN leads to fragmentation of the MPO-positive material and its spreading in granular pattern throughout the cytoplasm of the MAC to the right and the MAC in the centre of Fig. 10. The same figure shows also an MAC with MPO-positive granules in the cytoplasm.

Our modification of the staining for MPO allowed the staining of cell nuclei in MGL and thus facilitated the identification of apoptotic PMN by their blue, spherical and pyknotic nuclei. The visualisation of PMN nuclei also allowed the identification of recently phagocytosed apoptotic PMN before their degradation in the cytoplasm of MAC (Fig. 11). The MPO-positive material of PMN and MAC got stained brown or grey (Figs. 10 and 11). Since the cytoplasm of the MPO-positive MAC contained phagocytosed material, their size was larger than that of the MPO-negative, nonphagocytising MAC. Both single and multiple phagocytosed apoptotic PMN could be observed in the individual MAC. The size of MAC was proportional to the number of phagocytosed PMN (Figs. 11 and 12). The percentages of MPO-positive MAC and MAC containing recently phagocytosed PMN are given in Tab. II.

II. Characteristics of the macrophage population after staining for myeloperoxidase

Cell category	%
MPO-negative MAC	90.7
MPO-positive MAC*	5.3
MAC with APMN**	4.0

* MAC containing diffuse MPO-positive material in cytoplasm
** MAC with recently phagocytosed apoptotic PMN in cytoplasm

Confocal microscopy of apoptotic PMN

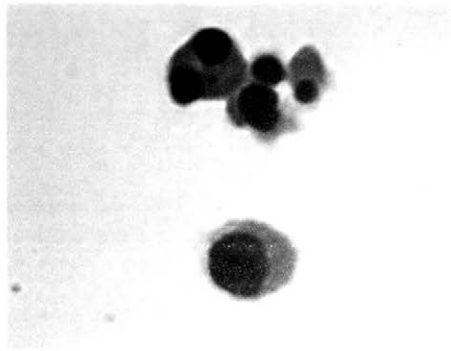
CM of preparations stained for MPO by the modified method allowed us to observe all stages of apoptosis of PMN as well as the process of phagocytosis and degradation by MAC of the apoptotic PMN. Fig.12 shows two MAC with phagocytosed PMN with structures analogous to those seen in Fig. 11.

DISCUSSION

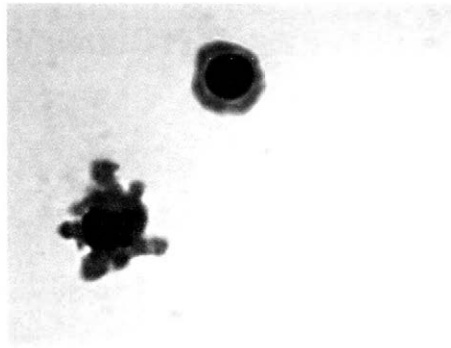
This paper deals with morphological characteristics of apoptosis of polymorphonuclear leukocytes collected from juvenile bovine mammary glands.

The morphology of PMN the juvenile bovine mammary gland, including structural abnormalities, was described in our previous papers (Sládek and Ryšánek, 1999a, b) which already pointed out two possible forms of death of PMN of the juvenile bovine mammary gland, i.e. necrosis and apoptosis. Both the forms were observed in the cavitary system of the mammary gland.

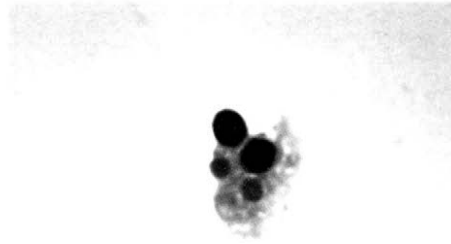
PMN for the morphological studies of apoptosis were collected by lavage of the cavitary system of the mammary gland stimulated with a synthetic derivative of



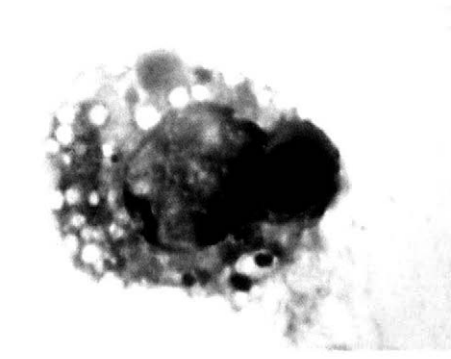
1. Apoptotic PMN: karyopyknosis (bottom); nuclear fragmentation (upper left); fragmentation into apoptotic bodies (upper right); magn. 1 000x



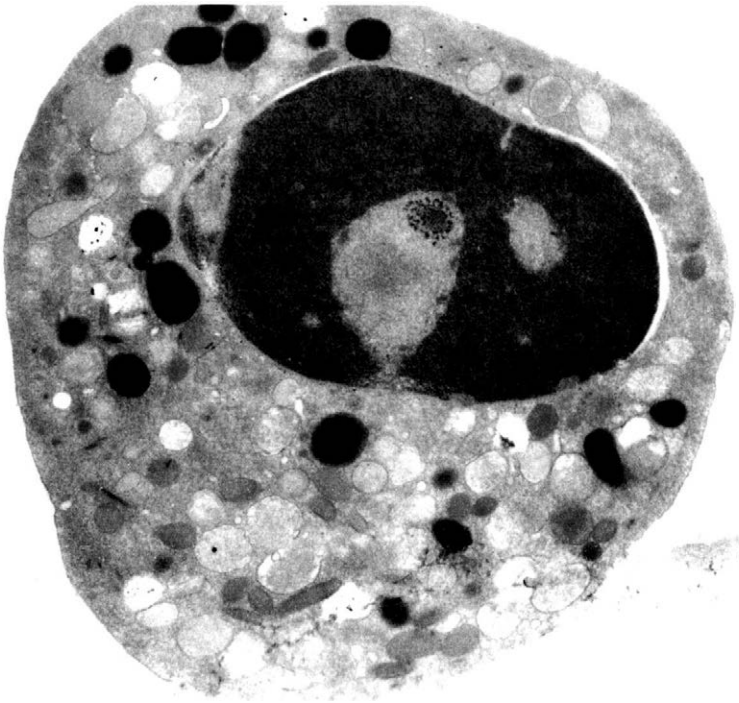
2. Apoptotic PMN: karyopyknosis (upper); zeiosis (bottom); magn. 1 000x



