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EXCRETION OF OXYTETRACYCLINE RESIDUES BY MILK OF COWS WITH CLINICAL MASTITIS DURING LACTATION PERIOD

VYLUČOVANIE OXYTETRACYKLÍNU MLIEKOM DOJNÍC S KLINICKÝMI PREJAVMI MASTITÍDY POČAS LAKTAČNÉHO OBDOBIA

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ABSTRACT: The objective of this study was to determine the oxytetracycline residues in milk from cows with clinical mastitis dosed with two extra-label routes of oxytetracycline administration not only during antibiotic's treatment (5 days), but also six days after treatment by use of a liquid chromatography method of testing with a detection limit of 20 ppb. Both groups of animals were treated once daily for five milkings at 24-hour intervals following morning milkings. Composite milk samples (equal volumes of foremilk from each quarter) were collected during morning and afternoon milkings, mixed together (1 : 1), and stored until analyzed. Milk samples were analyzed just before the first treatment (0 hour) and ten times at 24-hour intervals. Residue studies in milk cows indicate that oxytetracycline passes into milk. Residues in milk were higher for the cows receiving oxytetracycline by intramammary route (Tab. I) than for the cows receiving oxytetracycline intramuscularly (Tab. II). The highest mean data were 195.68 mg/kg after intramammary infusion (Fig. 2) and 2.74 mg/kg after intramuscular injection (Fig. 3) on the 5th day of the treatment beginning. The analysis data showed that oxytetracycline persisted in milk for as long as two days after both treatments at the concentration 0.03 mg/kg versus 0.02 mg/kg, respectively. No residues were detected in milk of any animal from the 4th day of the cessation of the therapy (Fig. 1) as detected by the HPLC method.

mastitis; cows; milk; oxytetracycline; residues; HPLC

ABSTRAKT: Na 12 dojniciach červenostrakatého plemena s klinickými prejavmi mastitídy rozdelených do dvoch skupín sme sledovali vylučovanie oxytetracyklínu (OTC) mliekom po jeho intramamárnom a intramuskulárnom podaní nielen počas liečby (jedenkrát denne ihneď po rannom vydojení počas piatich dní), ale aj ďalších šesť dní po ukončení liečenia. Z výsledkov sledovania rezíduí OTC vyplýva, že tieto boli vyššie u dojníc liečených OTC intramamárne než u dojníc po jeho intramuskulárnej aplikácii. Najvyššie zaznamenané koncentrácie boli 195,68 mg/kg u Oxymykoinu a 2,74 mg/kg u Engemycinu na piaty deň od začiatku liečenia. Výsledky ukázali, že OTC perzistuje v mlieku dojníc dlhšie ako dva dni po ukončení liečenia (koncentrácia 0,03 mg/kg a 0,02 mg/kg). Reziduá neboli zistené v mlieku ani jednej dojnice od štvrtého dňa po ukončení liečenia.

mastitída; dojnice; mlieko; oxytetracyklín; rezíduá; HPLC

ÚVOD

Antibiotiká, a to aj na báze oxytetracyklínu (OTC) sa používajú nielen vo svete, ale aj u nás (Fletouris a i., 1990; Sokol a Matisová, 1994; Anderson a i., 1995) pri liečení mastitíd – ochorení, považovaných z ekonomického, ale aj hygienického hľadiska za veľmi náročné.

Rozšírené používanie antibiotík vyvoláva potenciálne problémy s výskytom rezíduí v mliečnych produktoch určených pre ľudský konzum (Nagy a i., 1994). Od roku 1900 sa v celosvetovom meradle zvýšila výroba fermentovaných mliečnych výrobkov, takže v dnešnej dobe sa ich vyrába viac ako tisíc druhov (Mäirä-Mäkinen, 1995). Výroba fermentovaných mliečnych výrobkov vyrábaných za použitia čistých mliekárenských kultúr môže byť narušená prítomnosťou už aj stopových množstiev antibiotík v surovom kravskom mlieku ako východiskovej základnej suroviny, v dôsledku čoho dochádza k vzniku rôznych chýb a nedostatkov pri ich výrobe (Uhnák, 1988; Nagy a i., 1994). Mäirä-Mäkinen (1995) popisuje napr. horkú, neslanú chuť pri výrobe syrov s nízkodohrievanou syreninou; cestovitú konzistenciu, mäknutie, chyby pri tvorbe charakteristických ôk u syrov s vysokodohrievanou syreninou; niekedy sa nepodarí určitý výrobok vyrobiť vôbec.

Pri riešení hygienických ako aj zdravotných problémov dôležitou sa javí aj otázka voľby analytických metód za účelom stanovenia malých množstiev jedného alebo viacerých antibiotík vedľa seba (Barker a Walker, 1992; Sokol a Matisová, 1993).

Mikrobiologické metódy sú rozšírené v mnohých krajinách. Ich nevýhodou je, že sú citlivé na dôkaz beta laktámových antibiotík; citlivosť na inú skupinu antibiotík a sulfonamidov je nedostatočná (Suhren a Heeschen, 1993). Citlivosť mikrobiologických metód pre tetracyklíny sa pohybuje v rozmedzí 400 až 500 µg/kg (Idf, 1991). Tieto detekčné limity sú nedostatočné s ohľadom na možné zdravotné riziká vyplývajúce z konzumácie potravín. Z uvedených dôvodov vystupuje do popredia potreba rýchlych a citlivých metód na dôkaz rezíduí antimikrobiálnych látok v potravinách. Do skupiny takýchto metód patrí aj vysokotlaková kvapalinová chromatografia (HPLC). Sokol a i. (1995) udávajú detekčný limit pre tetracyklíny simultánne stanovené metódou HPLC v kravskom mlieku 20 ppb.

Vzhľadom k tomu, že sa na Slovensku pri liečbe zápalov mliečnej žľazy dojníc používa aj oxytetracyklín, zaoberali sme sa v našej práci štúdiom vylučovania oxytetracyklínu mliekom dojníc s klinickými prejavmi mastitídy po jeho intramamárnom a intramuskulárnom podaní, a to nielen počas liečby (päť dní), ale aj ďalších šesť dní po ukončení liečenia metódou vysokotlakovej kvapalinovej chromatografie.

MATERÁL A METÓDY

Do experimentu sme zaradili 12 dojníc červenostrakatého plemena v rôznom štádiu laktácie, u ktorých klinickým vyšetrením bol zistený chronický zápal mliečnej žľazy s postihnutím celého vemena. Zvieratá boli rozdelené do dvoch skupín po šiestich kusov dojníc, individuálne ustajnených na hlbokej podstielke. Prvej pokusnej skupine dojníc bol aplikovaný intramamárne Oxymykoin foam ad us.vet. (2,2 g oxytetracyklínu v 46 g peny, 0,44 g oxytetracyklínu na jednu štvrťku; Galena Opava, Česká republika). Pri intramamárnej aplikácii Oxymykoinu foam liečivo bolo aplikované do všetkých štyroch štvrtí vo forme peny jedenkrát denne ihneď po rannom vydojení počas piatich dní. Udávaná ochranná lehota pre mlieko je päť dní. Druhej skupine experimentálnych zvierat bol aplikovaný intramuskulárne do krku Engemycin 10 % L. A. inj. ad us. vet. obsahujúci 100 mg oxytetracyklínovej bázy v 1 ml prípravku (10 mg/kg; Intervet, Holandsko) jedenkrát denne ihneď po rannom vydojení počas piatich dní. Udávaná ochranná lehota pri tomto spôsobe aplikácie pre mlieko je 2,5 dňa. Prvú dávku obidvoch druhov liečiv sme experimentálnym skupinám zvierat aplikovali ihneď po nultom odbere mlieka (0 h) na laboratórne vyšetrenie.

Vzorky surového mlieka (rovnaké diely z každej štvrťky) získané počas ranného a večerného dojenia boli zmiešané *anapartes*. Koncentrácie sledovaných OTC v mlieku boli stanovené metódou HPLC v nasledujúcich časových intervaloch: pred začiatkom liečby (0 h), za 24, 48, 72 a 96 h počas liečenia a šesť dní po ukončení aplikácie obidvoch testovaných prípravkov. Citlivosť metódy bola 20 ppb.

Vzorky mlieka na analýzy boli pripravené metódou podľa Sokola a i. (1995).

VÝSLEDKY

V tab. I a II udávame výsledky koncentrácie oxytetracyklínov v mlieku dojníc v sledovaných časových intervaloch. Každá priemerná hodnota je doprevádzaná najnižšou a najvyššou individuálnou hodnotou, dokazujúcou rozdiely medzi pokusnými dojnicami.

Kontrolné vzorky mlieka odobraté pred prvou aplikáciou testovaných antibiotík, boli použité na dôkaz toho, že mlieko experimentálnych dojníc neobsahuje rezíduá týchto látok.

Prvých 24 h od začiatku liečby, všetky vzorky mlieka boli pozitívne na prítomnosť rezíduí OTC. Priemerná koncentrácia OTC bola nižšia než po 48 h: 59,13 ±

I. Koncentrácia oxytetracyklínu (OTC) vo vzorkách mlieka šiestich dojníc po intramamárnej aplikácii Oxymykoinu foam – Oxytetracycline (OTC) concentration in milk samples from six cows infused by intramammary route with Oxymykoin foam

Dĺžka trvania experimentu[1]	Koncentrácia OTC (mg/kg)[2]			Pozitívne/Celkom[6]	Percento[7]
	Rozpätie[3]	Priemer[4]	SD[5]		
0*	ND[8]	ND	ND	0/6	0
24	7,37–185,92	59,13	70,63	6/6	100,00
48	7,26–105,47	62,56	33,45	6/6	100,00
72	35,09–92,45	57,70	22,67	6/6	100,00
96	80,78–351,58	195,68	94,29	6/6	100,00
24**	0,72–7,11	2,78	2,46	6/6	100,00
48	ND-1,58	0,39	0,60	4/6	67,00
72	ND-0,18	0,03	0,07	1/6	17,00
96–144	ND	ND	ND	0/6	0

* – doba aplikácie liečiva (h) – hours of drug administration
** – doba po poslednom podaní liečiva (h) – hours after the last drug administration

[1]hours of experiment, [2]concentration of oxytetracycline, [3]range, [4]mean, [5]standard deviation, [6]positive/total, [7]percentage, [8]not detected

II. Koncentrácia oxytetracyklínu (OTC) vo vzorkách mlieka šiestich dojníc po intramuskulárnej aplikácii Engemycinu – Oxytetracycline (OTC) concentration in milk samples from 6 cows injected intramuscularly with Engemycin

Dĺžka trvania experimentu[1]	Koncentrácia OTC (mg/kg)[2]			Pozitívne/Celkom[6]	Percento[7]
	Rozpätie[3]	Priemer[4]	SD[5]		
0*	ND[8]	ND	ND	0/6	0
24	0,25–0,46	0,35	0,09	6/6	100,00
48	1,03-3,56	1,73	0,97	6/6	100,00
72	0,90-1,24	1,07	0,20	6/6	100,00
96	0,88-11,52	2,74	4,32	6/6	100,00
24**	0,13-0,64	0,49	0,19	6/6	100,00
48	ND-0,23	0,09	0,09	4/6	67,00
72	ND-0,06	0,02	0,03	2/6	33,00
96–144	ND	ND	ND	0/6	0

* – doba aplikácie liečiva (h) – hours of drug administration
** – doba po poslednom podaní liečiva (h) – hours after the last drug administration

[1]hours of experiment, [2]concentration of oxytetracycline, [3]range, [4]mean, [5]standard deviation, [6]positive total, [7]percentage, [8]not detected

70,63 mg/kg oproti 62,56 ± 33,45 mg/kg pre Oxymykoin foam (tab. I) a 0,35 ± 0,09 mg/kg oproti 1,73 ± 0,97 mg/kg pre Engemycin). Na štvrtý deň podávania antibiotík (72 h) došlo k poklesu koncentrácie rezíduí v mlieku oboch pokusných skupín zvierat: 57,70 ± 22,67 mg/kg pre Oxymykoin foam (tab. I) a 1,07 ± 0,20 mg/kg pre Engemycin (tab. II). K progresívnemu zvýšeniu hladín rezíduí OTC (obr. 1) v mlieku dojníc došlo v posledný deň aplikácie (195,68 ± 94,29 mg/kg oproti 2,74 ± 4,32 mg/kg).

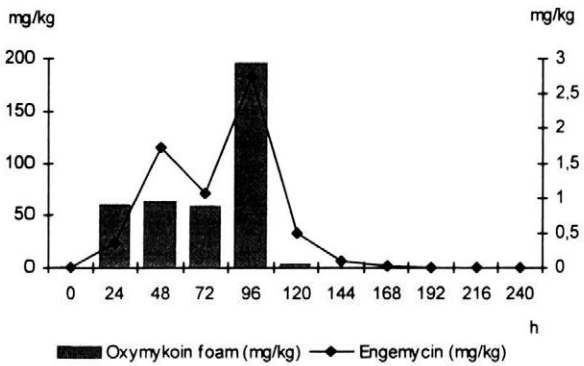
Vnútrosvalová aplikácia Engemycinu vykazovala nižšie koncentrácie OTC v mlieku, zatiaľ čo koncentrácie OTC po intramamárnej aplikácii boli vyššie počas celého experimentu. Podobne sme počas celého experimentu zaznamenali aj široké rozpätie prítomných rezíduí antibiotík oboch sledovaných produktov (obr. 2, 3). Koncentrácie OTC vo vzorkách mlieka oboch pokusných skupín zvierat sme nezistili vo všetkých vzorkách mlieka 48 h od ukončenia terapie. Štyri zo šiestich dojníc (67 %) oboch pokusných skupín zvierat boli pozitívne na prítomnosť rezíduí testovaných látok. Priemerná koncentrácia Oxymykoinu foam bola 0,39 mg/kg a Engemycinu 0,09 mg/kg. U jednej dojnice sme pozorovali vylučovanie Oxymykoinu foam pomalšie ako u ďalších piatich dojníc tejto pokusnej skupiny. Mlieko tejto dojnice obsahovalo rezíduá Oxymykoinu foam tri dni po ukončení antibiotickej liečby (koncentrácia pozitívnej vzorky bola 0,18 mg/kg).

Mlieko dojníc liečených Engemycinom bolo pozitívne počas celej ochrannej lehoty tohto prípravku (2,5 dňa). Dve dojnice (33 %) tejto experimentálnej skupiny vylučovali rezíduá Engemycinu dokonca dlhšie ako je stanovená jeho ochranná lehota. Koncentrácia rezíduí tohto antibiotika bola 0,041 mg/kg a 0,056 mg/kg.

