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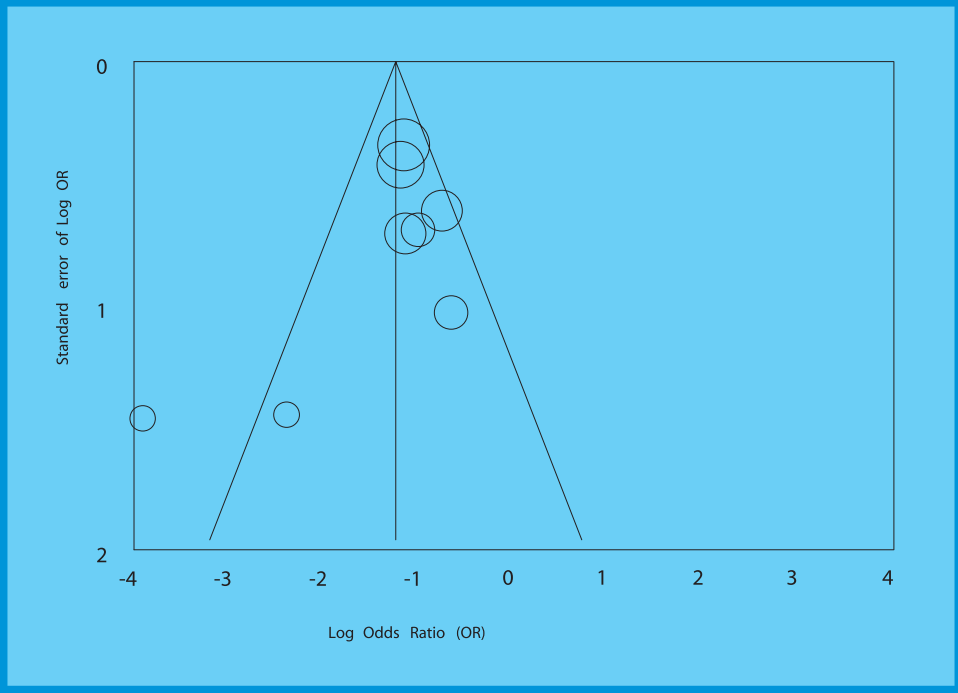

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Original Research Paper

MAST CELLS IN THE SUBEPIDERMAL LAYER OF THE BUDGERIGAR (*MELOPSITTACUS UNDULATUS*) DURING MOULTING

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Summary: We used histological methods to examine skin from the head region of budgerigars (*Melopsittacus undulatus*) during their feather regeneration (moulting period). Sixteen birds were included in the study; 8 were treated with thyroxine to accelerate moulting and another 8 represented the control group. Paraffin-embedded tissue samples were stained with haematoxylin and eosin, trichrome stain (after Goldner) and toluidine blue. Light microscopy was used to locate mast cells and their numbers were assessed by a Lucia-M computer-assisted image analysis system (Optoteam, Vienna).

Mast cells, which were found to be a regular component of budgerigar skin, were predominantly located in subepidermal connective tissue; however, they were also found near the dermal blood vessels. Thyroid hormones are elevated during moulting and lymphocytic infiltrations can be found in the skin, however, we found that the number of mast cells was significantly higher ($P < 0.01$) in thyroxine-treated animals. Although clinically healthy, 37 % of the animals investigated were infected with ectoparasites of the *Cnemidocoptes* genus. In those animals, which were found in both the thyroxine-treated and control groups, the number of mast cells was also significantly higher, probably as a consequence of their immune responses.

Key words: mast cells; budgerigar; skin; feather regeneration; parasitic infection

Introduction

Mast cells are a regular component of loose connective tissue, especially along blood vessels. They are migratory and are of various forms and sizes (1). They are relatively large cells with a centrally-located spherical nucleus and numerous cytoplasmic granules. Immature granules are small and orthochromatic with basic dyes, while the larger, mature granules are metachromatic (2, 3). Mast cells produce, store and excrete various biogenic amines and play an important role in an organism's immediate, delayed, local and systemic hypersensitivity. In mammals, two populations of mast cells are known: mucosal mast cells – associated with gut and lung mucosae, and the ubiquitous connective-tissue mast cells. Besides tissue distribution, they differ in their staining characteristics, receptor numbers and protease content (4). Increased numbers of connective-tissue mast cells are regularly found in the granulated tissue of healing wounds and in various pathological changes such as chronic dermatoses and many tumours (5).