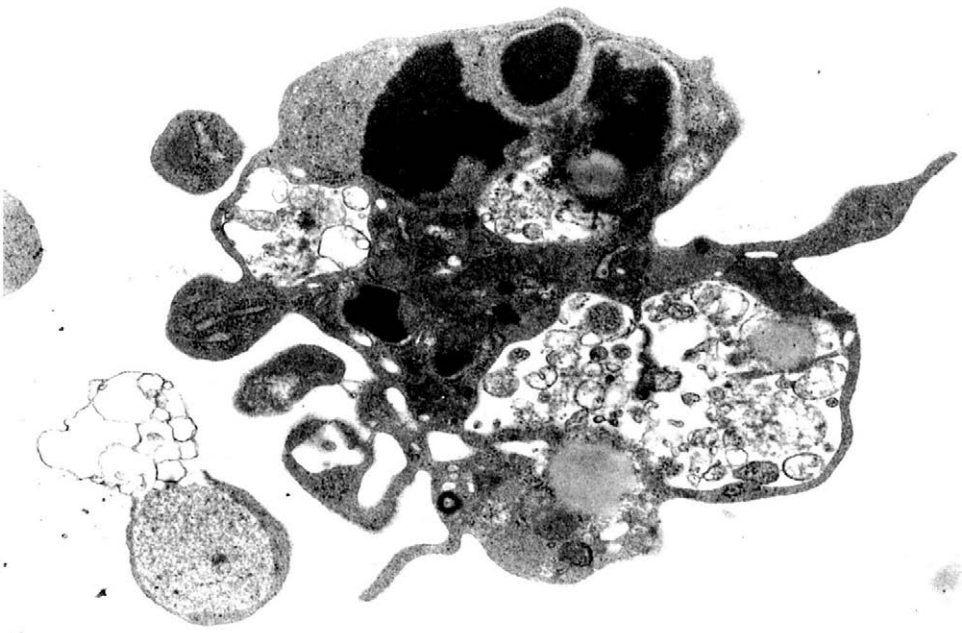
3. Apoptotic PMN: fragmentation of nucleus and whole cell; vacuolisation of cytoplasm; magn. 1 000x



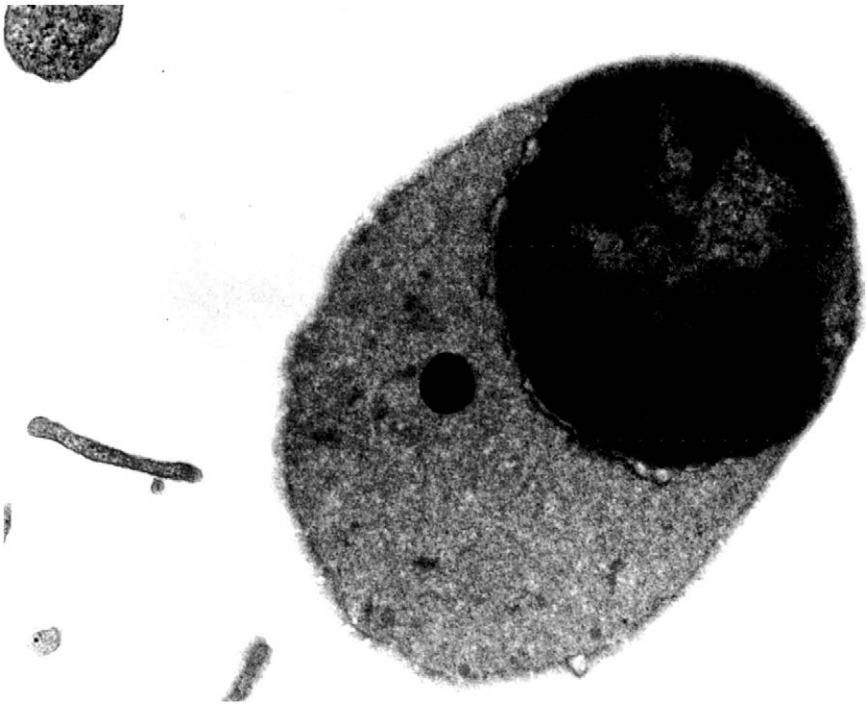
4. MAC with engulfed apoptotic PMN with distinct pyknotic nucleus and neutrophilic cytoplasm; magn. 1 000x



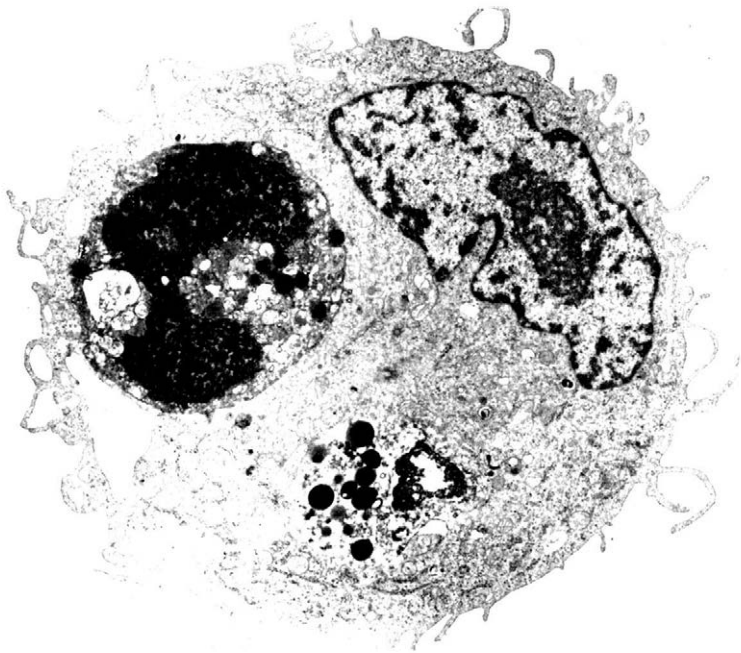
5. Apoptotic PMN: fusion of nuclear segments; nucleolar prominence; intact organelles; vacuolisation of cytoplasm; magn. 15 000x