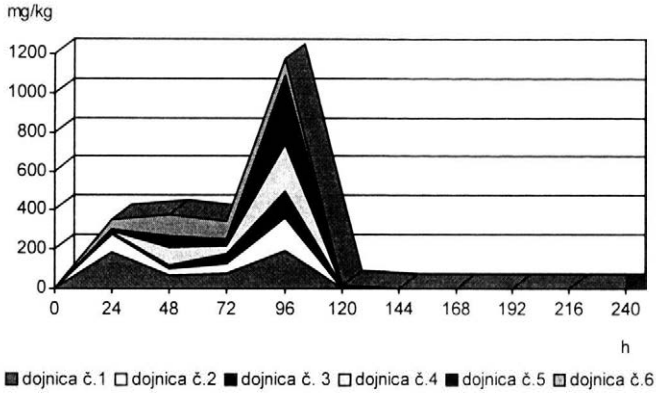
Posledné tri dni experimentu sme použitím metódy HPLC nezistili výskyt rezíduí testovaných antibiotík ani u jednej experimentálnej dojnice.

DISKUSIA

Antibiotiká sa na liečbu mastitíd u dojníc používajú už viac ako tri desaťročia. Pri liečbe zápalov mliečnej žľazy sa u dojníc počas laktácie odporúča podanie lie-

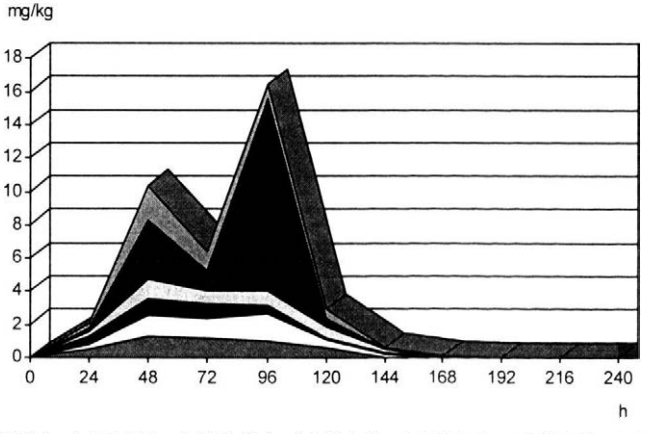


1. Koncentrácia oxytetracyklínu vo vzorkách mlieka dojníc po intramamárnej aplikácii prípravku Oxymykoin foam a intramuskulárnej aplikácii prípravku Engemycin – Oxytetracycline concentration in milk samples from cows infused by intramammary route with Oxymykoin foam and injected intramuscularly with Engemycin



2. Koncentrácia oxytetracyklínu v individuálnych vzorkách mlieka šiestich dojníc po intramamárnej aplikácii prípravku Oxymykoin foam – Oxytetracycline concentration in individual milk samples from 6 cows infused by intramammary route with Oxymykoin foam

dojnica č. – dairy cow no.



3. Koncentrácia oxytetracyklínu v individuálnych vzorkách mlieka šiestich dojníc po intramuskulárnej aplikácii prípravku Engemycin – Oxytetracycline concentration in individual milk samples from 6 cows injected intramuscularly with Engemycin

dojnica č. – dairy cow no.

čiva v 24- až 48-hodinových intervaloch s najviac troma aplikáciami liečiva, a to z ekonomických dôvodov – možnosť použiť mlieko na spracovanie čo najskôr (Owens a i., 1988). V našej práci sme dojniciam aplikovali liečivá v 24-hodinových intervaloch počas piatich dní jedenkrát denne.

Je všeobecne známe, že antibiotiká sa môžu dojniciam aplikovať rôznymi cestami, na základe čoho potom dochádza k rozdielnej perzistencii antibiotík v mlieku. Dôkazom toho sú aj nami zistené rozdiely hladín rezíduí sledovaných OTC v kravskom mlieku nielen medzi dvomi pokusnými skupinami zvierat, ale aj u individuálnych dojníc zaradených do pokusu. Na druhej strane vylučovanie antimikrobiálnych látok cez bunkovú membránu z plazmy do mlieka závisí od viacerých faktorov: napr. pH mlieka, bodu topenia tuku, stavby bielkovín, väzby na plazmatické a tkanivové bielkoviny. Dôležitá je aj dávka liečiva, jeho koncentrácia v krvnej plazme, frekvencia dojenia, zdravotný stav – indikácia liekovej terapie, dĺžka terapie, atď. (Šimko a Kaňuščáková, 1992). Priebeh ochorenia mliečnej žľazy môže zmeniť aj farmakokinetiku antimikrobiálnych látok, čo môže viesť k narušeniu prechodu týchto látok do mlieka. Týmto si vysvetľujeme v našom experimente zaznamenané prudké zvýšenie koncentrácie oxytetracyklínu v posledný deň aplikácie u oboch testovaných produktov.

Väčšina nových liečiv je určená na liečenie patologických stavov. V praxi ale dochádza k tomu, že v jednotlivých fázach predregistračného klinického hodnotenia sa lieky skúšajú na zdravých zvieratách, vrátane stanovenia ochrannej lehoty (Šimko a Kaňuščáková, 1992). Z týchto dôvodov sme sa zaoberali dôkazom OTC v mlieku dojníc s klinickými prejavmi mastitídy v priebehu laktácie.

Počas celého experimentu široké rozpätie hladín rezíduí OTC v mlieku dojníc obidvoch experimentálnych skupín mohlo byť spôsobené zhoršenou penetráciou antibiotík mliečnou žľazou v dôsledku prítomných zápalových procesov v samotnej mliečnej žľaze, pri ktorej zápal a blokáda vyvolaná infekciou, opuch a indurácia mliečnej žľazy bránia úplnej difúzii antibiotík týmto tkanivom (Owens a i., 1988). Okrem toho je známa aj limitovaná distribúcia OTC mliečnou žľazou po intramamárnej aplikácii na rozdiel od parenterálnej aplikácii, kedy sa OTC distribuje v mliečnej žľaze dobre (Saran, 1995).

Koncentrácia sledovaných oxytetracyklínov nebola pod detekčným limitom 48 h po ukončení liečenia, po-

hybovala sa ale pod hladinou maximálneho limitu stanoveného pre oxytetracyklín v mlieku, t. j. 0,1 mg/kg (Vyhláška MZ SR, 1993).

Všetky dojnice liečené Oxymykoinom foam intramamárne produkovali mlieko bez antibiotík 24 h pred koncom doporučenej ochrannej lehoty. Rezíduá oxytetracyklínu po podaní prípravku Engemycin boli prítomné v mlieku dojníc nielen pred koncom doporučenej ochrannej lehoty, ale aj dlhšie.

Naše výsledky sme nemohli porovnať s inými literárnymi údajmi, pretože v takomto rozsahu a s použitím metódy HPLC nie je popísaný podobný experiment. Nows a i. (1985) vo svojej experimentálnej práci použitím mikrobiologickej metódy stanovenia rezíduí oxytetracyklínu v mlieku uvádzajú relatívne vysoké hladiny rezíduí tohto antibiotika v mlieku, pričom tieto rezíduá neboli v mlieku pozorované dlhšie ako 41 hodín. Hamann (1983) popisuje po jednorázovej aplikácii 1 500 mg oxytetracyklínu jednej dojnici perzistenciu rezíduí tohto antibiotika viac ako 13 dní po tejto aplikácii. Šimko a Kaňuščáková (1992) vo svojej práci uvádzajú pretrvávanie rezíduí liečiv v mlieku oviec a kôz rôzne dlhý čas, pričom spravidla došlo k ich vylučovaniu dlhšie ako sú úradne stanovené ochranné lehoty pre mlieko.

Ako z výsledkov našej práce vyplýva, sledovanie rezíduí antibiotík, a teda aj OTC v mlieku dojníc, je závažné nielen z hľadiska zníženia rizika pre zdravie človeka, ale aj z dôvodov negatívneho pôsobenia týchto antimikrobiálnych látok pri výrobe výrobkov, kde sa využívajú čisté mliekárenské kultúry (jogurty, syry). Preto je bezpodmienečne potrebné vyžadovať u výrobcov surového kravského mlieka určeného na priemyselné spracovanie a následne na ľudskú výživu správne dodržiavanie liečebného postupu pri liečení zápalov mliečnej žľazy, ako aj ochranných lehôt použitých liečiv po ukončení liečby.

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Pod redakčním vedením PharmDr. Josefa Suchopára za spoluúčasti dalších 41 odborníků daného lékařského oboru (z nichž bylo 14 doktorů věd) vzniklo dílko, které se stane nepostradatelnou pomůckou a rádcem každého kdo bude potřebovat podrobnější seznámení s lékem, jeho účinnou látkou a především možností, jak dané látky terapeuticky použít. Knihu recenzovalo 43 specialistů a odborné spolupráce se účastnilo dalších šest odborníků.

Na 614 stranách jsou v 16 kapitolách popsány terapeutické možnosti současné moderní medicíny. Jednotlivé kapitoly se věnují léčivům pro terapii skupin orgánů v logických celcích: Léčiva používaná k terapii nemocí gastrointestinálního ústrojí, Léčiva používaná k terapii nemocí kardiovaskulárního systému, Léčiva používaná k terapii nemocí respiračního systému, Léčiva používaná k terapii nemocí centrální nervové soustavy, Léčiva používaná k terapii infekčních a parazitárních nemocí, Léčiva používaná k terapii nemocí endokrinního systému, Léčiva používaná k terapii gynekologických nemocí a v porodnictví a léčiva používaná k terapii nemocí močových cest, Léčiva používaná k terapii nádorových nemocí, Léčiva používaná k terapii nemocí krve a krvetvorného systému a léčiva používaná k nutriční podpoře, Léčiva používaná k terapii nemocí pohybového ústrojí, Léčiva používaná k terapii očních nemocí, Léčiva používaná k terapii ORL nemocí, Léčiva používaná k terapii kožních nemocí, Léčiva používaná k ovlivnění imunitního systému, Léčiva používaná v anesteziologii, Základní informace o terapii některých intoxikací.

Kapitoly jsou rozděleny do subkapitol a začínají vždy obecným úvodem, který popisuje problematiku dané kapitoly z obecného hlediska, vymezuje léčebné použití léčiva a jeho obecné mechanismy působení. Subkapitoly jsou zahájeny úvodem, který je konkrétnější podrobnějším vysvětlením mechanismů působení dané látky. Každá část týkající se dané účinné látky má přesně stanovenou posloupnost. Daná látka je charakterizována chemicky a mechanismem působení, pak následuje na prvním místě mezinárodní nechráněný název (pokud tedy existuje). Pokud není stanoven, je název odvozen z Evropského lékopisu. Dále je každá účinná látka charakterizována ACT klasifikací.

Následují jednotlivé rubriky. Charakteristika: podrobněji popisuje účinky a mechanismy působení účinné látky (samozřejmě pokud jsou známy). Indikace: indikační použití, tj. rozhodný důvod či soubor okolností vyžadující určitý léčebný postup. Kontraindikace: uvádí kontraindikace pro použití daného léčiva, jsou vybrány samozřejmě takové, které mají významnost a důležitost. Nežádoucí účinky: vybrány jsou takové, které mají vyšší incidenci, významnost a důležitost. Dávkování: popisuje terapeutické použití dané léčebné látky s ohledem na množství této účinné látky v preparátu. Upozornění: není uvedeno u všech léčiv, pouze v případech, kdy je nutné provádět specifické testy či kontroly. Poznámka: odkazy na jiné kapitoly, kdy se stejné látky používá pro léčbu jiného orgánového systému. Předepisování: uvádí preskripční, případné indikační omezení.

Léčiva jsou řazena abecedně. V pořadí podle lékové formy infuzní, injekční, perorální, pevné, perorální tekuté, rektální a lokální. Následuje seznam všech v ČR registrovaných přípravků obsahující danou látku, a to jak monokomponentní, tak kombinované přípravky. Před názvem léku je uveden symbol označující, zda je volně prodejný, na lékařský předpis, zda je látka psychotropní a omamná nebo zda slouží pro přípravu magisraliter. Dále je uvedena velikost balení, kód přípravku stanovený SÚKL, orientační cena a kdo přípravek hradí, případně schvaluje jeho použití (revizní lékař, antibiotické středisko).

V závěru knihy je podrobný rejstřík umožňující vyhledání preparátu podle jeho názvu nebo podle účinné látky. Takto bylo podáno sdělení o téměř 13 500 různých formách léčivých přípravků více jak 60 000 údajů týkajících se ATC klasifikace. Odborné veřejnosti se tak dostává do rukou přehled léčiv, která jsou orgánově tříděna, přehled různých variant léčby určitého onemocnění, seznam všech léčiv, která jsou registrovaná a lze je v dané situaci použít.

Podle mého mínění by u jednotlivých skupin léčiv mohl být uveden přesněji chemický status dané látky, sumární a stechiometrický vzorec a možná se mohlo více místa věnovat také mechanismům působení daných látek v organismu.

O kvalitě této knihy se musí přesvědčit každý sám, ale zcela jistě se jedná o velmi přínosnou a precizně zpracovanou knihu, kterou doporučuji všem alespoň k nahlédnutí.

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PREVENTION OF NECROTIC ENTERITIS IN PIGLETS BY VACCINATION OF PREGNANT GILTS WITH A *CLOSTRIDIUM PERFRINGENS* TYPE C AND D BACTERIN-TOXOID

PREVENCE NEKROTICKÉ ENTERITIDY U SELAT VAKCINACÍ BŘEZÍCH PRASNIČEK BAKTERIN-TOXOIDEM *CLOSTRIDIUM PERFRINGENS* TYPU C A D

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ABSTRACT: On a large pig farm with a known history of necrotic enteritis 12 pregnant gilts were vaccinated s. c. 7 and 2 weeks before expected farrowing with a commercial bacterin-toxoid preparation of toxigenic strains *C. perfringens* type C and D (Dizevak^{R}-Pliva, Zagreb). At the farrowing the titers of β-antitoxins in serum samples from vaccinated gilts ranged from 9.0 to 26.0 IU/ml with a mean value of 14.16 IU/ml. Colostral titers varied from 12.0 IU/ml with a mean of 16.12 IU/ml. On the second day of life the mean serum titers between litters differed greatly from 4.75 to 24.0 IU/ml. By the age of 7 days the average serum titers were commonly lower and varied between the litters from 2.25 to 15.0 IU/ml, with a low of 1.5 to a high of 16.0 IU/ml in single animals. Ten (8.47%) out of a total of 118 piglets from vaccinated gilts died during the first 7 days of life but the losses were not caused by *C. perfringens* infection. In unvaccinated control animals 18 (15.9%) of 113 piglets died, eleven of them with clinical and pathoanatomical signs of necrotic enteritis. The affected piglets predominantly succumbed in the first 4 days of life. These data indicate that the investigated bacterin-toxoid can be successfully used in immunprophylaxis of necrotic enteritis in piglets.