Ehrlich drew attention to the specific metachromatic staining characteristics of these cells as early as 1877 and gave them the name "Mastzellen". Among many species, he described them in pigeons. Danchakoff (6) later described them in connective tissue along the blood vessels of the omentum in domestic fowl and Arvy (7) also described them in the connective tissue of *Gallus domesticus*. They are sparse in fowl compared to other domestic animals according to Boseila (8).

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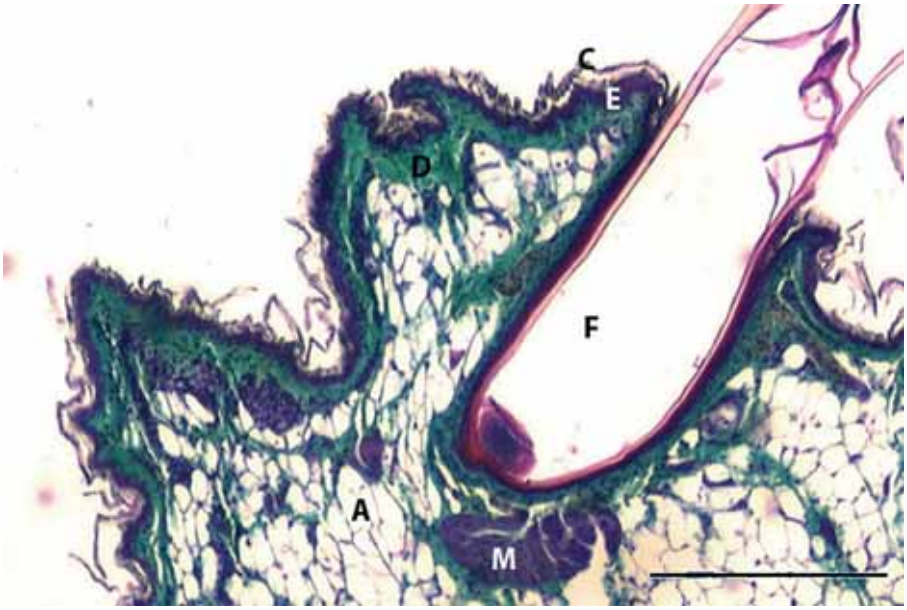

Figure 1: Skin from the head region of a budgerigar. Epidermis (E) with *stratum corneum* (C), dermis (D), feather muscles (*musculi pennarum*, M), feather follicle (F) and fat tissue in the hypodermis (*corpora adiposa*, A). Trichrome staining after Goldner, scale bar = 200 μm

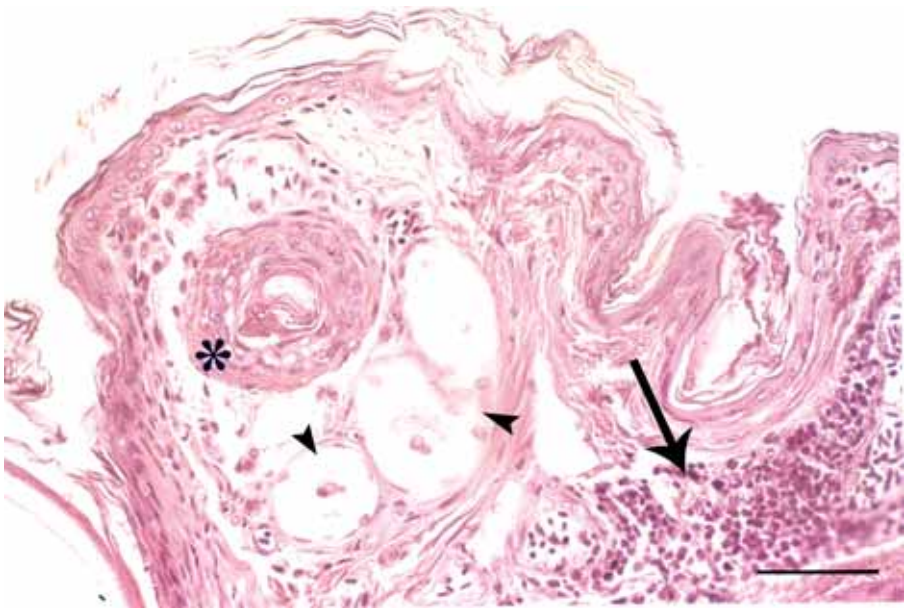

Figure 2: Skin from the head region of a budgerigar during moulting. Lymphocyte infiltrations during feather regeneration can be seen in the dermis (arrow) as can lamellar bodies (Herbst bodies, arrowheads) around a thread feather (filoplume, asterisk). H&E stain, scale bar = 50 μm

Wight (9) extensively researched *Gallus domesticus* and reported that mast cells are mainly concentrated in the infundibulum of the oviduct, the ovary, the periphery nerves and the alimentary tract, particularly the proventriculus and floor of the mouth. He observed that, in contrast to mammals, they are relatively infrequent in loose subcutaneous tissue. He also described the distribution and ultrastructure of the mast cells; their morphological characteristics indicated that there was only one type of mast cell in fowl.