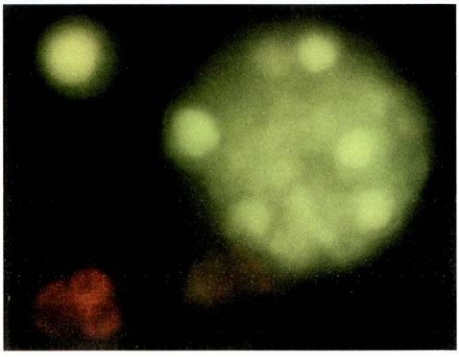
6. Apoptotic PMN: zeiosis with surface protuberances and fragmented nucleus; magn. 10 000x



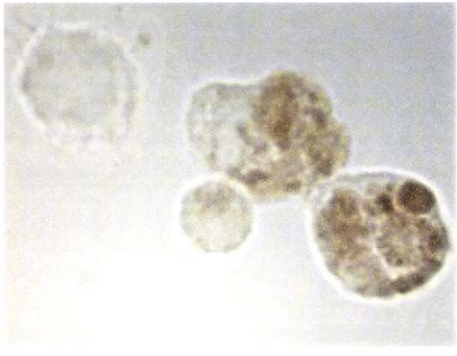
7. Apoptotic PMN: apoptotic body with a nuclear fragment containing electron-dense chromatin and a cytoplasmatic granule; magn. 25 000x



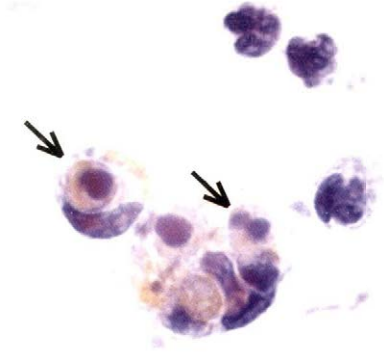
8. MAC with two phagocytosed apoptotic PMN; the upper, relatively intact, PMN shows a distinct pattern of apoptosis (condensation of nuclear chromatin, intact organelles, vacuolisation of cytoplasm); the lower is in an advanced stage of degeneration of cell structures; magn. 5 000x



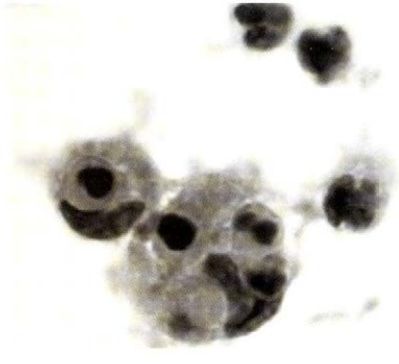
9. FM of apoptosis and necrosis of PMN: apoptotic PMN with spherical nucleus containing condensed chromatin (upper left); necrotic PMN with a red, segmented nucleus (bottom left); MAC with cytoplasm saturated by phagocytosed apoptotic PMN (right); magn. 1 000x



10. Staining for MPO: MPO-negative nonphagocytising MAC (upper left); MAC containing diffuse MPO-positive material in cytoplasm (upper, mid); MAC with both diffuse and aggregated MPO-positive material in cytoplasm (right); PMN with PMO-positive material in granules (centre); magn. 1 000x



11. Two MAC with phagocytosed apoptotic PMN (➔) containing MPO-positive material in cytoplasm (bottom left); MPO-positive material released by lysis of phagocytosed apoptotic PMN can also be seen in cytoplasm of both the MAC; inactive PMN (right); magn. 1 000x



12. CM of the same object as imaged in Fig. 11; magn. 1 000x

muramyl dipeptide. Such method for inducing an influx of PMN into the cavitary system of the mammary gland with the aim to obtain phagocytising cells for *in vitro* studies was first described by Wardley et al. (1976).

The cell population obtained by this method included PMN with a normal structure, as well as PMN showing morphological alterations typical of both necrosis and apoptosis. Such finding is not mentioned in the paper by Wardley et al. (1976).

Our findings indicating that the first signs of apoptosis appear in a part of PMN already 24 h after stimulation correspond to the short life span of this cell type in tissues which is estimated to 1 and 2 days after leaving the blood vessels (Squier et al., 1999).

Morphological alterations in necrotic PMN were described in a previous paper (Sládek and Ryšánek, 1999a, b). The apoptotic PMN showed the major structural features of cell apoptosis as defined by Wyllie et al. (1980), including chromatin condensation (karyopyknosis), cell shrinking, loss of pseudopodia, and spherical cell shape in light and confocal microscopy, and chromatin aggregation, intact organelles and cytoplasmatic membrane, and vacuolisation of cytoplasm in transmission electron microscopy. No description of PMN isolated from the bovine mammary gland *in vivo* and showing morphological characteristics of programmed cell death were found in the available literature.

Apoptotic PMN are promptly phagocytosed and ingested by MAC (Savill et al., 1989). Such event was also observed in our studies of apoptotic PMN isolated from juvenile bovine mammary glands. Our electron microscopic studies indicate that PMN are phagocytosed at various stages of apoptosis. This is a clear evidence that MAC can identify and phagocytise PMN at both the early and the late stage of apoptosis (apoptotic bodies). A confrontation of the slow development of apoptosis with the rapid *in vitro* recognition and phagocytosis of apoptotic PMN by MAC, as demonstrated by Wyllie et al. (1980) and Savill et al. (1989), might explain the relatively low percentage of apoptotic PMN in the cell population of MGL of stimulated juvenile mammary glands.

Like the recognition and phagocytosis, the degradation of apoptotic PMN by MAC is apparently a very rapid process. This assumption is indicated by the distribution pattern of the MPO-positive material in the phagocytising MAC. *In vitro* studies of Savill et al. (1989) demonstrated the onset of interaction of MAC with apoptotic PMN already after 10 min and fragmented or granular appearance of the MPO-positive material in MAC after one hour. Newman et al. (1982) and Savill et al. (1989) interpret such a finding as a result of degradation of engulfed apoptotic PMN. In our preparations, the MPO-positive material was distributed in whole cytoplasm in more than one half of the MPO-positive MAC. This finding allows us to assume that apoptotic PMN are present in the cavitary system of the mammary gland sooner than 24 h after the stimulation.