Clostridium perfringens type C and D; prevention of necrotic enteritis in piglets; gilt vaccination

ABSTRAKT: Na velkokapacitní farmě s výskytem nekrotické enteritidy jsme provedli sedm a dva týdny před očekávaným porodem vakcinaci s. c. 12 březích prasniček komerčním bakterin-toxoidním preparátem připraveným z toxigenních kmenů *Clostridium perfringens* typu C a D (Dizevak^{R}-Pliva, Záhřeb). V době porodu se titry beta-antitoxinů ve vzorcích sér odebraných z vakcinovaných prasniček pohybovaly od 9,0 do 26,0 m. j./ml se střední hodnotou 14,16 m. j./ml. Titry v kolostru se pohybovaly od 12,0 do 27,0 m. j./ml se střední hodnotou 16,12 m. j./ml. Střední titry v sérech u jednotlivých vrhů druhý den značně kolísaly od 4,75 do 24,0 m. j./ml. Do sedmi dní věku zpravidla došlo ke snížení průměrných titrů v sérech a u jednotlivých vrhů se pak pohybovaly od 2,25 do 15,0 m. j./ml, od nízké hodnoty 1,5 do vysoké hodnoty 16,0 m. j./ml u jednotlivých zvířat. Během prvních sedmi dní uhynulo deset (8,47 %) z celkového počtu 118 selat pocházejících od vakcinovaných prasniček, tyto ztráty však nebyly způsobeny infekcí *C. perfringens*. Z nevakcinovaných 113 selat uhynulo 18 (15,9 %), z nichž 11 mělo klinické a patologicko-anatomické příznaky nekrotické enteritidy. Postižená selata hynula převážně v prvních čtyřech dnech života. Tyto údaje naznačují, že testovaný bakterin-toxoid lze úspěšně použít k imunoprofylaxi nekrotické enteritidy u selat.

Clostridium perfringens typu C a D; prevence nekrotické enteritidy u selat; vakcinace prasniček

INTRODUCTION

Since the first description in England (Field and Gibson, 1955) and Hungary (Szent-Ivanyi and Szabo, 1956) necrotic enteritis (NE) caused by *Clostridium perfringens* type C has been diagnosed among piglets with increasing frequency all over the world (Hogh, 1967, 1969; Bergeland, 1986; Okazaki et al., 1993) and also in Croatia (Udovičić et al., 1977). Once introduced into a herd this disease, characterized by severe haemorrhagic or necrotic enteritis tends to become enzootic with high morbidity and mortality (Azuma et al., 1983; Ripley and Gush, 1983; Perea et al., 1987) in affected litters. Piglets are most susceptible to the infection in the first week of life when due to the severity of the disease, antibiotic treatment is hardly effective (Nillo, 1988). Administration of a *C. perfringens* type C an-

titoxin after birth is highly effective in protection from clostridial enterotoxaemia, but this method of passive immunization is expensive and stressing because it requires extensive handling of newborn piglets. The only reasonable method of preventing the disease in piglets is vaccination of pregnant sows or gilts with a *C. perfringens* type C toxoid and colostral transfer of antibodies to their offspring (Ripley and Gush, 1983; Barker and van Dreumel, 1985; Ostle and Welter, 1987).

In this study the possibility of use of a modified commercial vaccine Dizevak^{R}-Pliva containing *C. perfringens* type C and D bacterins and toxoids in prevention of NE was investigated. The vaccine has been primarily intended for prevention of enterotoxaemia in ruminants.

MATERIAL AND METHODS

Animals

The test animals were 12 vaccinated pregnant Swedish Landrace gilts and 118 of their piglets in the experimental group and 12 nonvaccinated pregnant gilts and 113 of their piglets in the control group. All the animals were housed under the same conditions within the same building on a large commercial pig farm with a known history of NE (Bohm et al., 1987; Udovičić et al., 1990a) and were observed daily during the experiment.

Vaccine and immunization schedule

Dizevak^{R}-Pliva vaccine is a bacterin-toxoid preparation containing an inactivated culture of high toxigenic strains of *C. perfringens* type C and D and their detoxicated β- and ε-toxins, isolated from dead pigs. The strains were separately grown in a meat digest medium under optimum conditions in a fermentor, using submerged cultivation. After being inactivated with merthiolate and detoxicated with formaldehyde during 21 days at 37 °C, the bacterins and toxoids were adsorbed on aluminium hydroxide and mixed in ratio 65% v/v of *C. perfringens* type C to 35% v/v of *C. perfringens* type D. The final mixture contained 2 mg aluminium hydroxide in 1 ml of vaccine (Udovičić et al., 1990b; Kelnerić et al., 1990). Before use, the quality of vaccine was tested according to the British Pharmacopoeia (Veterinary) (1985) regulations.

Each of 12 pregnant gilts were immunized twice, being injected subcutaneously behind the ear with 5 ml of vaccine at six and two weeks before the expected farrowing day.

Serology

Blood samples for serological investigation were taken in the experimental group from each gilt before primary vaccination and then again before the second vaccination, and at the time of farrowing. Their colostral samples were collected on the day of farrowing. Blood samples were also taken from 4 to 5 randomly selected piglets from each litter of vaccinated gilts when the neonatal animals were 36 to 48 hours and 7 days of age. In the control unvaccineted group of animals only primary blood samples were taken from gilts six weeks before farrowing and were serologically examined. As the titers were less than 0.5 IU/ml of β-antitoxins (see the results!), serological examination of their colostral samples or blood samples of their piglets was not performed.

The *C. perfringens* β-antitoxin titers in serum and colostrum samples were estimated by a standard toxin/antitoxin neutralization assay strictly following the British Pharmacopoeia (Veterinary) (1985) and the recommendation of WHO (Anonym, 1968). The international standards for *C. perfringens* β- and ε-antitoxin were obtained from Central Veterinary Laboratory, Weybridge, Eng-

I. Serum and colostral β-antitoxin titers of vaccinated gilts and serum titers of their piglets (IU/ml)

Gilt No.	Serum titers in gilts		Colostral titers	No. of piglets examined in litter	Serum titers in piglets			
	two weeks before farrowing	at the farrowing			at 2 days		at 7 days	
					range	mean	range	mean
1	2.5	12.5	14.0	5	5.0–6.0	5.5	1.5–3.0	2.25
2	2.5	26.0	27.0	7	12.0–14.0	13.0	3.0–5.0	4.0
3	4.0	12.0	12.0	5	4.0–5.5	4.75	2.0–3.0	2.5
4	2.0	13.0	15.0	5	10.5–12.5	11.5	5.5–8.0	6.75
5	4.5	11.0	13.5	5	4.0–8.0	6.0	2.5–4.0	3.25
6	3.0	11.5	12.5	5	5.5–7.0	12.5	4.0–4.5	4.25
7	3.5	9.0	13.0	4	5.0–10.0	7.5	7.0–10.0	8.75
8	3.0	12.5	13.0	5	8.0–10.5	9.25	4.0–5.0	4.5
9	4.0	11.0	12.5	5	5.5–7.0	6.25	4.0–4.5	4.25
10	4.5	14.0	21.0	5	10.0–16.0	13.0	4.0–13.0	8.5
11	3.0	11.5	13.0	5	8.0–11.0	9.5	4.0–5.0	4.5
12	6.5	26.0	27.0	6	23.0–25.0	24.0	14.0–16.0	15.0

II. Etiology of piglet losses in the vaccinated group

Gilt No.	No. of live--born piglets	No. of piglets which died	Etiology (age when died)
1	8	0	
2	9	1	1 died at blood sampling
3	9	0	
4	11	1	1 squeezed
5	10	2	2 cannibalism
6	7	1	1 colibacillosis (7th day)
7	10	0	
8	11	2	1 died at blood sampling
			1 squeezed
9	11	0	
10	10	0	
11	14	2	2 low birth weight
12	8	1	1 colibacillosis (7th day)
Total	118	10 (8.5%)	2 colibacillosis

III. Etiology of piglet losses in the control group

Gilt No.	No. of live--born piglets	No. of piglets which died	Etiology (age when died)
1	11	1	1 squeezed
2	8	1	1 NE (1st day)
3	9	2	2 NE (4th day)
4	9	2	1 colibacillosis (7th day)
			1 died at blood sampling
5	12	4	3 NE (4th and 7th day)
			1 died at blood sampling
6	11	0	
7	7	1	1 died at blood sampling
8	9	2	1 squeezed
			1 NE (4th day)
9	11	3	1 squeezed
			2 NE (4th day)
10	7	0	
11	9	2	2 NE (1st day)
12	10	0	
Total	113	18 (15.9%)	11 NE, 2 colibacillosis

land. In the titration of antitoxins six NMRI mices were used for each dilution.

Bacteriological examinations

All the piglets that died during the experiment were necropsied and their intestinal contents cultured of standard bacteriological nutrient media under aerobic and anaerobic conditions. The isolated strains of *C. perfringens* and *Escherichia coli* and their antigenic and toxigenic properties were determined according to Berghoff and Redenbarger (1976) and Holt et al. (1996). Bacteriological and pathoanatomical examinations were performed by experts of Veterinary Faculty, University of Ljubljana, Slovenia.

RESULTS

The β-antitoxin levels in the serum and colostrum of vaccinated pregnant gilts and in the serum of their piglets are presented in Tab. I.

Primary serum samples from all the pregnant gilts of the vaccinated and control groups contained less than 0.5 IU/ml of β-antitoxins.

Ten (8.47%) out of a total of 118 piglets from vaccinated gilts died during the first 7 days of life. Only two deaths were from microbial infection but these were not caused by *C. perfringens*. At the same time in unvaccinated control, 18 (15.9%) of 113 farrowed piglets died. Eleven of them succumbed having characteristic clinical and pathoanatomical signs of necrotic enteritis, and one with signs of colibacillosis. Both presumptive diagnoses were confirmed by isolation and characterization of toxigenic strains of *C. perfringens* type C and *E. coli*, respectively.

The etiology of piglets losses in the experimental and control is detailed in Tabs. II and III.

DISCUSSION AND CONCLUSIONS

It is well known that on large pig farms much greater attention should be paid to prevention of NE in piglets farrowed by gilts than those by sows, because of their greater susceptibility to infection (Bergeland, 1986). Using a commercial bacterin-toxoid preparation of toxigenic strains *C. perfringens* type C and D, relatively high levels of neutralizing antibodies in twice vaccinated gilts and their offspring were produced. The titers of β-antitoxins in gilt serum samples at farrowing ranged from 9.0 to 26.0 IU/ml with a mean value of 14.16 IU/ml. Colostral titers were still higher, varying from 12.0 to 27.0 IU/ml, with the mean 16.12 IU/ml. At the second day of life the mean serum titers in single litters varied greatly from 4.75 to 24.0 IU/ml, with the lowest 4.0 IU/ml to the highest 25.0 IU/ml in some piglets. At the age of 7 days the mean serum titers were generally lower and varied between the litters from 2.25 to 15.0 IU/ml with the lowest 1.5 to the highest 16.0 IU/ml in single animals. These results are basically in agreement with previously published reports (Ripley and Gush, 1983; Matisheck and McGinley, 1986) considering the fact that the experiments were performed with gilts and not sows.

There is still not a general agreement on the protective level of β-antitoxins, but minimal colostral titers of 10 IU/ml (Hogh, 1976), and titers greater than 0.5 IU/ml in piglet serum are recommended (Kennedy et al., 1977). The present levels of antitoxins mainly exceed these values, indicating that in the prophylaxis of NE in piglets, a bacterin-toxoid combination is at least equally good, if not better than a preparation containing toxoid only (Hogh, 1988). Significantly higher serum titers at parturition (mean 14.16 IU/ml) compared to the titers before the second vaccination (mean

3.58 IU/ml), clearly indicate the need of a booster dose which should be given to the pregnant gilts approximately 2 weeks before the expected farrowing.

The good immunogenic characteristics of Dizevak[R] are strongly supported by pathoanatomical and microbiological observations. Piglets from vaccinated gilts exposed to environmental infection with *C. perfringens* type C were completely protected from NE. At the same time this disease was the cause of death of 9.7% of the total number of piglets in the control group. The majority of affected piglets succumbed during the first 4 days of life when therapy is usually not successful due to the severity of the disease (B e r g e l a n d , 1986).

The given data show that the bacterin-toxoid preparation used in the vaccination of pregnant gilts, and the resulting colostral transfer of antitoxins are highly effective in the prevention of NE enteritis in piglets.

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MORPHOLOGICAL CHANGES IN THE EWE UTERINE TUBE (OVIDUCT) EPITHELIUM DURING PUERPERIUM

MORFOLOGICKÉ ZMENY NA EPITELI VAJCOVODU OVIEC V PUERPÉRIU

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ABSTRACT: The aim of our investigations was to describe the morphological changes in the epithelium of the ampulla region of the ewe uterine tube ampulla on days 2, 7, 17, 25 and 34 *post partum*. Shortly after the parturition the epithelium was low. Ciliated cells were dark with few kinocilia and secretory cells were light in colour and contained no secretory granules. On days 17 and 25 *post partum* the ciliated cells were high. They had well-developed granular endoplasmic reticulum, ribosomes, and developing basal corpuscles and kinocilia. Secretory cells contained a well-developed Golgi complex, granular endoplasmic reticulum and numerous secretory granules of varied size, shape, and density. On day 34 *post partum*, the ciliated cells had numerous kinocilia on their apical surface. Secretory cells released their product into the oviduct lumen through pores or high protrusions of cells detached, decomposed, and became the part of the secretion.

uterine tube (oviduct); puerperium; morphology; ewe

ABSTRAKT: Študovali sme morfologické zmeny na epiteli ampuly vajcovodu oviec na 2., 7., 17., 25. a 34. deň po pôrode. Pre puerperium, ako acyklické obdobie, je charakteristické fyziologické zníženie hladín estrogénu a progesterónu, čo výrazne vplýva na štruktúru ciliárnych a sekrečných buniek vajcovodu. Krátko po pôrode bol epitel nízky. Ciliárne bunky boli tmavé a mali málo kinocílií, sekrečné bunky boli svetlé a neobsahovali sekrečné granuly. Na 17. a 25. deň po pôrode boli ciliárne bunky vysoké, tmavé aj svetlé. Mali dobre vyvinuté granulované endoplazmatické retikulum, ribozómy, vytvárali sa bazálne telieska a kinocílie. Sekrečné bunky obsahovali výrazný Golgiho komplex, granulované endoplazmatické retikulum a v cytoplazme pribúdali sekrečné granuly rôznej veľkosti, tvaru a denzity. Na 34. deň po pôrode ciliárne bunky mali na apikálnom povrchu veľa kinocílií. V cytoplazme sa hromadil glykogén, cisterny granulovaného endoplazmatického retikula boli mierne dilatované. Sekrečné bunky vylučovali sekrét do lumenu vajcovodu cez póry, alebo vysoké protrúzie bunky sa oddelili, rozpadli a stali sa súčasťou sekrétu. Nepozorovali sme mitózy bazálnych buniek, ani premenu ciliárnych buniek na sekrečné a opačne.

vajcovod; puerperium; morfológia; ovca

INTRODUCTION

During the oestrous cycle the oviduct epithelium undergoes pronounced qualitative and quantitative changes which affect most of all the ovarian and hypophysis hormones. The microscopical and submicroscopical structure of the oviduct epithelium during the oestrous cycle in cows was described by Uhrín (1983), Uhrín and Kliment (1983a, b), Nayk and Ellington (1977); in ewes by Abdalla (1968), Willemse and Vorstenbosch (1975), Nayk et al.,(1976); in pigs by Nayk and Zimmerman (1973); in bitches by Verhage et al.,(1973); in monkeys by Brenner (1970) and in humans by Patek et al., (1972, 1973).