Although physiological, moulting is stressful for birds. Since thyroid hormones are typically elevated, synthetic thyroxine is often used to induce or accelerate moulting for clinical purposes. During feather regeneration there is extensive activity in the skin and it becomes more exposed to environmental influences. While an activation of the immune system during moulting is to be expected there is no systematic description of mast cells in avian skin during moulting to be found in the available literature. Therefore, we added the study of mast cells, in particular the concentration and location of mast cells in the dermis of the head region, to the extensive research already conducted on the characteristics

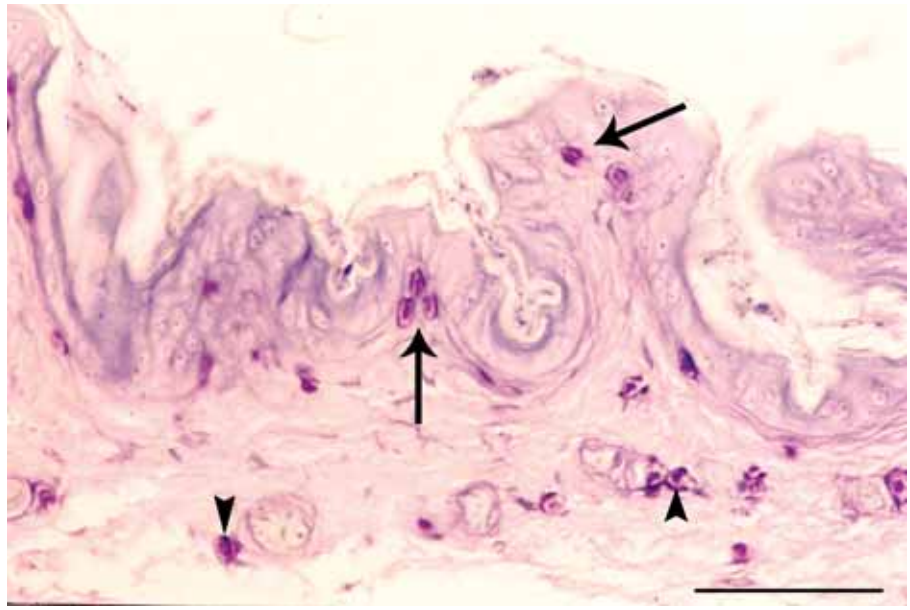

Figure 3: Skin of an L-thyroxine-treated animal. Numerous mast cells can be seen just beneath the epithelium - along the dermo-epidermal juncture (arrows) and in the connective tissue of the dermis near blood vessels (arrowheads). Toluidine blue staining at pH 5.2, scale bar = 50 µm

of budgerigar (*Melopsittacus undulatus*) moulting (10, 11).

Materials and methods

The research was conducted between the 10th and 27th of September on 16 budgerigars during their physiological moulting period. The animals were between 6 and 8 months old and clinically healthy. They were fed the same food and kept in identical climatic conditions with an average temperature of 23.6 °C.

We accelerated the moulting of 8 animals by giving them thyroxine and the other 8 were given a placebo (saline). At 7 p.m. for seventeen consecutive days, 500 µg/kg of body weight of levothyroxin L-T4 (Lek, Ljubljana, Slovenia), a synthetic, thyroid-gland hormone, was applied by cannula into the crop of the treatment group.

Four treated and 4 control animals were sacrificed on September 21st, and the remaining animals on September 27th – at the peak of the moult. Skin samples from the sacrificed animals were then taken from the head region (*pteryla capitalis*), fixed in Bouin's solution and embedded in paraffin. A Leica SM 2000R microtome was used to cut 5 µm tissue sections that were then stained with haematoxylin & eosin as well as with trichrome stain – after Goldner. Metachromasy of mast cells was confirmed by Toluidine blue, in a pH range from of 4.0 to 5.2.