It is apparent that MAC participate actively in the removal of apoptotic PMN from the cavitary system of the juvenile mammary gland. Our current observations (data in preparation) confirm the important role played by apoptosis and subsequent phagocytosis by MAC of PMN in the resolution of acute damage to the mammary gland. The same conclusion was arrived by authors dealing with acute arthritis of the knee joint in the man (Savill et al., 1989), nephritis (Savill et al., 1992), pneumonia (Grigg et al., 1991), experimentally induced peritonitis in guinea pigs (Yamamoto et al., 1993), experimentally induced pneumonia in rats (Cox et al., 1995; Hussain et al., 1998; Ishii et al., 1998), experimental glomerular capillary injury in rats (Hughes et al., 1997) and experimental injury in rats (Meszaros et al., 1999).

Considering the predisposition of PMN to apoptosis (Raff, 1992), no degenerating PMN should be hypothetically found in the cavitary system of the juvenile mammary gland. However, since the apoptotic programme is triggered only in PMN unaffected by signals alterating the programme, PMN perish also by sudden death, i.e. necrosis preceded by degenerative alterations (Cooter et al., 1994; Haanen and Vermes, 1995). The percentages of necrotic and apoptotic PMN in MGL of the juvenile mammary gland are suggestive of probable activity in the cavitary system of signals which partly block the apoptotic programme of PMN. Therefore, degeneration and necrosis replace apoptosis in a part of PMN. The necrosis-inducing signals include a number of well known factors, of which some are specific for the bovine mammary gland (Russell et al., 1976, 1977; Paape et al., 1977; Paape and Guidry, 1977; Paape and Wergin, 1977; Nickerson et al., 1985; Sládek and Ryšánek, 1999a, b).

Further studies will be necessary to quantify the inhibitory or inducing effects of such signals on the apoptosis of PMN.

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A COMPARISON OF THE SERUM LEVEL KINETICS AND BIOLOGICAL HALF-LIFE OF OXYTETRACYCLINE AFTER LONG ACTION PREPARATIONS (TETRAVET 20% L.A. INJ. AND ENGEMYCIN 10% L.A. INJ.) IN SHEEP AND THEIR TOLERANCE*

POROVNANIE KINETIKY SÉROVÝCH HLADÍN A BIOLOGICKÝ POLČAS OXYTETRACYKLÍNU U OVIEC PO PRÍPRAVKOCH S PREDĹŽENÝM ÚČINKOM (TETRAVET 20% L.A. INJ. A ENGEMYCIN 10% L.A. INJ.) A ICH ZNÁŠANLIVOSŤ

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ABSTRACT: Blood serum concentrations and biological half-life of oxytetracycline after long action preparations were investigated in adult sheep of the Slovak Merino breed. Contemporarily their local tolerance has also been observed. In the first group oxytetracycline was administered in form of the preparation Tetravet 20% L.A. inj. a.u.v. (Sanofi, Ltd., France) and in the second group in form of the preparation Engemycin 10% L.A. inj. a.u.v. (Intervet, Ltd., The Netherlands). Oxytetracycline was administered intramuscularly at a single dose of 20 mg per kg of live weight. The blood serum concentrations of oxytetracycline were studied in the intervals of 1st, 6th, 24th hours and 2nd, 3rd, 4th, 5th, 6th, 7th and 8th days after single administration of preparations. Oxytetracycline was determined by high HPLC chromatography (Sokol and Matisová, 1994). The significantly higher serum concentrations of oxytetracycline ($p < 0.05$) were recorded after preparation Tetravet L.A. from 24th hour to 5th day. Concentrations of oxytetracycline were detected on the 6th day after Tetravet L.A. preparation and in the case of Engemycin L.A. on the 5th day. Longer half-life was registered after Tetravet L.A. preparation (40–42 hours). After Engemycin L.A. this time was registered as 37–39 hours. Tetravet L.A. does not induce any local reactions at the site of administration. In contrast to Tetravet L.A., Engemycin L.A. caused inflammatory edematisation in 50% of animals which persisted 3 days only, without other complications.

oxytetracycline; sheep; Tetravet L.A.; Engemycin L.A.; serum concentrations and half-life of oxytetracycline; local tolerance

ABSTRAKT: Na dospelých ovciach plemena Slovenské merino sa sledovali sérové koncentrácie a biologický polčas oxytetracyklínu po prípravkoch s predĺženým účinkom. Súčasne sa sledovala aj ich lokálna znášanlivosť. Oxytetracyklín sa jednej skupine podával vo forme prípravku Tetravet 20% L.A. inj. a.u.v. (Sanofi Ltd., Francúzsko) a druhej vo forme prípravku Engemycin 10% L.A. inj. a.u.v. (Intervet Ltd., Holandsko). Oxytetracyklín bol aplikovaný intramuskulárne do stehenného svalstva v dávke 20 mg/kg živej hmotnosti. Koncentrácie oxytetracyklínu v krvnom sére sme sledovali v 1., 6. a 24. hodine a na 2., 3., 4., 5., 6., 7. a 8. deň po jednorázovej aplikácii prípravkov. Oxytetracyklín sa stanovoval chromatograficky podľa autorov Sokol a Matisová (1994) na kvapalinovom chromatografe. Po Tetravete L.A. sa zistili významne vyššie sérové koncentrácie oxytetracyklínu ($p < 0,05$) od 24. hodiny až po 5. deň sledovania. Merateľné koncentrácie boli zachytiteľné ešte na 6. deň po Tetravete L.A. a na 5. deň po Engemycine L.A. Dlhší biologický polčas bol zaregistrovaný po Tetravete L.A. (40 až 42 hodín). Pri Engemycine L.A. činil 37 až 39 hodín. V mieste vpichu Tetravet L.A. nevyvolal lokálne reakcie, kým Engemycin L.A. u 50 % jedincov vyvolal zápalové opuchy pretrvávajúce tri dni bez ďalších komplikácii.

oxytetracyklín; ovce, Tetravet L.A.; Engemycin L.A.; sérové koncentrácie a biologický polčas oxytetracyklínu; lokálna znášanlivosť