Pronounced histological changes in the heifer oviduct epithelium during the postnatal period were observed and described by Šťastný (1993), and those in dogs by Myers et al., (1984). Qualitative and quantitative changes occur even after the administration of gestagens used for synchronization of oestrus (Maráček et al., 1978, 1984), or after experimental X-ray irradiation when the decrease of estrogen levels can be observed (Staníková et al., 1980).

The available literature presents few data characterizing the morphological structure of the oviduct epithelium during the postparturition period (Seki et al., 1978a; Patek et al., 1973). The postparturition acyclic period, the length of which varies considerably, is affected by numerous factors. It depends on the breed, nutrition, season of the year, length and intensity of suckling, and others (Kudláč and Minář, 1969). Most of these factors affect the reproductive functions by means of a neuroendocrine system. According to Karg and Schallenberger (1983), the physiological puerperium is characterized by decreased concentrations of progesterone, 17-beta estradiol and luteinizing hormone – LH. The low level of progesterone immediately after the parturition and during consider-

able portion of the postparturition period is related to the termination of the function of *corpus luteum graviditatis* (Erb et al., 1971). The dynamics of the steroid hormones in cows in the course of physiological and pathological puerperium was observed by Zralý et al., (1989).

In this study, we investigated morphological changes in the ewe oviduct epithelium on days 2, 7, 17, 25 and 34 *post partum*. We directed our attention on the investigation of the submicroscopical structure of individual cell types of the oviduct ampulla epithelium.

MATERIAL AND METHODS

The experiment was carried out on 15 ewes of the Slovak Merino breed, 3 to 5 years old, weighing 40 to 50 kg on average. The animals were clinically healthy and were slaughtered ($n = 3$) on days 2, 7, 17, 25, and 34 *post partum*.

The material for light and electron microscopy examination was sampled from the oviduct ampulla. Specimens for light microscopy were fixed in 10% neutral formol and embedded in paraffin. Histological sections were stained with haematoxylin-eosin. Excisions from the organ, 1 cm^3 in size, for observation under a transmission electron microscope, were fixed by immersion in 3% glutaraldehyde for 3 hours and post-fixed in 2% OsO_4 in a phosphate buffer (pH 7.4) for 1 hour. Afterwards, the specimens were gradually dehydrated in an increasing series of alcohol, rinsed in propylene oxide and embedded in Durcupan ACM Fluka. After polymerization ultrathin sections were obtained by means of an ultramicrotome Tesla BS 490 and LKB Nova and stained in two stages with uranyl acetate and lead citrate (Reynolds, 1963; Venable and Coggeshal, 1965). The sections were observed under a JEOL 1200 electron microscope.

Examination by a scanning electron microscopy was carried out on 5 x 5 x 3 mm samples fixed in 3% glutaraldehyde for 3 hours and post-fixed in 1% OsO_4 in the phosphate buffer (pH 7.4) for 1 hour (Mráz and Polónyi, 1988).

After rinsing in the phosphate buffer they were dehydrated in an increasing series of ethyl alcohol. Excisions were dried by the critical point method (CPDO--20 Baltzers). The dry samples were fixed to the holders by means of a conductive Silver print and gold-encoated. They were examined by means of a JEOL 1 200 scanning electron microscope.

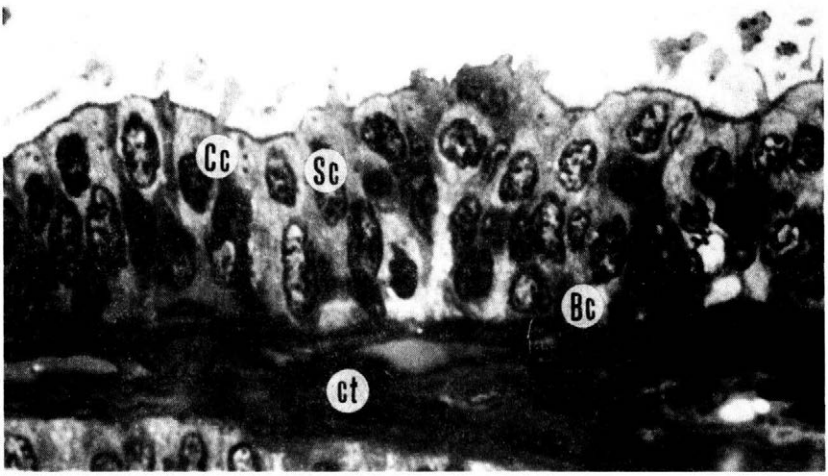
Semithin sections were stained with toluidine blue and examined and photographed under a light microscope Olympus CH2 with photographic equipment.

RESULTS

Examination under a light microscope, particularly in semithin sections, revealed that the multiple row cylindrical oviduct ampulla epithelium consists of cells of two types. Ciliated cells are tall, thin, of cylindrical shape, and rest on the basal membrane. They exhibit variable density of cytoplasm and their apical portion is covered with kinocilia. Secretory cells are found among ciliated cells, individually or in groups. They are tall, of cylindrical shape, and their wide base rests on the basal membrane. They have electron-dense cytoplasm and secretory granules are found in their apical portion. Their shape, structure, and secretory granules change according to the stage of their oestrous cycle. Wedge shaped cells are wedged between ciliated and secretory cells. They are thin, their cytoplasm does not extend to the basal membrane. Its major club-shaped cytoplasmic portion protrudes above the surface and extends above the level of the surrounding epithelium. Some of them also contain secretory granules. These cells do not form an individual group and are considered to be worn-out secretory cells. Rod shaped cells are thin, dark, rest on the basal membrane and can also contain secretory granules. They are also considered to be regressive forms of main cell types (Martínek et al., 1984). The third type of cells are basal cells. They are low, have relatively large spherical nuclei and lie on the lamina basalis. They are regarded to be stem cells from which the restoration of ciliated and secretory cells occurs and some of them are capable of mitotic division.

On days 2 and 7 *post partum*, the oviduct ampulla epithelium, observed under a light microscope, was low (Fig. 1). It consisted of above mentioned ciliated, secretory, and basal cells. Ciliated cells were present in relatively small numbers and were deciliated on day 2 *post partum*. The epithelium consisted mostly of differentiating secretory cells. Their cytoplasm was electronlucent and contained numerous cisternae of granular endoplasmic reticulum, ribosomes, polyribosomes and mitochondria. Ciliated cells were present in relatively low numbers on day 7 *post partum*. Secretory cells showed no signs of secretion and contained microvilli on their free surface. Examination by means of a scanning electron microscope showed similar findings (Fig. 2).

On days 17 and 25 *post partum*, the epithelium of the oviduct ampulla was significantly higher (Fig. 3). Ciliated cells were tall, thin, some with light, other with darker cytoplasm (Fig. 4). Basal corpuscles formed in the apical part of cells, the number of cilia increased considerably and reciliation of these cells occurred. Cisternae of the granular endoplasmic reticulum were moderately dilated. Higher variability was also observed in the shape and structure of secretory cells (Figs. 5 and 6). Secretory cells with electrondense cytoplasm and accumulating secretory granules in the apical and supranuclear parts of the cytoplasm were most frequently observed. Secretory cells varied in size and density which reflects probably different stages of their development. They were coated with a single membrane. The content of those which developed recently is light and fine granulated. They change gradually to dense granules. The most striking feature of secretory

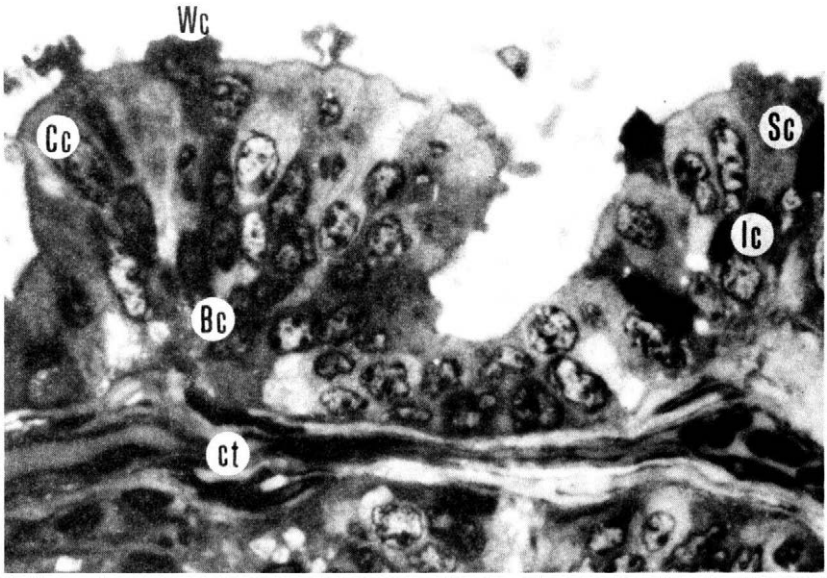


1. Epithelium of the uterine tube ampulla on day 2 *post partum*. Semithin sections stained with toluidine blue. Sc – secretory cells, Cc – ciliated cells, Bc – basal cells, ct – connective tissue x 1 000

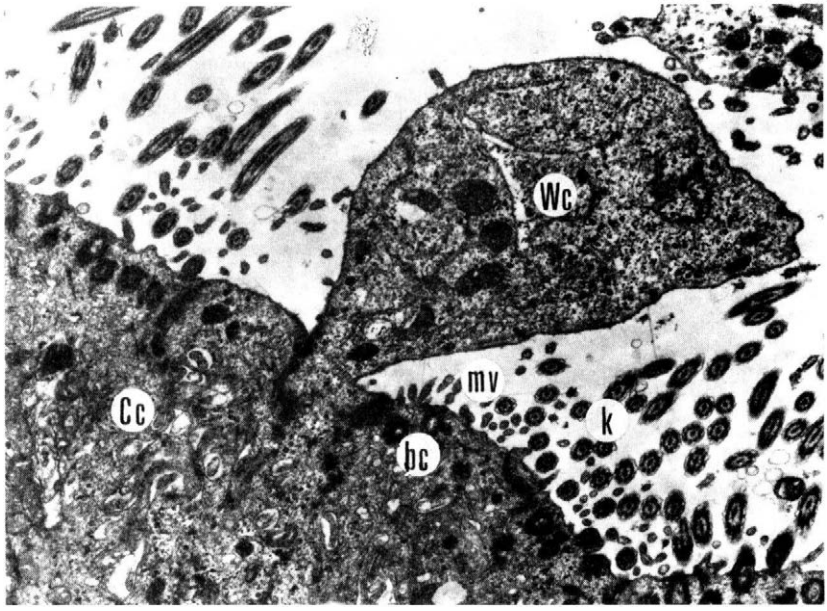
2. Submicroscopic picture of an apical part of ampullar secretory and ciliated cells on day 7 *post partum* under the scanning microscope. Ciliated cells are few, secretory cells show no signs of secretion and contain microvilli on their free surface. Sc – secretory cells, Cc – ciliated cells x 7 000

granules is their membranous structure consisting of concentric lamellae of equal dimensions (Fig. 7). The apical surface of secretory cells is covered with microvilli. From among organelles we can observe the pronounced Golgi complex, polyribosomes, mitochondria and cisternae of granular endoplasmic reticulum. Some secretory cells lose contact with the basal membrane and they are gradual extruded. We were also able to find rod shaped cells and wedge shaped-intercalary cells.

On day 34 *post partum* the epithelium of the oviduct ampulla was high (Fig. 8). The number of ciliated cells increased significantly and the secretory cells released their secretion into the oviduct lumen. Ciliated cells contained a number of kinocilia on the apical surface. They had the well-developed Golgi complex and slightly dilated cisternae of granular endoplasmic reticulum. Secretory granules are most frequently released in such a way that they accumulate in the apical



3. Epithelium of the ewe uterine tube ampulla on day 25 *post partum*. Semithin section stained with toluidine blue. Sc – secretory cells, Cc – ciliated cells, Ic – intercalary (rod shaped) cells, Bc – basal cells, Wc – wedge shaped cells, ct – connective tissue x 1 000

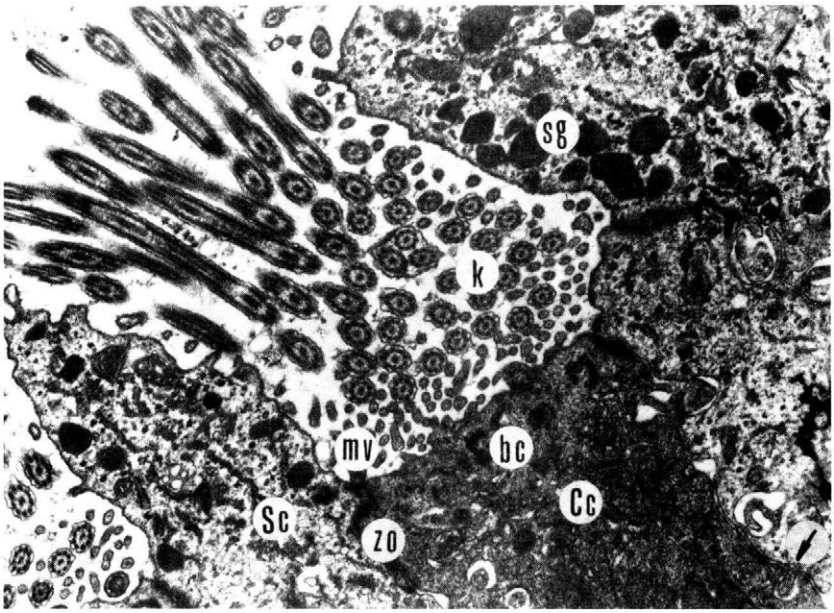


4. Electron micrograph of an apical part of ampullar ciliated cells from ewe on day 17 *post partum*. Wc – wedge shaped cells, Cc – ciliated cells, k – kinocilia, bc – basal corpuscles, mv – microvilli x 12 000

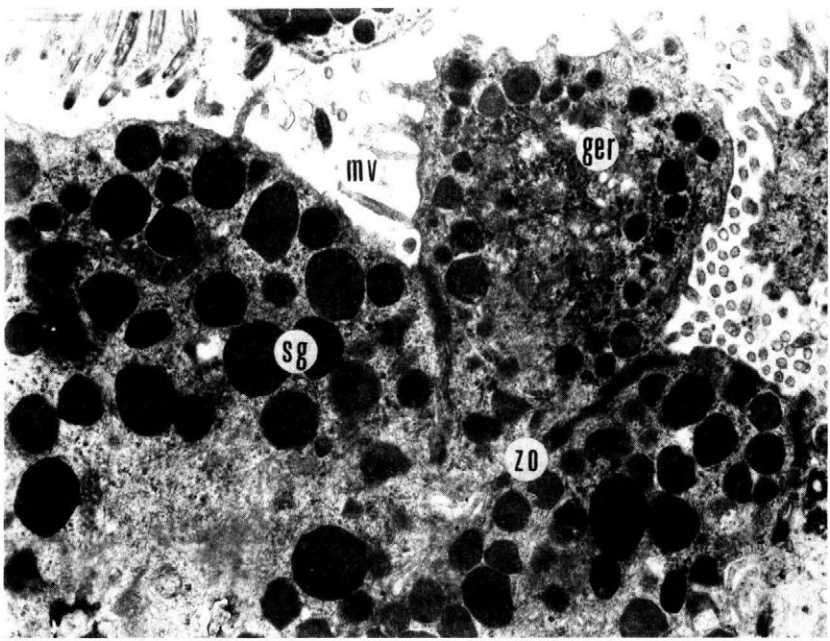
part of the cell and form high protrusions. They constrict and detach from the cell surface and the cytoplasmic cell membrane closes. The detached part of the cell disintegrates and the granules become part of the secretion. Less frequently, the membrane of the secretory granule adheres to the internal surface of the cytoplasmic membrane and the content of the granule, which exhibits most frequently a lamellar arrangement, is released through the developed pore. The cytoplasmic membrane forms microvilli of different size in the apical portion of the cells. Extrusion of the whole secretory cells was also observed (Fig. 9).