A Nikon Microphot FXA microscope was used to perform the histological analyses and to take microphotographs. The histometry was performed using a Lucia M image analysis system (Optoteam Vienna). Mast cells were enumerated in the subepithelial layer of the dermis, which was 30 µm thick, and a total of 210,000 µm² of skin from each animal was analysed. The statistical differences between the mast cell numbers of each group were analysed with a Student 't'-test.

Results

A general histological analysis of the skin on the head region revealed a typical, thin epidermis with a keratinous layer and a dermis with feather follicles (Fig. 1). The animals whose moulting was accelerated (treatment group) had numerous lymphocyte infiltrations (Fig. 2). There were two locations where mast cells were primarily encountered: near the dermo-epidermal juncture and around the blood vessels (Fig. 3).

The histometric analyses revealed little difference between the animals in the treatment group but varying results within the control group. Some of the control animals had significantly lower numbers of mast cells than others. No differences were observed between the first and second groups of sacrificed animals within either the treatment or control group. An additional histological analysis of histological slides revealed a parasite infection in many of the animals used in our study. Developmental stages of the *Cnemi-*

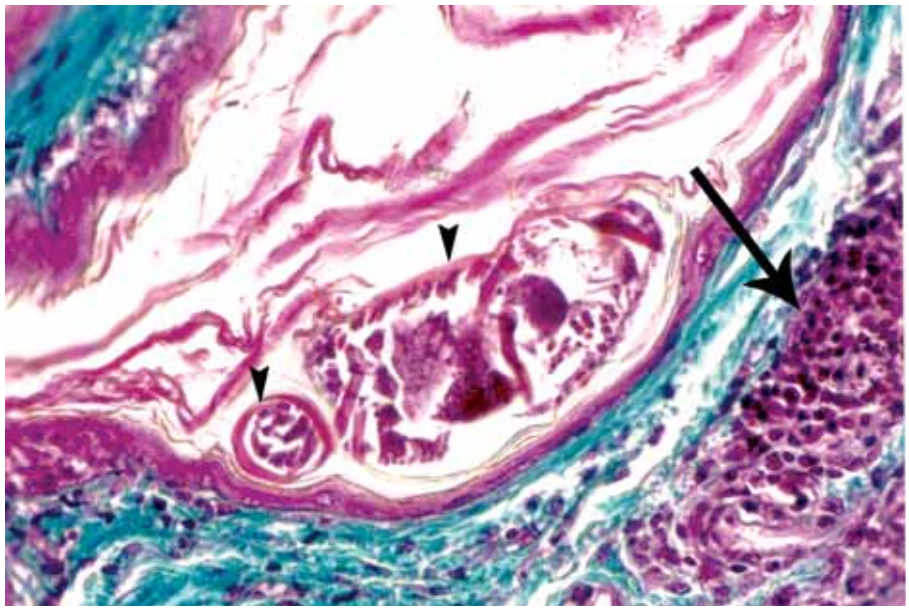

Figure 4: Skin from the head of a budgerigar. Many of the otherwise healthy animals were invaded by parasites of the *Cnemidocoptes* genus, which were found in the epithelial layer (arrowheads). A lymphocyte infiltration can be seen in the nearby dermis (arrow). Trichrome staining after Goldner, magnification x 200

Table 1: Number of mast cells in a 210000 μm² section of dermis taken from budgerigars in a treatment group, a control group and a group of animals invaded by ectoparasites
1 = first sacrifice, 2 = second sacrifice, C = control, T = treated

Treated		Control		Invaded by ectoparasites	
group	No. mast cells	group	No. mast cells	group	No. mast cells
1	86	1	30	1C	111
1	80	1	35	1C	73
1	112	2	29	2C	75
2	91	2	31	2C	91
2	113			1T	120
2	73			2T	178
average	92.5*		31.25		108*
SD	15.2		2.3		35.7

* The number of mast cells in the treated animals and those invaded by parasites (treated or control) were significantly higher than those in the uninfected control group ($P < 0.01$, Students t-test).

docoptes spp. ectoparasite were found in the epidermis of many of the animals (Fig. 4).

Following the diagnosis of ectoparasites and on the basis of experimental procedures, the animals were reclassified into 3 groups for further analysis: animals from the treatment group without parasites, control animals without parasites and animals with parasites. The animals in the control group without parasites had significantly lower numbers of mast cells in the dermis than those in the other two groups (Table 1).