INTRODUCTIONS

Equivalent drugs, i.e. drugs containing identical medical substance with the same purity (quality) and the same mass (dose) turned out not to have necessarily the same efficiency (Chalabala and Mandák, 1977; Rak and Chalabala, 1984; Švec, 1998). Equivalent drugs can differ also in general or local tolerance (Hirtz, 1972). These findings were the basis for introduction of the problem concerning bioavailability of drugs-level

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determination of drug applicability. Successive studies aimed at clarification of factors affecting this important pharmacokinetic phenomenon contributed to the fact that an increased attention started to be paid to the drug form because of its special importance for drug effectiveness (Zathurecký et al., 1989). The result of this effort is development of drug forms of higher generations. Drugs of the second generation are preparations with a controlled release of drug. Adjuvant substances which are added slow up the release of drug, which makes it possible to keep the concentration of drug in blood at a desired level for a longer time (Zathurecký et al., 1989; Chalabala et al., 1983). Tetracycline preparations with protracted effect (long action – L.A.) are also the result of the above mentioned biopharmaceutical and pharmacological studies. The advantages of tetracyclines L.A. against classical ones are in general known. The advantages of preparations with protracted effect (long action – L.A.) come from changed pharmacokinetics of tetracycline antibiotic as a result of the use of viscous vehicle on the basis of modern polymers (polyvinylpyrrolidone, dimethylacetamide, glycerol-formaldehyde, N-methylpyrrolidone, aluminium monostearate), or other adjuvant substances. Not only pharmacokinetic parameters, but also irritability, oedema, or possible occurrence of necrotic changes at the site of i.m. application of L.A. preparations may be influenced by the type of solvent (Nouws et al., 1990; Svendsen, 1989). Concentration of preparations also plays an important role. Tetravet 20% L.A. inj. and Engemycin 10% L.A. inj. often used in practice are on the basis of oxytetracycline. The former contains the vehicle dimethylacetamide and Engemycin 10% L.A. polyvinylpyrrolidone. On the basis of the above mentioned fact we started to compare kinetics of serum levels of oxytetracycline and biological half-life in sheep after one application of the mentioned long action preparations: Tetravet 20% L.A. inj. and Engemycin 10% L.A. inj. The aim of the work was to judge local reactions at the site of application and tolerance of the medications.

MATERIAL AND METHODS

Characteristics of preparations

Tetravet 20% L.A. inj. is a pure dark brown liquid. According to the producers data the preparation contains 200 mg of oxytetracycline per 1 ml and 1.85 g of magnesium oxide per 100 ml of preparation. Dimethylacetamide is indicated as a solvent.

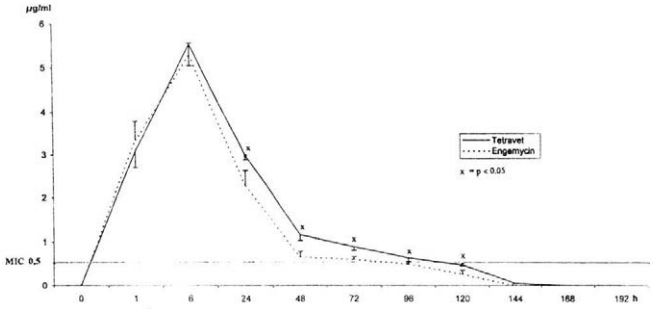
Engemycin 10% L.A. inj. is a pure yellow liquid. According to the producer s data it contains 100 mg of oxytetracycline per 1 ml. Polyvinylpyrrolidone is indicated as a solvent.

Experimental set

The experiment was conducted with a set of 8 adult sheep, Slovak merino breed weighing 40–55 kg. The experimental set was divided into two groups. In the first group oxytetracycline was administered in the form of Tetravet 20% L.A. inj. a.u.v. (Sanofi Ltd., France). In the second group oxytetracycline was administered in the preparation form of Engemycin 10% L.A. inj. a.u.v. (Intervet Ltd., The Netherlands). Oxytetracycline was administered to animals in a single dose of 20 mg/kg of live weight i.m. to thigh muscle. Engemycin was administered in a volume of 2 ml/10 kg of live weight and Tetravet in a volume of 1 ml/10 kg of live weight. At one site of application Engemycin was administered in volume to 10 ml and Tetravet in volume to 5 ml. Concentrations of tetracycline in blood serum were in the 1st, 6th and 24th hour, then on day 2, 3, 4, 5, 6, 7 and 8 after application of preparations. Oxytetracycline was determined on the liquid chromatograph of Hewlett Packard firm (Avondale, PA, USA) series 1050, at wave length 360 nm with sensitivity 0.05 μg/ml. Concentration of oxytetracycline was given in μg/ml. Constants of elimination were calculated using the least squares method according to one-compartment pharmacokinetic model. Constants of elimination from serum levels were computed from a decreasing curve from the interval 1 up to 120 hours. Biological half-life was calculated like this: mean constant of elimination ln 2. Statistical evaluation was provided by Student's *t*-test. Local reaction in the site of drug application was evaluated according to painful reaction of animals for palpation, presence of oedema and increased local temperature. Tolerance of preparations was evaluated according to the general behaviour of animals, feed intake and droppings consistence.

RESULTS

Fig. 1 demonstrates dynamics of changes in oxytetracycline serum levels in sheep after a single Tetravet 20 % L.A. inj. and Engemycin 10 % L.A. inj. application. It is seen from the figure that there are differences in serum levels hight (quantitative differences), but dynamics of level changes in the individual observed time periods is substantially the same. In the first observed time interval (first hour) we notice unimportant higher concentrations of oxytetracycline (3.34 μg/ml) after Engemycin application compared to Tetravet (3.09 μg/ml). In all other time intervals we observe, on the contrary, higher concentrations after Tetravet application. These differences are not statistically significant in 6th hour, but in 24th hour and on day 2, 3, 4, and 5 of observations they are statistically significantly higher ($p < 0.05$) after Tetravet application compared to Engemycin. Maximum serum concentrations are recorded in 6th hour after application of both preparations. In this time interval the level of oxytetracycline is 5.52 after Tetravet application and 5.28 μg/ml after Engemycin application. Therapeutic concentrations of oxytetracycline (MIC above 0.5 μg/ml) are still recorded on day 4 after Tetravet (0.63 μg/ml) and on day 3 after Engemycin (0.60 μg/ml) application. Measurable concentrations after Tetravet



1. Blood serum concentrations of oxytetracycline in sheep after simple administration of preparations Tetravet 20% L.A. inj. and Engemycin 10% L.A. inj.