DISCUSSION

Puerperium is an acyclic period characterized by a physiological decrease in the levels of progesterone,

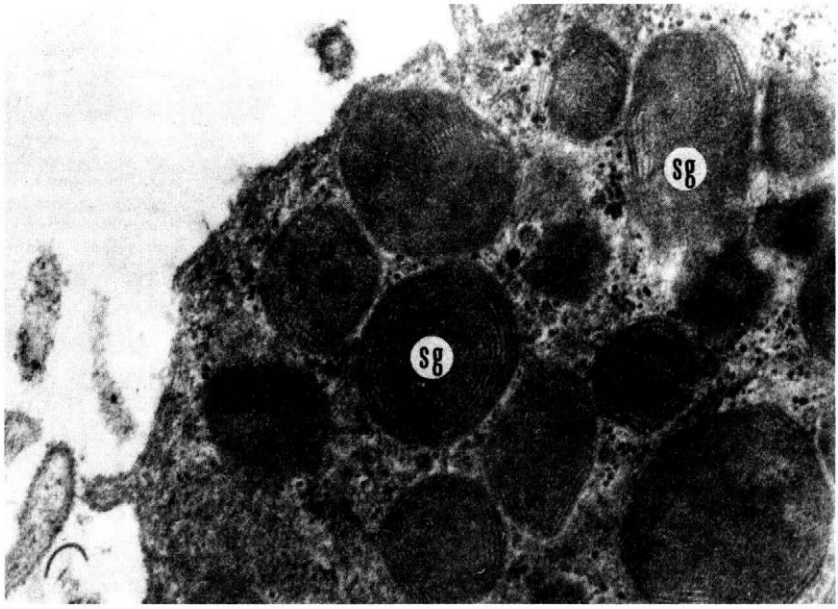


5. Electron micrograph of an apical part of ampullar secretory and ciliated cells on day 17 *post partum*. Sc – secretory cells, Cc – ciliary cells, k – kinocilia, bc – basal corpuscles, mv – microvilli, sg – secretory granules, zo – *zonulae occludentes*, interdigitation (arrow) x 12 000

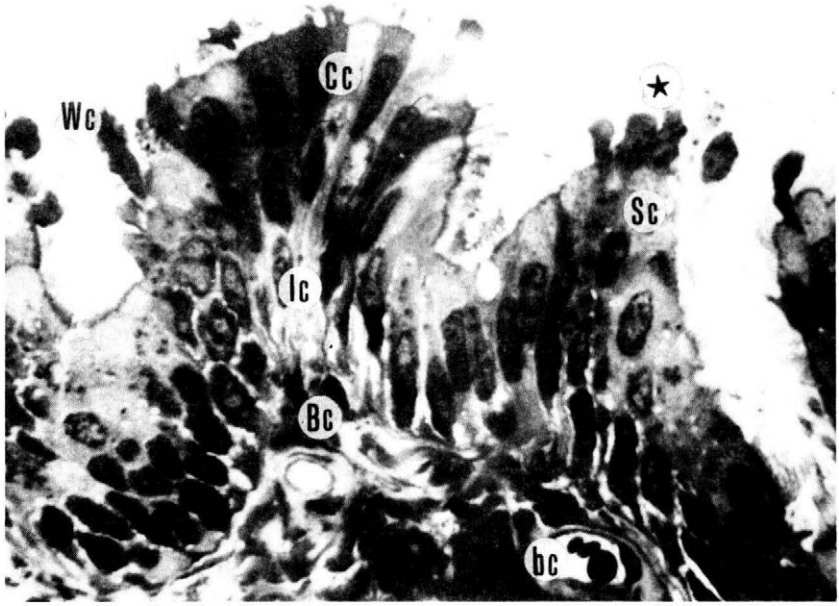


6. Electron micrograph of an apical part of ampullar secretory cells on day 25 *post partum*. sg – secretory granules, mv – microvilli, zo – *zonulae occludentes*, ger – granular endoplasmic reticulum x 12 000

estradiol and luteinizing hormone (Karg and Schallenberger, 1983; Zralý et al., 1989). The low level of progesterone is related to the involution of *corpus luteum graviditatis* (Erb et al., 1971). The endocrine state and the development of sexual functions after the parturition depends directly on the dynamics of hormonal changes in the last phase of gravidity and after parturition. The hormonal changes mentioned affect considerably the morphological structure of oviduct epithelium cells during the puerperium, which was the subject of investigations of the present study. They prove that the differentiation of cells, ciliation, deciliation as well as formation of secretory granules and their release are controlled by hormones. While estrogens have stimulative effect on ciliation, progesterone, on the other hand, induces deciliation (Uhrín, 1983;



7. Electron micrograph of an apical part of an ampullar secretory cell on day 25 - *post partum*. Notice the concentric lamellae and moderately electron-dense material in the secretory granules (sg) x 20 400

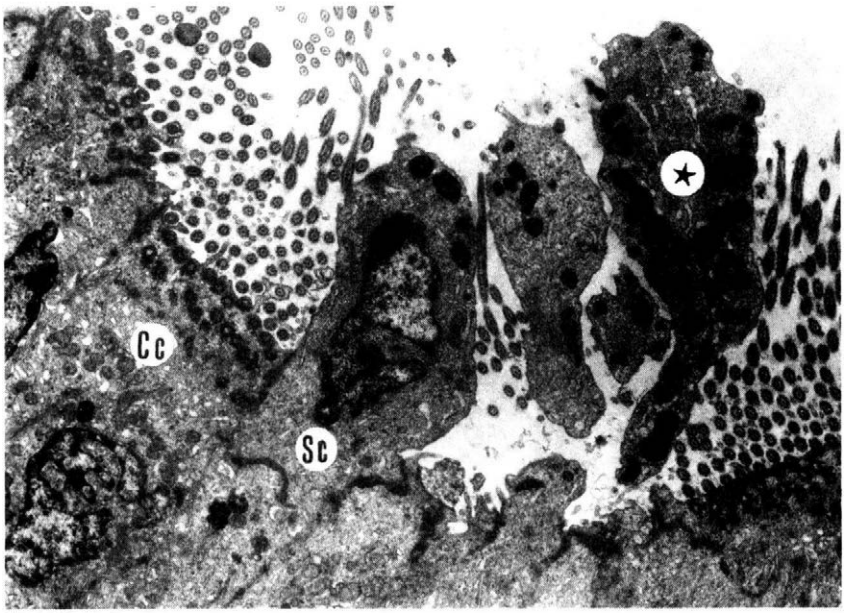


8. Epithelium of the ewe uterine tube ampulla on day 34 *post partum*. A semithin section stained with toluidine blue. Sc – secretory cells, Cc – ciliated cells (light and dark), Bc – basal cells, Ic – intercalary (rod shaped) cells, Wc – wedge shaped cells, bc – blood capillaries in the connective tissue. Notice the highly protruding apical portions of the cytoplasm of secretory cells with secretory granules and the detached portions of secretory cells in the oviductal lumen (asterix) x 1 000

Uhrín and Kliment, 1983a). Estrogens stimulate the formation of secretory granules while progesterone inhibits this process (Willemse and Van Vorstenbosch, 1975; Uhrín and Kliment, 1982b).

On the basis of the results of our study we can state that shortly after the parturition the oviduct ampulla epithelium was low. Ciliated cells were dark, they had few kinocilia on their surface, and microvilli along their perimeter. We assume that deciliation occurred as early as during gravidity. In this context, very interesting is the statement of Nayak et al., (1976) who observed cyclic changes in the epithelium of the ewe oviduct infundibulum. He reported that the pronounced lack of cilia was not apparent either in the follicular or in the luteal phase of the oestrous cycle.

On day 17 and especially on day 25 *post partum* pronounced changes were observed in ciliated cells. The cells were tall, either light or darker, had well-de-



9. Electron micrograph of an apical part of ampullar secretory and ciliated cells on day 34 *post partum*. Like in Fig. 3, we can observe high protrusions and separated parts of secretory cells with granules in the oviduct lumen (asterix). Sc – secretory cell, Cc – ciliated cell x 8 200

veloped granular endoplasmic reticulum, ribosomes, polyribosomes and mitochondria with cristae. Their occurrence suggests active proteosynthesis. Basal corpuscles and kinocilia formed in the cells. We assume that the occurrence of light and dark cells with cilia, frequently in their immediate neighbourhood, is related to the degree of their differentiation. The mechanism of kinocilia formation was studied in detail by Brenner (1970), Jirsová et al. (1979), and Uhrín and Kliment (1983a). Shortly after the parturition the secretory cells were abundant, their cytoplasm was electronlucent and contained microvilli on their free surface. However, they did not contain any secretory granules. The synthesis and gradual accumulation of granules in supranuclear and apical portions was observed as late as on days 17 and 25 *post partum*. Secretory cells had well-developed granular endoplasmic reticulum, Golgi complex, numerous ribosomes, mitochondria and secretory granules of various size, shape and density. On day 34 *post partum* glycogen accumulated in ciliated cells and cisternae of granular endoplasmic reticulum were dilated. The most remarkable feature of the granules was their membranous structure, consisting of concentric lamellae of equal dimensions. Typical extrusion of secretory granules into the oviduct lumen was apparent on day 34 *post partum*. Nuclei containing cytoplasmic projections protruded into the tubal lumen while some of them were found free in the lumen. Less frequently, the secretory granules were extruded through the pores in the cytoplasmic membrane. Neither mitosis of basal cells nor the transformation of ciliated cells to secretory ones, and vice versa, was observed.

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EFFICACY OF FLUBENDAZOLE AGAINST *MUELLERIUS CAPILLARIS* IN MOUFLON

ÚČINNOST FLUBENDAZOLU PROTI *MUELLERIUS CAPILLARIS* MUFLONÍ ZVĚŘE

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ABSTRACT: *Muellerius capillaris* is the most important pneumonematode of the mouflon kept in the Czech Republic. Particularly high values of prevalence and intensity of infection are recorded in the mouflon stocks kept in preserves. Numerous anthelmintics can be used for pharmacotherapy. Adulticide effect of the drug in verminous lung foci should be the aim of administrations. The aim of this study was to test anthelmintic efficacy of flubendazole (FBZ) against *M. capillaris* in mouflon. The study was conducted in 16 mouflons in a small game preserve. An FBZ dose of 3 x 15 mg/kg live weight was chosen to be tested. Samples of mixed droppings were collected before treatment, during it and after its termination. LPG values were determined by larvoscopic examinations. Four (2 x 2) mouflons were shot on day 7 and 14 after treatment termination and they were subjected to detailed helminthologic examinations (macroscopic description of pulmonary verminous lesions, larvoscopy of verminous foci and mucus smears from the respiratory tract, larvoscopy of individual droppings). The game were extremely willing to ingest the drug applied with feed, the dosing schedule being confirmed. Pre-treatment LPG values of mixed droppings (Fig. 1) fell rapidly after the treatment started, and the excretion of *M. capillaris* larvae completely terminated beginning on day 7 after treatment termination. Pulmonary and coprological LPG findings in the shot mouflons (their description is in Tab. I) were minimum, in one animal only (Tab. II), in the others the findings were zero. Macroscopic findings (Fig. 2) showed that all shot mouflons had suffered from the infection to a large extent before treatment. The therapeutic efficacy of FBZ administered at a dose of 3 x 15 mg/kg of body weight can be in general evaluated highly positively. The drug administration quickly stopped larvae excretion in the mouflon droppings due to adulticide effect in the verminous foci of the lungs.

flubendazole; *Muellerius capillaris*; mouflon

ABSTRAKT: *Muellerius capillaris* je obligátním pneumonematodem mufloní zvěře. Farmakoterapeutický přístup k řešení infekce je třeba vést s cílem dosažení helmintocidního (adulticidního) účinku léčby. S tímto záměrem byla zorganizována studie účinků flubendazolu (aplikační schéma 3 x 15 mg/kg ž. hm.) proti *M. capillaris* mufloní zvěře. Studie byla uskutečněna na skupině 16 kusů mufloní zvěře. Parazitologickými metodami (především larvoskopie) byl vyšetřen směsný trus zvěře před léčbou, v průběhu a po ukončení léčby, plíce a individuální trus čtyř kusů (2 + 2) zvěře ulovených v termínech 7 a 14 dnů po ukončení léčby. Z výsledků všech vyšetření vyplývá, že flubendazol v ověřovaném aplikačním schématu působí kompletně adulticidně proti *M. capillaris* mufloní zvěře.

flubendazol; *Muellerius capillaris*; muflon

ÚVOD

Muellerius capillaris je v současnosti všudypřítomným pneumonematodem chovů mufloní zvěře v České republice. Patogenita malých plicnivek (*Protostrongylidae*), kam *M. capillaris* patří, je považována za podstatně nižší než je tomu u plicnivek velkých (*Dictyocaulidae*), (Lochman aj., 1979; Páv aj., 1981; Kotrlá aj., 1984). Zvěř mufloní však obecněji k hostitelům velkých plicnivek nepatří (Kotrlá aj., 1984), *M. capillaris* proto zaujímá mezi pneumonematody nejvýznamnější postavení. Hodnoty prevalence i intenzity infekce (především v oborních chovech) jsou často v absolutních vyjádřeních značně vysoké, což se projevuje nepřímými, méně i přímými, ztrátami na chované zvěři.