1. Blood serum concentrations of oxytetracycline in sheep after simple administration of preparations Tetravet 20% L.A. inj. and Engemycin 10% L.A. inj.

Administration of preparations	Values in µg per ml of blood serum in time intervals									
	1h	6h	24h	48h	72h	96h	120h	144h	168h	192h
Tetravet 20% L.A. inj.	3.09 ± 0.38	5.52 ± 0.47	2.98 ± 0.10	1.16 ± 0.12	0.89 ± 0.08	0.63 ± 0.08^x	0.47 ± 0.01^x	0.06 ± 0.01^x	0	0
Engemycin 10% L.A. inj.	3.34 ± 0.44	5.28 ± 0.27	2.26 ± 0.36^x	0.65 ± 0.13^x	0.60 ± 0.05^x	0.49 ± 0.05^x	0.25 ± 0.08^x	0	0	0

x = $p < 0.05$

application were still perceivable on day 6 (0.06 µg/ml) and after Engemycin application on day 5 (0.26 µg/ml).

Tab. I. shows a survey of serum oxytetracycline levels in the observed time intervals. No changes in general behaviour, feed intake and in droppings consistence elevated registered after any preparation. We recorded painful reactions for palpation and raised temperature at the site of puncture in all animals one hour after application of Engemycin. Significant warm oedema was gradually developed in two of animals and it persisted for 3 days. We did not record any local changes at the site of puncture in three animals but short-time hyperaesthesia for palpation (within 12 hours) was registered in one sheep after Tetravet application.

DISCUSSION

As the pharmacotherapeutic effect of drugs depends mainly on their bioavailability (relative drug amount which can get from the site of application to systemic circulation in unchanged form in a certain time) and on drug concentration in biophase (i.e. in the environment, in which drug comes into direct contact with receptors (Holomáň, 1984), we decided to find out if and to what extent various vehicles comprised in Tetravet L.A. and Engemycin L. A. influence oxytetracycline kinetics and local tolerance of tested preparations. Pharmaceutical adjuvants have also an important function in biological accessibility and drug survival in the organism besides dose, drug form, the way of application, metabolism speed, resorptive processes and chemical properties of the drug.

Parenteral repository preparations are possible to gain by more ways. One of the ways of their preparation is realized by using modern adjuvants. Various synthetic polymers belonging to adjuvant pharmaceutical substances have an important position in their preparation. These polymers are dissociated into nontoxic components in the organism in a hydrolytic or enzymatic way (Rak et al., 1985). Biodegradable polymers are the carriers of so called long action effect. Long action preparations on the basis of oxytetracycline tested by us also contain biodegradable polymers. It follows from the fact that producers indicate dimethylacetamide in Tetravet L.A. and polyvinylpyrrolidone in Engemycin L.A. as a solvent. At single *i.m.* oxytetracycline application in dose 20 mg/kg of live weight they indicate its therapeutical levels (over 0.5 µg/ml) from 30 minutes after application for 4–5 days in Tetravet L.A. and at least for 3 days in Engemycin L.A. They also indicate minimal irritability at the site of *i.m.* application. It results from our comparative studies that there are quantitative differences in serum levels of oxytetracycline between Tetravet L.A. and Engemycin L.A., but the dynamics of level changes in individual observed time periods is basically the same. In the first observed time interval (first hour) we recorded a nonsignificantly higher oxytetracycline concentration (3.34 µg/ml) after Engemycin against levels after Tetravet application (3.09 µg/ml). In all other time intervals, on the contrary, we recorded higher concentrations after Tetravet. In 6th hour the differences were statistically nonsignificant while in 24th hour and on day 2, 3, 4, and 5 of observations they were statistically significantly higher

($p < 0.005$) after Tetravet application compared to Engemycin. The highest levels after application of both preparations were recorded in 6th hour. In this time interval the measured oxytetracycline level after Tetravet application was 5.52 μg/ml and after Engemycin 5.28 μg/ml.

Therapeutic concentrations after Tetravet application were still recorded on 4th day (0.63 μg/ml) and after Engemycin on 3rd day (0.60 μg/ml). Measurable concentrations were perceivable still on day 6 after Tetravet and on day 5 after Engemycin application. Different course of serum levels accords with different levels of another pharmacokinetic index – biological half-life. It is 40–42 hours in Tetravet and 37–39 hours in Engemycin. Higher serum level of oxatetracycline registered in the first hour after Engemycin shows its faster resorption from the application site compared to Tetravet. But a steeper decrease of oxytetracycline serum levels recorded from 6th hour after Engemycin and its survival in therapeutical concentrations for a shorter time than after Tetravet shows a faster start of eliminative phase in Engemycin. The stated statistically significantly higher serum levels of oxytetracycline and its survival in therapeutic concentrations one day longer after Tetravet L.A. application compared to Engemycin L.A. at the same dose in the form of basis, witnesses about the probably different influence of polyvinylpyrrolidone and dimethylacetamide on the kinetics of oxytetracycline especially in the phase of resorption. It is not possible to exclude a factor of higher oxytetracycline concentration in Tetravet from probable causes, mainly quantitatively different kinetics of oxytetracycline besides different vehicles between two compared preparations. Considering that a higher drug concentration in a smaller volume of injected dissolvent rather accelerates resorption, it is interesting that in the first time period (1st hour) a lower serum concentration of oxytetracycline after Tetravet application was recorded. It is not possible to unambiguously deduce from these facts what is the contribution of different oxytetracycline concentrations in preparations or different vehicles in diverse oxytetracycline kinetics. We do not know studies concerning with oxytetracycline kinetics observation in tetracycline long action preparations depending on the type of polymer contained in them. From the results of studies dealing with pharmacokinetic problems of long action preparations it is seen that differences in oxytetracycline serum levels or in its survival in blood there are differences also in the same species of animals depending on the used long action preparation (Cohen et al., 1993; Vyhnálek and Hera, 1994; Escudero et al., 1994; Arqum et al., 1995). The above mentioned studies substantially correspond to our findings. No general side reactions were observed after using the tested preparations and they were very well tolerated. In local irritability we recorded differences between the preparations. Tetravet did not cause any reactions at the site of puncture, while Engemycin induced inflammatory oedema persisting for 3 days without other complications in 50% of animals. In general a higher drug volume especially at *i.m.* application causes the development of painful reaction. But also injected solution with higher concentration induces higher local irritability. Our results suggest that the type of vehicle contained in preparations participated in higher level in differential degree of local irritability of tested preparations than factor of applicated volume. It is confirmed by the fact that at one site of application Engemycin was used in higher volume (up to 10 ml) than Tetravet (up to 5 ml) but in lower concetration (10%) compared to Tetravet (20%). Irritability degree of tested preparations found by us is not the same as findings of Nouws (1990).

Our findings suggest that Tetravet L.A. appears more beneficial in observed pharmacokinetic parameters as well as in tolerance compared to Engemycin L.A. It was proved that after Tetravet L.A. application oxyteracycline serum concentrations ($p < 0.05$) in all observed time intervals, except in 1st and 6th hours, were statistically significantly higher compared to Engemycin L.A. The same was proved by finding that Tetravet L.A. MIC survives one day longer and is also locally better tolerated.

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EFFECTS OF SEX AND AGE UPON CADMIUM RETENTION IN THE ORGANISM OF CHICKENS*

VLIV POHLAVÍ A VĚKU NA RETENCI KADMIA V ORGANISMU KUŘAT

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ABSTRACT: Effects of sex and age of chickens upon cadmium retention were investigated in an experiment with 8 groups of chickens receiving 0.404 mg of Cd and another 8 groups receiving only 0.348 mg of Cd per 1 kg of consumed dry matter. In the mixture with a lower level of Cd, fish meal, blood flakes and torula yeast were replaced by meat-and-bone meal. During the growing period from Day 12 to Day 56, the experiment was divided into 15 balance periods. From the mixture with a lower level of Cd, a significantly ($P < 0.05$) higher amount of this element was retained; percentage of deposited Cd in the organism was dependent on the source of Cd. Retention of cadmium and the content of this element in live body gain increased highly significantly ($P < 0.01$) with age. The growth rate of the total amount of Cd in the organism was higher than that of body weight. Allometric coefficients were 1.016 ± 0.0129 and 1.099 ± 0.0215 for chickens fed on diets with 0.404 and 0.348 mg of Cd, respectively. The difference was highly significant ($P < 0.01$). The effect of sex on the values under study was not significant.

chickens; effect of sex; effect of age; cadmium retention; growth allometry

ABSTRAKT: Vliv pohlaví a věku kuřat na retenci kadmia jsme sledovali v pokusu s osmi skupinami kuřat, která přijímala 0,404 mg Cd a osmi skupinami kuřat, která přijímala pouze 0,348 mg Cd na 1 kg spotřebované sušiny. Ve směsi s nižším obsahem kadmia byla rybí moučka, krevní vločky a torula nahrazeny masokostní moučkou. Experiment byl rozdělen do 15 bilančních období od 12. do 56. dne života. Ze směsi s nižším obsahem kadmia se ukládalo průkazně ($P < 0,05$) větší množství tohoto prvku; procento kadmia uloženého v organismu záviselo na zdroji Cd. Retence kadmia i jeho obsah v přírůstku živé hmotnosti se s věkem vysoce průkazně ($P < 0,01$) zvyšoval. Relativní rychlost růstu celkového množství Cd v organismu byla vyšší než rychlost růstu tělesné hmotnosti. Alometrické koeficienty pro kuřata krmená dietou obsahující 0,404 mg Cd byly 1,016 ± 0,0129 a pro kuřata s příjmem 0,348 mg Cd 1,099 ± 0,0215. Rozdíl byl vysoce průkazný ($P < 0,01$). Vliv pohlaví na sledované ukazatele byl neprůkazný.

kuřata; vliv pohlaví; vliv věku; retence kadmia; alometrie růstu

INTRODUCTION

For its adverse effect, above all on the fertility of animals and people, cadmium belongs to the most hazardous environmental pollutants. As defined by Regulation No. 194/1996 of the Ministry of Agriculture of the Czech Republic, complete feed mixtures for poultry shall not contain more than 0.5 mg Cd/kg at the dry matter content of 88%.

The retention of cadmium from feedstuffs is not always constant when fed to the same type of animals and may be dependent on a number of factors. In an experiment conducted by Bundscherer et al. (1985) feeding zinc-deficient diet resulted in a 40% higher cadmium retention. Addition of vitamin D, dietary calcium and iron reduced a cadmium burden in the organism while a dramatic increase in the pollutant level was observed with high dietary supplements of copper (Rothe et al., 1992). In a trial carried out by Rambeck and Guillot (1996), the addition of ascorbic acid lowered cadmium accumulation in kidney and liver by about 40% and phytase decreased the bioavailability of Cd by as much as 60%. Srebocan et al. (1988) managed to reduce cadmium deposition in the chicken tissues with hydrated aluminium silicate montmorillonite and Herzig et al. (1994) with a sodium humate supply in the diet. Cadmium retention can be influenced by various forms of its bonds (Bundscherer, 1984).

The objective of this experiment was to determine if there was a sexual dimorphism in the retention of this element and to study how the retention of cadmium from the feed mixture changed in the course of chick

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feeding. To evaluate the effect of age as exactly as possible, it was necessary to carry out a great number of estimations in very short time intervals during a longer period of life.

MATERIAL AND METHODS

Effects of sex and age on cadmium retention were investigated within 15 successive three-day balance periods using Ross hybrid chickens during the growth period from the 12th to the 56th day of age.

At the age of 7 days, the sexed chicks were distributed according to their body weight into 16 groups of 8 birds each. Eight groups of females and eight of males were kept in balance cages. Four groups of female and four of male chickens were fed on a commercial broiler starter diet (BR1) during the whole experimental period, while the remaining ones were fed BR2, a practical type of finisher diet (Tab. I). Chromic oxide was added to the diet as an indicator. The mixture BR1 contained 228 g crude protein per kg dry matter and the average content of nitrogen-corrected apparent metabolisable energy (AME_n), estimated in 120 balance experiments, was 12.64 MJ/kg. The contents of crude protein and AME_n per kg dry matter of BR2 were 211 g and 13.88 MJ, respectively. Pelleted feed was supplied *ad libitum*. Chickens fed on BR1 received an average of 0.404 mg of Cd per kg of consumed dry matter in the feed mixture while birds fed on BR2 only 0.348 mg of Cd. In the mixture with the lower level of Cd, fish meal, blood flakes and torula yeast were replaced by meat-and-bone meal. Cadmium was not present in drinking water. The body weight of chickens was estimated at the end of each balance period and the values were used in allometric calculations.