Farmakoterapie infekce *M. capillaris* je uskutečnitelná u muflonů mnohými léčivy. Jejich účinnost byla posuzována při použití různých dávkovacích schémat pomocí koprologických metodik. Těmito přístupy je dosahována a také prokazována pouze helmintostatická účinnost použitých léčiv (Kalivoda a Chroust, 1971; Chroust, 1982, 1985). Helmintocidní účinnost, která je u *M. capillaris* žádoucí vzhledem k loka-

lizaci dospělců ve verminózních centrech a ložiscích plic, byla doposud prokázána jen u ivermektinu (L a m k a aj., 1995).

Cílem této studie bylo a) vytipovat aplikační schéma pro flubendazol (FBZ) s předpokladem adulticidního účinku léčiva proti *M. capillaris*, b) experimentálně jednoznačně prokázat dosažený charakter účinku FBZ.

MATERIÁL A METODA

Lokalita a termín ověření

Náhodně vyčleněných 16 kusů mufloní zvěře z celooborní populace (Obora Opočno) bylo po celou dobu ověřování chováno v obůrce o rozloze 5 ha. Ověření bylo uskutečněno v termínu leden–únor 1996.

Podání FBZ, termíny sběru vzorků trusu

FBZ (Janssen Pharmaceutica) byl získán ve formě 50% premixu, do krmné směsi byl zamíchán dvoustupňově. Medikovaná krmná směs byla předkládána tři dny po sobě v denní dávce FBZ 15 mg/kg živé hmotnosti. Před léčbou, v průběhu léčby a po jejím ukončení byly opakovaně sbírány vzorky směsného trusu. Termíny sběrů jsou uvedeny v obr. 1.

Metodika zajištění a vyšetření parazitologických materiálů

Směsný trus od živé zvěře byl sbírán vždy čerstvý od nejméně tří čtvrtin kusů sledované skupiny, vyšetřován byl opakovaně a kvantitativně dle obvyklých postupů (Baermanova metoda) a doporučení (P o w e r s aj., 1982). Byly stanoveny hodnoty počtu larev na 1 g vyšetřovaného materiálu (LPG). V termínech 7 a 14 dnů po ukončení léčby byly odloveny vždy dva kusy zvěře označené A až D (tab. I). Plíce ulovené zvěře byly makroskopicky posouzeny na přítomnost verminózních změn a vzorky postižené tkáně byly larvoskopicky vyšetřeny. Kultivace parazitologických materiálů (plicní tkáň, veškeré koprologické materiály) byla prováděna ve vzorcích o hmotnosti 3 g. Celkový počet vzorků vyšetřované plicní tkáně byl dán rozsahem postižení plic jednotlivých kusů zvěře (A – 18 g, B – 27 g, C – 27 g, D – 9 g). Vyšetření plic byla doplněna i larvoskopickým vyšetřením vždy tří stěrů hlenu z různých míst trachey. Od ulovených kusů zvěře byl zároveň vyšetřen i trus odebraný z koncové části tlustého střeva. Tato vyšetření byla provedena v triplete-tech.

VÝSLEDKY

Larvoskopická vyšetření směsných vzorků trusu

Předléčebné LPG hodnoty byly počínaje prvním dnem léčby FBZ výrazně redukovány, poslední nález byl prokázán osmý den od zahájení léčby. V pozdějších intervalech byly nálezy pouze negativní (obr. 1).

Helmintologické nálezy v plicích a trusu odlovených kusů zvěře

Vyšetřením plic byly makroskopicky objeveny u všech zvířat příznaky infekce *M. capillaris*. Rozsahy postižení plic byly u jednotlivých zvířat rozdílné (obr. 2). Opakovaným vyšetřením tkáně verminózních center a ložisek bylo pouze u jediného kusu nalezeno malé reziduum larev (tab. II), ostatní tři kusy zvěře byly zcela bez larvoskopických nálezů. Výsledky larvoskopických vyšetření stěrů dýchacích cest byly ve všech případech negativní.

Trus ulovených kusů zvěře byl ve třech případech bez nálezu larev *M. capillaris*, pouze u jediného kusu byla prokázána velmi nízká hodnota LPG (tab. II).

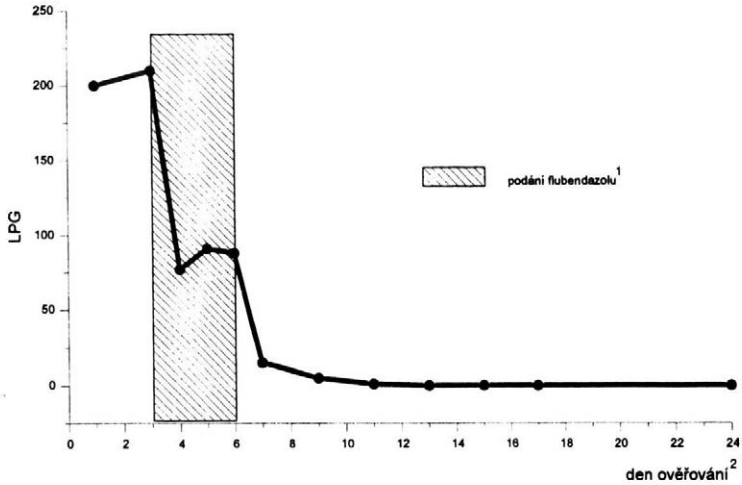
DISKUSE

Zkušenosti s využitelností FBZ u spárkaté zvěře jsou velmi omezené. Známy nám jsou pouze výsledky tří firmou Janssen zadaných studií u jelení, srnčí a mufloní zvěře uskutečněné Kutzerem v letech 1984 až 1986 v Rakousku. V nich byl FBZ ověřován (metodicky použita larvoskopie trusu) i v případě infekce mufloní *M. capillaris* s velmi dobrými výsledky. Na základě uskutečněných studií Kutzer doporučuje ze všech ověřených aplikačních schémat (2 x 15 mg/kg. ž. hm., 5 x 5 mg/kg ž. hm. a 10 x 3 mg/kg ž. hm.) první z uvedených. Výsledky neposkytují informaci o dosaženém charakteru účinku proti dospělcům *M. capillaris*. Dosažené zkušenosti Kutzer později částečně publikoval a ještě doplnil doporučením dávkovat FBZ 3 x

I. Charakteristika zvěře ulovené pro helmintologické vyšetření – Characteristics of the mouflons shot for helminthologic examinations

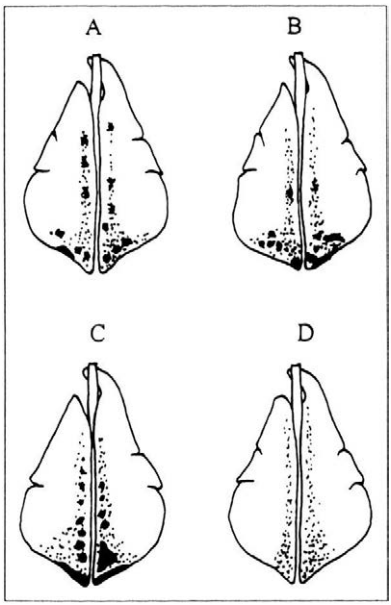
Označení zvěře[1]	Pohlaví zvěře[2]	Věk zvěře (měsíce)[3]	Celková hmotnost[4] (kg)	Termín odlovu (den po ukončení léčby)[5]
A	♀	71	32	7
B	♂	11	21	7
C	♀	11	18	14
D	♀	11	17	14

[1]mouflon designation, [2]sex, [3]age (months), [4]body weight, [5]day of shoot (the day after treatment termination)



1. Průběh larvoskopických nálezů ve směsných vzorcích trusu před, v průběhu a po aplikaci fubendazolu – The pattern of larvoscopic findings in mixed samples of droppings before, during and after flubendazole administration

[1]flubendazole administration, [2]day of experiment



2. Grafické znázornění makroskopických změn v plicích ulovené zvěře – Diagrams of macroscopic lesions in the lungs of shot mouflons

15 mg/kg živé hmotnosti (Kutzer, 1995). Pro naše ověření jsme po předpokusu dali přednost také doposud nejvyššímu ověřovanému dávkování FBZ.

Rozhodující podmínkou objektivního ověřování léčiv u spárkaté zvěře je úroveň jejich příjmu a udržení plánovaného dávkovacího schématu. FBZ byl v našem ověření ochotně přijímán všemi kusy zvěře po celou dobu studie, dávkování FBZ bylo dodrženo. V průběhu studie ani později nebyly zaznamenány žádné vedlejší účinky použitého dávkování FBZ a to ani u pěti vysokobřezích muflonek (obvyklý termín kladení mufloňčat v oboře je 5.–20. 3. běžného roku).

Terapeutickou účinnost FBZ hodnocenou ze směsných koprologických nálezů lze charakterizovat jako velmi dobrou. Již během léčby nastal prudký pokles LPG hodnot, od sedmého dne po ukončení léčby bylo vylučování larev *M. capillaris* trusem plně zastaveno.

Odlov čtyř kusů zvěře umožnil podrobné helmintologické vyšetření plic a stanovení charakteru účinku FBZ. Byla nalezena pouze zcela nepatrná larvoskopická rezidua u jediného kusu. Pro vyšetření bylo přitom použito vždy maximum dostupné plicní tkáně postižené přítomností *M. capillaris*. Pokud provádíme obdobná larvoskopická vyšetření plic u zvěře neléčené, LPG hodnoty dosahují řádově hodnot 10^2 až 10^3. Přibližně takový by musel být i nález u námi ulovené zvěře před

II. Výsledky pitevních helmintologických vyšetření – The results of post-mortem helminthologic examinations

Označení zvěře[1]	Stěr dýchacích cest (larvoskopie)[2]	Larvoskopie verminózních ložisek[3] (LPG)	Larvoskopie trusu[4] (LPG)
A	B.N. (n = 3)	6 (n = 6)	2 (n = 3)
B	B.N. (n = 3)	B.N. (n = 9)	B.N. (n = 3)
C	B.N. (n = 3)	B.N. (n = 9)	B.N. (n = 3)
D	B.N. (n = 3)	B.N. (n = 3)	B.N. (n = 3)

B.N. = bez nálezu – no finding
n – počet vyšetření – number of examinations

[1]mouflon designation, [2]smear from the respiratory tract (larvoscopy), [3]larvoscopy of verminous foci, [4]droppings larvoscopy

léčbou. Z tohoto pohledu je možné účinnost FBZ hodnotit opět vysoce pozitivně. Charakter účinku lze označit za helmintocidní. Larvoskopická vyšetření plicní tkáně doplňují a potvrzují i negativní nálezy larvoskopických vyšetření stěrů dýchacích cest i nepatrné LPG nálezy (a to pouze v kratším intervalu odlovu) individuálního trusu ulovených kusů zvěře.

Závěrem lze konstatovat, že dávkovacím schématem FBZ 3 x 15 mg/kg ž. hm. bylo dosaženo velmi dobrého anthelmintického účinku proti *M. capillaris*. Léčivo působí adulticidně ve verminózních centrech a ložiscích plic mufloní zvěře.

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MUTAGENICITY OF FEEDS AND BOVINE MILK IN DISTRICTS WITH DIFFERENT LEVELS OF ENVIRONMENTAL POLLUTION*

MUTAGENITA KRMIVA A MLÉKA SKOTU V OBLASTECH S ROZDÍLNOU ÚROVNÍ ZNEČIŠTĚNÉHO PROSTŘEDÍ

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ABSTRACT: Mutagenic activities of feedstuffs and bovine milk were investigated by the Ames test in two districts of the Czech Republic (Teplice and Prachatice), differing in the levels of industrial pollution. *Salmonella typhimurium* strains TA 98 and TA 100 with or without metabolic activation were used in the tests. Samples of currently fed feedstuffs and milk were collected in both districts in the spring 1992, autumn 1992, spring 1993, and autumn 1993. The Teplice and Prachatice sets included 12 and 11 representative samples, respectively. The samples were rinsed and extracted with acetone and methanol. Thus each sample was divided into four separate samples. The total number of revertants obtained in the industrial district Teplice was significantly higher than the corresponding value for the Prachatice district. Some between-the-seasons differences were also found within each of the two districts. The strain TA 98 proved to be more sensitive in the mutagenicity tests than the strain TA 100. Thirty-eight and thirty-one milk samples were collected in the districts Teplice and Prachatice, respectively. The overall evaluation demonstrated a significant between-the-districts difference in the total number of revertants induced by the milk samples in the strains TA 98 and TA 100.

mutagenicity; feeds; milk; *Salmonella typhimurium*; Ames test; cattle

ABSTRACT: Ve dvou okresech České republiky, v Teplicích a Prachaticích, byla s použitím Amesova testu sledována mutagenní aktivita krmiva a mléka skotu. K testování byly použity indikátorové kmeny *Salmonella typhimurium*, TA 98 a TA 100 s metabolickou aktivací i bez této aktivace. Odběrová období byla: jaro 1992, podzim 1992, jaro 1993 a podzim 1993. V Teplicích bylo odebráno celkem 12 a v Prachaticích 11 reprezentativních vzorků krmiva. U jednotlivých krmiv byly prováděny oplachy a extrakty v acetonu a metanolu. Každé krmivo bylo v Amesově testu hodnoceno jako čtyři samostatné vzorky. Celková suma revertant získaných v okrese Teplice statisticky významně prověřila sumu revertant v okrese Prachatice. Zároveň jsme zjistili také některé mezisezonní rozdíly v rámci jednotlivých okresů. Mutagenita byla detekována ve větší míře na kmenu TA 98. V daných obdobích bylo v okrese Teplice odebráno 38 a v okrese Prachatice 31 vzorků mléka. Po celkovém zhodnocení všech období byl na kmenech TA 98 a TA 100 v sumě revertant prokázán mezi Teplicemi a Prachaticemi statisticky významný rozdíl pro vzorky mléka.

mutagenní aktivita; krmiva; mléko; *Salmonella typhimurium*; Amesův test; skot

INTRODUCTION

Environmental pollution may have adverse impacts not only on human health, but also on wild and domesticated animals and wild and cultural plants.

In response to the actual state of the environment, a comprehensive project known as „The Teplice Programme" was launched in 1991. The objectives of the project are to monitor in detail individual components of the environment and to assess their impacts on human health. The severely polluted district of Teplice was selected as a model area and the moderately polluted district of Prachatice as its counterpart for comparative purposes.

Environmental impacts on the human population can be assessed not only from results of extensive laboratory analyses of environmental samples and of measurements of biological effects, but also from results of examinations of other species, such as cattle, used as biological indicators. Cattle is an important component of the human food chain and cumulation of residues in meat and their shedding in milk pose additional hazards

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to man living in the affected area. Cytogenetic analyses of bovine circulating lymphocytes were carried out simultaneously in the two districts by Rubeš et al. (1994).

One of the ways of the penetration of xenobiotics into the food chain is the vegetation. Plants take up heterogeneous mixtures of genotoxic compounds from the air, water and soil, and cumulate them on the surface and/or metabolize them in their structures thus augmenting the environmental impacts on living creatures.