I. Formulation of diets (g/kg)

Ingredient	BR 1	BR 2
Maize meal	400	560
Wheat meal	186	78
Soybean meal	256	200
Fish meal	30	10
Meat-and-bonemeal	0	50
Lucerne meal	30	30
Shim milk powder	20	20
Torula yeast	20	10
Blood flakes	20	0
Cereal germs	15	0
Added fat	0	20
Supplementary premix[a]	10	10
Mineral premix[b]	10	10
Sodium chloride	3	2

a – the premix supplied the following (mg/kg diet): retinyl acetate, 3.44; cholecalciferol, 0.025; DL-α-tocopherol acetate, 10; menadione, 2; riboflavin, 3; hydroxycobalamin, 0.02; niacin, 10; pantothenic acid, 4; DL-methionine, 1000; ethoxyquin, 125 and a coccidiostat (Amprol Plus)

b – the premix supplied the following per kg diet: ground limestone 6.7 g; calcium phosphate 1.7 g; steamed bone meal 1.5 g; copper 4 mg; iron 12 mg; zinc 25 mg; manganese 28 mg; iodine 0.4 mg

Excreta were collected daily for each three-day balance period. The coefficients of cadmium utilization were estimated using the chromic oxide indicator method. The content of chromic oxide in feed and freeze-dried excreta was estimated iodometrically (Mandel et al., 1960). Cadmium was estimated after wet mineralization with nitric acid and perchloric acid by the flame atomic absorption spectrometry using Philips PU9200X Atomic Absorption Spectrometer at wavelength 228.8 nm (Regulation No. 222/1996 of the Ministry of Agriculture of the Czech Republic). There were three replications in each determination. The difference between parallel estimations was lower than 11 relative per cent. Our results on reference materials (feed mixtures for poultry for interlaboratory tests of the Central Institute for Supervising and Testing in Agriculture) are in agreement with certified values. The recovery was 95.2 ± 2.95 per cent (mean ± standard error of the mean; 10 determinations).

The analysis of variance and regression of determined values was performed according to Snedecor and Cochran (1967).

For the expression of a relation between cadmium retained in the body and live body weight of chicken a power function was used (Brody, 1945):

$$Y = aX^b,$$

where Y – content of Cd in the body in ng,
X – live body weight of chicken in g,
a – extrapolation of Y for $X = 1$,
b – allometric coefficient, ratio of the percentage change in Y to the corresponding percentage change in X.

RESULTS AND DISCUSSION

The average coefficients of cadmium retention from mixture BR1 estimated in females and males were 0.106 ± 0.0116 (mean ± standard error of the mean) and 0.099 ± 0.0124, respectively, and those of mixture BR2 were 0.143 ± 0.0106 and 0.156 ± 0.0139 in females and males, resp. Analysis of variance showed that the effect of sex was insignificant ($P > 0.05$). For diets containing 0.404 and 0.348 mg of cadmium, the average coefficients of retention, when involving values measured in both sexes into the mean, were 0.102 ± 0.0085 and 0.150 ± 0.0087, respectively. The difference was highly significant ($P < 0.001$). It means that 41.2 ± 3.43 ng (BR1) and 52.2 ± 3.03 ng (BR2) of cadmium per each gram of consumed dry matter were retained in the organism. From the mixture with a lower level of Cd, a significantly ($P < 0.05$) higher amount of this element was retained. The percentage of deposited Cd in the organism was dependent on the source of this element. It was confirmed that cadmium retention was influenced by various forms of its bonds (Bundscherer, 1984).

The effect of age was highly significant ($P < 0.01$). The dependence of Cd retention coefficients upon age of chickens in days was expressed by linear regression equations

$Y = -0.005 + 0.00320\ X$; $r = 0.465$, $P < 0.01$ for females,
$Y = 0.015 + 0.00238\ X$; $r = 0.324$, $P < 0.01$ for males,
$Y = 0.005 + 0.00279\ X$; $r = 0.392$, $P < 0.01$ for both sexes,

and

$Y = 0.044 + 0.00284\ X$; $r = 0.455$, $P < 0.01$ for females,
$Y = 0.046 + 0.00315\ X$; $r = 0.384$, $P < 0.01$ for males,
$Y = 0.045 + 0.00300\ X$; $r = 0.410$, $P < 0.01$ for both sexes

for mixtures BR1 and BR2, respectively. In all cases the retention of Cd increased with increasing age. Regression coefficients were highly significant ($P < 0.01$).

In chickens fed on BR1 and BR2, the average contents of Cd per 1 g of live body gain were 84.5 ± 7.88 ng and 104.8 ± 7.09 ng, respectively. The difference was not significant ($P > 0.05$). The relationship between Cd content (ng) in body weight gain and age (d) of chickens fed on a Cd higher diet, as estimated in 120 balance experiments, was expressed by the straight line equation

$$Y = -34.7 + 3.40\ X;\ r = 0.513,\ P < 0.01$$

and for those fed on a low Cd mixture by the equation

$$Y = -9.42 + 3.26\ X;\ r = 0.547,\ P < 0.01$$

Both regression coefficients were highly significant ($P < 0.01$).

Relations between the total amount of cadmium in the body (ng) and body weight of chickens (g) were expressed by the equation for all chickens fed on the mixture

BR 1 $Y = 0.0559\ X^{1.016}$; $I_{YX} = 0.922$, $P < 0.01$ and
BR 2 $Y = 0.0376\ X^{1.099}$; $I_{YX} = 0.913$, $P < 0.01$.

The growth rate of the total amount of Cd in the organism was higher than that of the body weight. The average allometric coefficients were 1.016 ± 0.0129 and 1.099 ± 0.0215 for eight groups of chickens fed on diets with 0.404 and 0.348 mg of Cd, respectively. The difference was highly significant ($P < 0.01$).

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