The investigations presented here are based on the fact that xenobiotics present in the environment affect live organisms at various interacting levels. Therefore, the objectives of the investigations were:

- to find out by the Ames test the mutagenicity of green fodder in the districts Teplice and Prachatice and to demonstrate possible differences in the mutagenicity between the two districts;
- to find out the mutagenicity of milk produced in the polluted area as an immediate risk to the food chain.

The Ames test (Ames et al., 1975) is the most widespread rapid test for the screening of genotoxic agents and their mixtures. The test uses a set of strains of *Salmonella typhimurium* with various mutation types in the histidine operon. This deficit is manifested by the failure to grow in histidine free media. Mutagens induce reversions which become phenotypically apparent by resumed production of histidine and, consequently, by the growth in histidine free media.

MATERIALS AND METHODS

SAMPLES

Feeds

Feed samples were collected in all selected herds in the districts of Teplice and Prachatice in the autumn 1992 and in the spring 1993 at the time of blood sampling for cytogenetic analyses. Only feed and no blood samples were collected in the autumn 1993. The samples represented the currently fed forages, i. e. fresh grass, alfalfa and mixtures in the autumn and silages and haylages in the spring. The Teplice and Prachatice sets included 12 and 11 representative samples, respectively.

Milk

The milk samples were collected from cooling tanks (400 ml of each milk sample) after the morning milking. The samples of milk were transported in the glass bottles (0.5 l) and stored at –20 °C. Thirty-eight and thirty-one milk samples were collected in the districts of Teplice and Prachatice, respectively.

SAMPLE PROCESSING

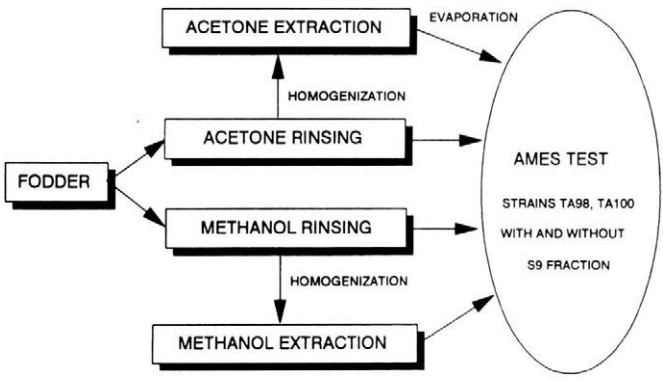
Feeds

Rinsings and extracts with a methanol and acetone as polar and non-polar solvents were prepared from each sample. Each sample (150 g) was first rinsed with 500 ml of each of the two solvents and the rinsed material (100 g) was homogenized and shaken with the two solvents (2 x 2 h). After filtration and centrifugation, the solvents were removed in a rotating vacuum evaporator and the residue was dissolved in DMSO (Scheme I).

Ames tests were done with 10 ml, 5 ml and 2.5 ml of each rinsing per 1 plate, and with extract aliquots corresponding to 200 mg, 100 mg and 50 mg of the dry matter per 1 plate (theoretical calculation of dry matter according to the Czech Standard CSN 46 7007).

Milk

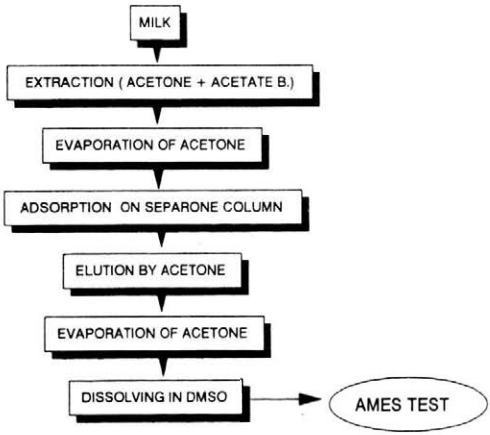
Milk samples (400 ml) were treated with acetone and acetate buffer. Acetone was removed in a rotating vacuum evaporator (40 °C) and the water fraction was passed through a column filled with the organic sorbent SEPARON SE (TESSEK, Prague, grain size 0.125–0.2 μm). The adsorbed pollutants were eluted with ace-



I. Treatment of feed samples

tone, the latter was removed in an evaporator and the residue was dissolved in 6 ml DMSO (Gajdůšková, 1983; Černá et al., 1989) – Scheme II.

Ames tests were done with aliquots corresponding to 14 ml, 7 ml and 3.5 ml of milk per plate.



II. Treatment of milk

AMES TEST

The Ames test (Maron and Ames, 1983; Černá et al., 1989) using the strains TA 98 (frameshift mutations) and TA 100 (substitutions of base pairs) with or without metabolic activation was used to detect mutagenicity. The two strains allow sensitive detection of a number of known mutagens and promutagens, including nitropolyaromatic and polyaromatic hydrocarbons, aflatoxin B1, etc.

The fraction S9 of a liver homogenate (mice C57 B1) and a mixture of cofactors (NADP + glucoso-6-phosphate) were used to simulate the metabolic activation *in vitro*.

Each dose was tested in triplicates.

EVALUATION OF RESULTS

Revertant colonies were counted after 72 hours of incubation at 37 °C and two ways of evaluation of the results were chosen:

1. A twofold or higher and dose-dependent increase in the number of revertants, compared with the negative control, was regarded as an evidence of mutagenicity (Ames et al., 1975).
2. The numbers of revertants for individual dosages were converted to numbers per volume (ml) or weight (100 mg dry matter) units and expressed as a sum for each district. The differences in the numbers of revertants obtained by the examinations of feeds, milk and urine were evaluated by the non-parametric Mann-Whitney or the Kruskal-Wallis tests using the software STAT Plus Vers. 1.10. (Matoušková et al., 1992).

RESULTS

FEEDS

Comparison of numbers of positive findings

The numbers of findings with mutagenic responses were 105 (55%) and 66 (38%) for the districts Teplice and Prachatice, respectively (Fig. 1). In both districts, the percentages of positive findings were higher in rinsings than in extracts. This applies particularly to the autumn sampling periods (Fig. 1). The numbers of positive findings in silage and haylage samples, collected in the spring, were lower than in the fresh fodder samples collected in the autumn. Toxicity was evident in some of the spring samples.

Overall between-the-districts comparison of sums of revertants

Sums of revertants per volume or weight units were calculated in both districts to compare all the results obtained by testing the feeds (both mutagenic and non-mutagenic). The sum of the revertants induced by rinsings or extracts in any of the test strains was significantly higher ($p \leq 0.01$) in the Teplice district than in the Prachatice district (Fig. 2).

Overall between-the-seasons comparison of sums of revertants

Comparisons of the sums of revertants induced by the samples collected in the autumn 1992 and the spring and autumn 1993 are shown in Fig. 3.

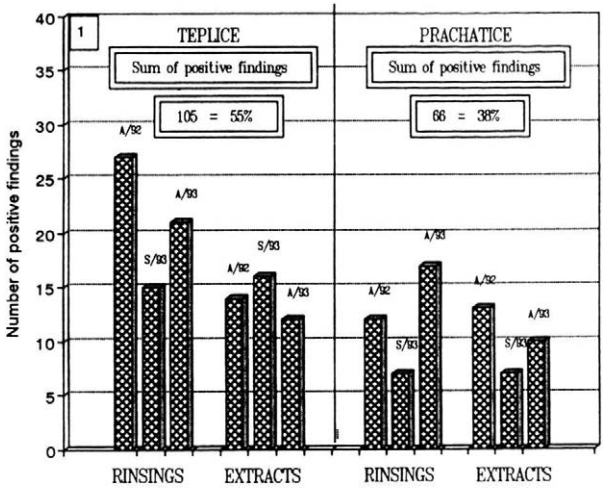
The Teplice district

The sum of the revertants induced by feed samples collected in the autumn 1992 was higher than the corresponding value for the samples collected in the spring 1993. The difference was highly significant for rinsings in the strain TA 98 and TA 100 and for extracts in the strains TA 100.

Also the number of revertants induced in the strain TA 98 by rinsings of feed samples collected in the autumn 1992 was higher than the corresponding value for the autumn 1993. The number of revertants induced in the strain TA 100 by feed samples collected in the autumn 1993 was higher than the corresponding value for the spring 1993. Both the latter differences were significant (Fig. 3).

The Prachatice district

The sum of revertants induced in the strain TA 98 by the feed samples collected in the autumn 1992 was



1. Positive findings in Teplice and Prachatice districts during the autumn 1992 (A/92), the spring 1993 (S/93) and the autumn 1993 (A/93). Positive findings in rinsings and extracts are shown separately

significantly lower than the corresponding values for the spring and autumn 1993.

A similar significant difference was found in the numbers of revertants induced in the strain TA 98 by extracts of feeds collected in the autumn 1992 and in the spring 1993.

On the other hand, the number of revertants induced by rinsings or extracts in the strain TA 100 was significantly higher in the autumn 1992 than in the spring 1993. The same was true also for extracts of feeds collected in the autumn 1992 and the autumn 1993 (Fig. 3).

Comparison of the districts Teplice and Prachatice

The sum of revertants induced by extracts or rinsings of feed samples collected in the autumn 1992 was significantly higher in the Teplice district than in the Prachatice district. A similar between-the-districts difference was found also for extracts of the autumn 1993 samples.

No differences in revertant numbers were found between the two districts in any of the strains in the spring 1993 and in the strain TA 100 in any of the sampling periods.

MILK

Mutagenic activity was not evident in any of the milk samples

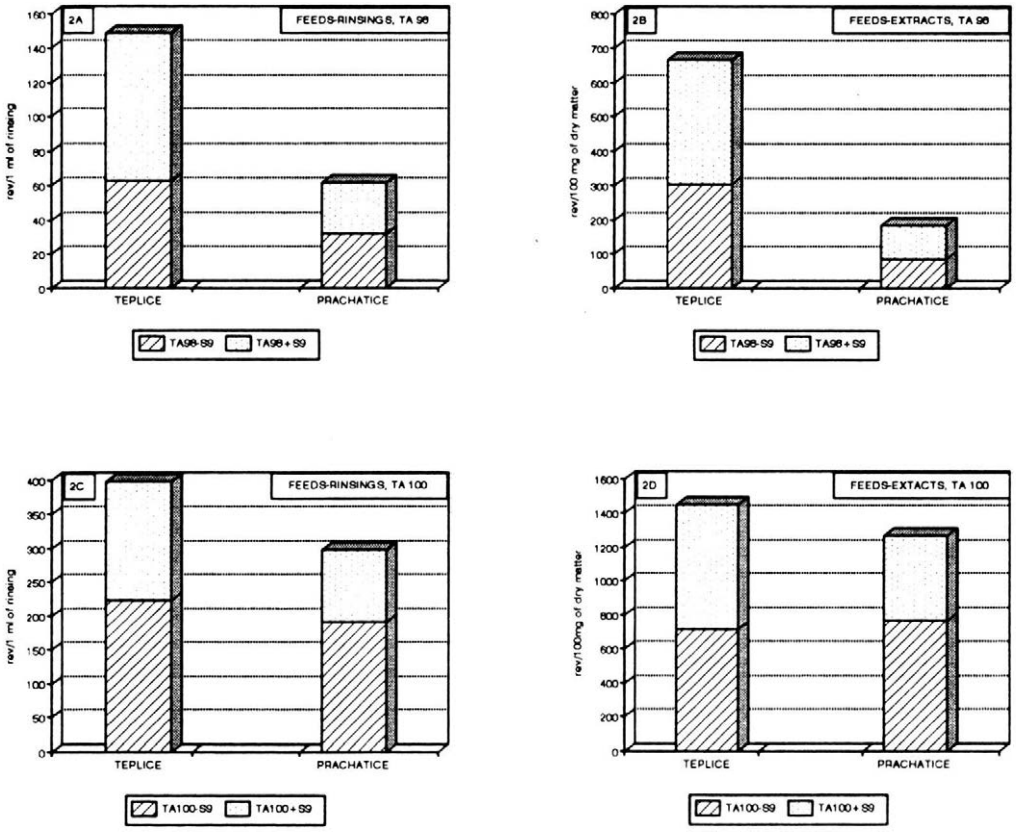
Total numbers of revertants per unit of volume of all milk samples collected in the three sampling periods were calculated to allow the comparison between the two districts. This sum was significantly higher ($p \leq 0.01$) in the strain TA 98 in the Prachatice district (Fig. 4), and in the strain TA 100 in the Teplice district (Fig. 5).

DISCUSSION

The aim of our investigations was to assess in both the districts the mutagenic potential of feeds consumed by cattle and the possible mutagenic activity of cow's milk.

The use of the Ames test for the determination of the mutagenic activity of plants was described by several authors. Mutagenicity of grain and sugar beet grown on soils polluted by wastes was tested by Hang et al. (1983). Van der Hoeven et al. (1983) published a comprehensive study of mutagenicity of several vegetable species consumed currently in the Netherlands. Suzuki et al. (1992) tested the mutagenicity of leaves of some wood species as an indicator of pollution rate of the urban environment. Separate testing and evaluation of rinsings and extracts of feeds allowed us to interpret the results more correctly because it eliminated possible misrepresentations resulting from the metabolic activity of plant tissues. The results of tests of rinsings express the mutagenicity of the fall-out cumulating on the surface of the plants, while those of extracts reflect the mutagenicity of the compounds that penetrate into plant structures, but can be transformed by the metabolic activity of natural components of plant tissues.

The results of tests of green plants can be affected by chlorophyll as an essential tissue component. Higher concentrations of chlorophyll can inhibit the mutagenic activity of certain compounds (4-nitro-o-phenylenediamine, e. g.), while lower concentrations can enhance it (Gentile and Gentile, 1991). Cabbage was eliminated from the feeds to be tested owing to its natural content of indolylglucosinolates (belonging to the indol group). As the grown cabbage is rich in nitrates, it is probable that indols present in cabbage are transformed endogenously into N-nitro compounds which then act as bacterial mutagens (Tiedink et al., 1989).



2. Comparison of all revertants induced by feed samples per volume or weight units in the districts Teplice and Prachatice

A – rinsings of feeds tested in TA 98, B – extracts of feeds tested in TA 98, C – rinsings of feeds tested in TA 100, D – extracts of feeds tested in TA 100

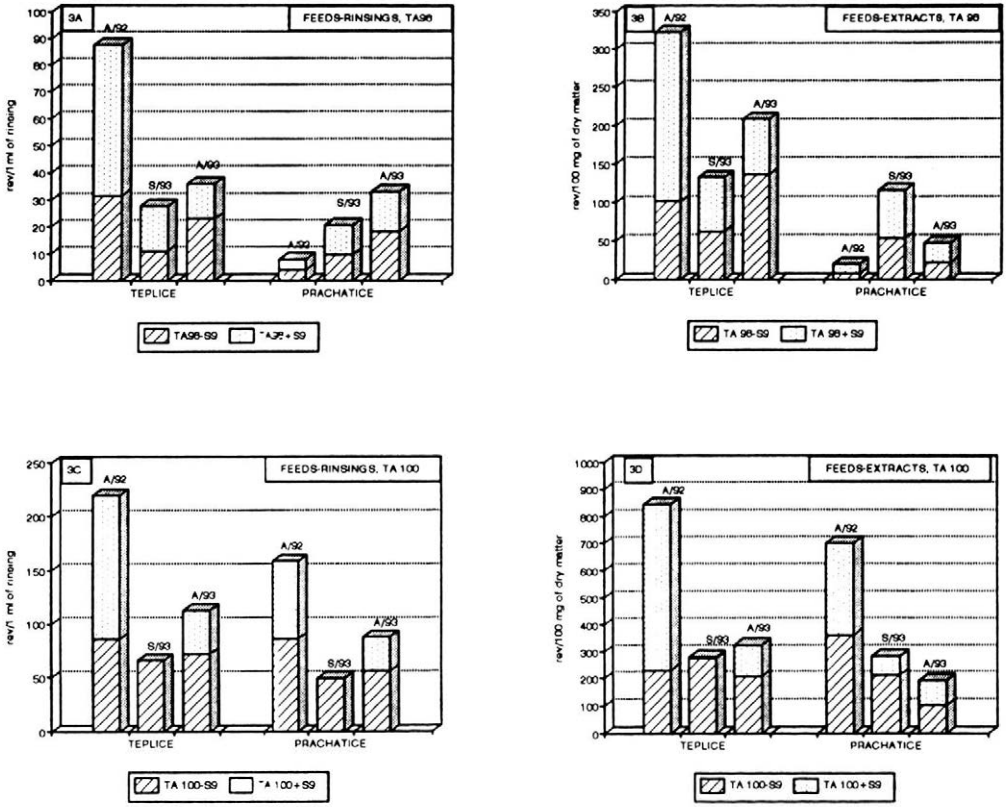
No qualitative or quantitative differences in the application of pesticides and fertilizers were found between the two districts. Nitrogen fertilizers at the rate of 200 kg per 10 000 sqm were used in both of them.

The sum of revertants induced by rinsings or extracts of feeds in any of the two test strains was significantly higher in the Teplice district than in the Prachatice district. The same was true also for the percentage of positive findings. These results are in accord with those of cytogenetic analyses carried out simultaneously in the same cattle herds by R u b e š et al. (1994).

More marked between-the-districts and between-the-seasons differences in the numbers of revertants were found in the strain TA 98 than in the strain TA 100. The same result was reported by D o b i á š et al. (1994), who investigated airborne mutagenicity in the Teplice district.

It was the strain TA 98, in which the sum of revertants in the Teplice district was high in the autumn 1992 and decreased in the spring 1993. On the other hand, the corresponding value for the Prachatice district was low in the autumn 1992 and increased in the spring 1993. Similar dynamics also reported for the percentages of aberrant cells in cattle in both districts by R u b e š et al. (1994).

Apparently, the autumn feed samples reflect the rate of air pollution in the summer and autumn 1992, when airborne mutagenicity was higher in the Teplice district than in the Prachatice district (D o b i á š et al., 1994). The low numbers of revertants induced by samples of ensiled feeds could be explained by extensive drainage of silage juices which removed a considerable part of the immissions from the surface of the plants. Possible degradation of certain mutagenic substances during fermentative processes in the ensiled mass should also be considered. On the other hand, the ensiling may be accompanied by the production of several toxins, particularly of mycotic origin. Indeed, we could occasionally observe toxic effects at high doses of some silage samples, mostly in the strain TA 100, which has been reported to be more sensitive to toxins than the strain TA 98 (J u r a d o et al., 1993).



3. Comparison of sums of all revertants induced by feed samples per volume or weight units in the districts Teplice and Prachatice during the autumn 1992 (A/92), the spring 1993 (S/93) and the autumn 1993 (A/93)

A – rinsings of feeds tested in TA 98, B – extracts of feeds tested in TA 98, C – rinsings of feeds tested in TA 100, D – extracts of feeds tested in TA 100

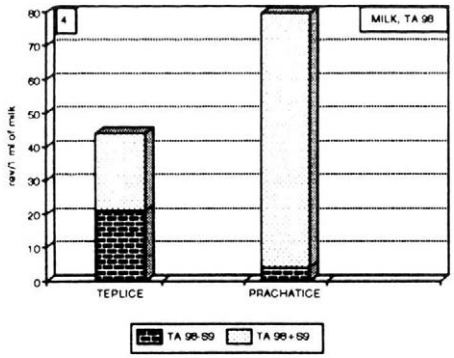
The industrial production was partly reduced in the Teplice district in 1993. This change could also be reflected in a significant decrease in the sum of revertants induced by feed rinsings in the strain TA 98. On the contrary, the sum of revertants induced by feed rinsings in the Prachatice district increased from 1992 to 1993. Although the results indicate that the between-the-districts differences diminished, the sum of revertants induced by extracts of the autumn 1993 samples continued to be significantly higher in the Teplice district than in the Prachatice district.

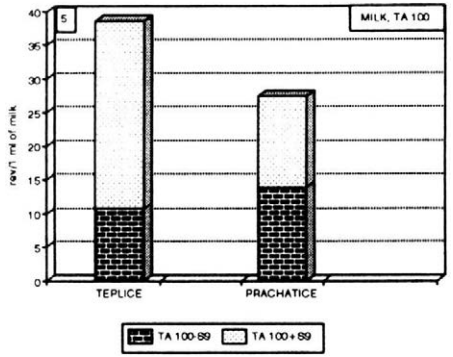
No mutagenic activity was demonstrable in any of the milk samples even the autumn 1992 when the highest percentages of aberrant cells in cattle and high numbers of revertants induced by feed rinsings or extracts were found. Chemical analyses (Gajdůšková and Ulrich, 1994) showed that the concentrations of lindane, DDE and HCB did not exceed permissible levels set by the Ministry of Health of the Czech Republic (Directive on Xenobiotics in Foods No. 50, vol. 431978).

The milk samples were also analysed for the concentration of PCB. However, this group, along with some solvents and metals, rank among compounds to which the Salmonella test is insensitive (Houk, 1992). The permissible concentration of 0.300 mg PCB sum per 1 kg milk fat was not exceeded in any of the samples. 3,5-dichlorophenol was demonstrated in two milk samples collected in the Teplice district at concentrations not exceeding 0.5 µg per 1 kg milk (Gajdůšková and Ulrich, 1994).

No between-the-districts differences were found in the concentrations of chlorinated agrochemicals in milk. The values ranged within one order of magnitude and were rather low. The permissible concentrations of mercury or cadmium were not exceeded in any of the samples (Gajdůšková and Ulrich, 1994).

Arai et al., (1982) and Green et al., (1980) demonstrated mutagenic activity of heat-processed milk and Berg et al. (1990) reported positive results of tests of combusted milk. On the other hand, no mutagenic activity could be demonstrated by Seki-





4 and 5. Comparison of sums of revertants induced by milk samples in TA 98 and TA 100 in the districts Teplice and Prachatice

zawa and Shibamoto (1986) in pasteurized, sterilized or UHT-processed milk. The method used in our experiments for the extraction of milk samples had been previously tested using the standard mutagen (promutagen) benzidine, which is active for TA 98+. Mutagenic activity and its dose-dependence were demonstrated in milk samples containing known concentrations of benzidine and processed by the same method. As reported by the author of the method, the recovery after separation from a Separon column is 85% (Gajdůšková, 1983).

The number of revertants induced by the tested milk samples was at or near the background level. This finding might be suggestive of the existence of one or more barriers preventing or retarding the penetration of xenobiotics through the organism. At the milking intervals of approx. 12 h, their concentration in milk might not reach the level detectable by mutagenicity tests. One of the barriers may also be the presence of antimutagenic substances in foods, such as vitamin C, tocopherols, sulphur compounds, etc. (Aeschbacher, 1990), but also casein which is known to bind mutagenic heterocyclic amines (Yoshida and Ye-Xiuyun, 1992). It is also probable that a number of other natural components of foods of animal origin, including milk, may bind with mutagenic particles and thus assist the defence system of the organism.

Screening by the bacteriological Ames test can detect the effects of complex substance mixtures or their reaction products on higher biological systems. The test can provide useful information in the detection of mutagenic components in rather complicated systems such as feeds and milk.

CONCLUSION

1. The percentages of positive findings and the sum of revertants of feeds were higher for the Teplice district.
2. The Teplice district showed a higher sum of revertants of feed samples in the autumn 1992 and its decrease in the spring 1993, while the Prachatice district showed lower value in the autumn 1992 than in the spring.
3. The results of feeds are in accord with the results of cytogenetic analyses carried out simultaneously in the same cattle herds by Rubeš et al. (1994).
4. The between-the-districts differences in feeds were more pronounced when the strain TA 98 was used.
5. Rinsing of feeds yielded a higher number of positive findings than extracts.
6. Mutagenic activity was not evident in any of the milk samples.

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SHORT COMMUNICATION – KRÁTKÉ SDĚLENÍ

RAPID METHOD FOR ISOLATION OF HIGH MOLECULAR WEIGHT PLASMIDS SUITABLE FOR DIGESTION WITH RESTRICTION ENDONUCLEASES*

MODIFIKOVANÁ METODA PRO IZOLACI PLAZMIDŮ O VYSOKÉ MOLEKULOVÉ HMOTNOSTI

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ABSTRACT: The described method is a modification of the hot alkaline method (K a d o and L i u , 1981). The modification lies in replacement of SDS with Triton X-100. The DNA purified by this method was readily digested with restriction endonucleases for a variety of applications, e. g. restriction analysis of plasmid DNA in epidemiological studies, restriction mapping and cloning of high molecular weight plasmids, and for identification of transpozon mutants.

Salmonella; *E. coli*; plasmid; SDS; Triton X-100; cloning

ABSTRAKT: V předložené práci je popsána rychlá metoda pro izolaci plazmidové DNA o vysoké molekulové hmotnosti. Modifikovaná metoda je založena na alkalické lyzi bakteriálních buněk za zvýšené teploty. Zvýšení kvality purifikované DNA bylo dosaženo nahrazením SDS Tritonem X-100. Takto purifikovaná DNA je snadno štěpitelná restrikčními endonukleázami a je vhodná pro restrikční analýzu plazmidové DNA při epidemiologických studiích, restrikčním mapování a klonování nebo lokalizaci transpozónových mutantů.

Salmonella; *E. coli*; plazmid; SDS; Triton X-100; klonování

The most widely used procedures for plasmid isolation are those described by B i r n b o i m and D o l y (1979) and K a d o and L i u (1981), with several recent modifications (K i b e n g e et al., 1991; M u s i c h and S h u , 1993; X i a n g et al., 1994). While the procedures derived from B i r n b o i m and D o l y (1979) are suitable for purification of plasmids of low molecular weight and high number of copies, e. g. plasmid vectors or recombinant molecules vector-foreign DNA, their use for isolation of plasmids of high molecular weight and low number of copies per cell was in our hands unreliable. The procedures derived from K a d o and L i u (1981), on the other hand, can be applied to plasmids of high molecular weight and low number of copies, however, the suitability of the isolated DNA for restriction endonuclease digestion is inconsistent. The aim of the study was to modify the method of K a d o and L i u (1981) in order to improve the reproducibility of restriction endonuclease digestion of high molecular weight, low copy number, plasmids.

The DNA isolated by the method of K a d o and L i u (1981) was in our hands reliably digested only with Bam HI, Pvu II and Sma I, and not with Eco RI, Hind III, Pst I, Cla I, Sal I, Kpn I, Bgl I and Xba I. The latter enzymes were either inactive or yielded non-reproducible results with a number of partial digests (Fig. 1). It was postulated that the substance inhibiting the activity of some of the restriction endonucleases was SDS, which, unlike the method of B i r n b o i m and D o l y (1979), is not precipitated by potassium acetate. SDS remains in the sample the whole time, and finally is partially precipitated by ethanol and blocks the activity of restriction endonucleases. Therefore, SDS was replaced by Triton X-100, which in most cases does not inhibit the activity of enzymes. Moreover, Triton X-100 is not precipitated by ethanol. Briefly, the described procedure was started by centrifugation of 1.5 ml overnight liquid bacterial culture. The sediment was resuspended in 50 µl of 50 mM EDTA. Two hundred microliters of the lysing solution

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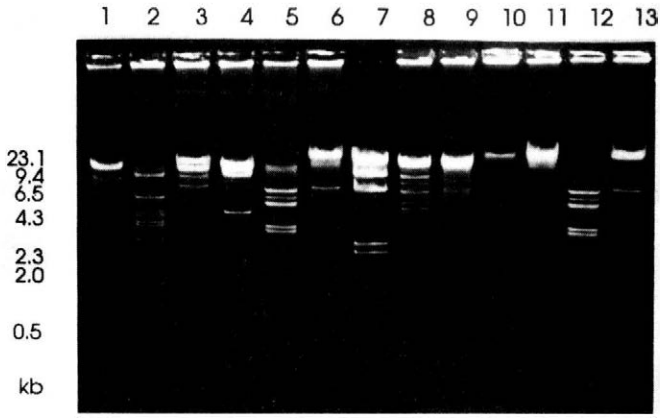

1. Virulence plasmid of *Salmonella typhimurium* LT2 isolated according to the described method (lanes 1–6) and according to Kado and Liu (1981) – (lanes 8–13). Plasmid DNA was digested with Sal I (lane 1 and 8), Pst I (2 and 9), Hind III (3 and 10), Eco RI (4 and 11), Pvu II (5 and 12) and Bam HI (6 and 13). Lane 7 – lambda DNA digested with Hind III as a molecular weight standard

were added (2% Triton X-100 and 0.2 M NaOH) and the mixture was incubated at 55 °C for 30 minutes. This was followed by one extraction with 200 µl of phenol : chloroform : isoamylalcohol (25 : 24 :1) and another one with chloroform : isoamylalcohol (24 :1). The plasmid DNA was precipitated with ethanol at 4 °C for 30 minutes. It was essential to reduce the pH of the sample prior to the precipitation by adding 1/10 of the volume 3M sodium acetate with pH adjusted by acetic acid to 5.0. The sediment was resuspended in 20 µl of TE solution (10 mM Tris-HCl pH 7,6 and 1 mM EDTA) and allowed to dissolve at room temperature for 10 minutes. Ten microliters were used for digestion with restriction endonucleases.

The described method proved to be time saving, and it facilitated reliable restriction endonuclease analyses of a number of plasmids for a variety of applications, e. g. characterisation of the virulence plasmids of Salmonella (Fig. 1), restriction analyses of plasmid DNA for epidemiological studies of Salmonella virulence plasmids (50–95 kb), restriction mapping and cloning of high molecular weight plasmids of wild type strains of *E. coli* and Salmonella, and for identification of transpozon mutants.

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