Discussion

Differences in the numbers of mast cells found in the different groups of birds were found in this study. Although clinically healthy, many of the animals were found to be infected with the *Cnemidocoptes* spp. ectoparasite. These animals had a higher number of mast cells in the subepidermal layer of their skin than the uninfected control group. A more complete interpretation of the results was hindered by the relatively small number of animals in the study. It can be concluded, however, that the number of mast cells in

the untreated animals (control group) remained approximately the same from the onset of the moult (first sacrifice) until the time when the moult was at its most intensive (second sacrifice). Also in the L-thyroxine treated group, no significant differences in the number of mast cells could be noticed between the first sacrifice and the second.

However, an important difference between the two groups (treated and control) was found. The group of budgerigars that received treatment had a statistically-significant ($P < 0.01$) higher number of mast cells in the subepidermal layer of the head skin. It could be concluded that the L-thyroxine application induced a proliferation of mast cells in the subepidermal layer of the skin or their migration into this layer. The most likely cause of this was the accelerated moulting (induced by the L-thyroxine application) that compromised the intensified metabolism needed for skin and feather regeneration. This process includes an intensified engagement of the immune system – as was noted in a previous experiment where there were significantly higher numbers of lymphocytes in the skin of treated animals compared to control (untreated) animals (12).

Mast cells are known to be engaged in local immune responses, which includes a response to parasites (4). There are reports of increased numbers of mucosal mast cells in the intestines of fowl following worm or Eimeria invasions (13, 14, 15) but to the best of our knowledge this is one of the first studies to report an in increase in the mast-cell population following parasitosis in avian skin.

References

1. Dorsche HH, Ferhmann P, Sulzmann R. Die mastzelle als einzellige Druse. Acta Anat 1977; 77: 560-9.

2. Combs JW. Maturation of rat mast cells: an electron microscope study. J Cell Biol 1966; 13: 563-75.

3. Mendonca VO, Vugman I, Jamur MC. Maturation of adult rat peritoneal and mesenteric mast cells. A morphological and histofluorescence study. Cell Tissue Res 1986; 234: 635-9.

4. Galli SJ, Dvorak AM, Dvorak HF. Basophils and mast cells: morphologic insights into their biology, secretory patterns and function. Prog Allergy 1984; 34: 1-118.

5. Lever WF. Histopathology of the skin. 4th ed. Philadelphia: J.B. Lippincott, 1967: 52-6.

6. Danchakoff V. The wandering cells in loose connective tissue of the bird and their origin. Anat Rec 1916; 10: 483-92.

7. Arvy L. Les labrocytes (Mastzellen). Rev Hemat 1955; 10: 55-94.

8. Boseila AW A. Basophil leucocytes and histamine. Acta Allergol 1959; 14: 411-5.

9. Wight PAL. The mast cell of *Gallus domesticus*. I. Distribution and ultrastructure. Acta Anat 1970; 75: 100-13.

10. Golob Z. The effect of L-T4 and TRH treatment on the T3 and T4 blood serum concentrations, elements in the plumage and excrement, and head epidermis height of the budgerigar (Melopsittacus undulatus). Slov Vet Res 2001; 38: 209-17.

11. Golob Z, Bavdek SV, Cestnik V. The thyroxine influence on the moulting budgerigars (*Melopsittacus undulatus*). In: 5th European AAV & 3rd ECAMS scientific meeting. Pisa: European Committee of the Association of Avian Veterinarians, 1999: 140-3.

12. Golob Z. Influence of the thyroxine on the moulting dynamic and plumage regeneration of the budgerigar (Melopsittacus undulatus). Ljubljana: Veterinary Faculty, 1996. Thesis.

13. Lillehoy HS, Trout JM. Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. Clin Microbiol Rev 1996; 9: 349-60.

14. Mucha KH, Huffman JE. Inflammatory cell stimulation and wound healing in *Sphaeridotrema globulus* experimentally infected mallard ducks (*Anas platyrhynchos*). J Wildl Dis 1991; 27: 428-34.

15. Petrone VM, Constantino CF, Pradal-Roa P. Identification and quantification of granulocytes in caecal mucosa and submucosa of chickens experimentally infected with *Eimeria tenella* and *Salmonella enteritidis*. Br Poult Sci 2002; 43 (5): 653-61.

TKIVNI BAZOFILCI V PODEPITELIJSKEM PASU PRI PAPIGI SKOBČEVKI (*Melopsittacus undulatus*) MED GOLJENJEM

M. Štrbenc, U. Grahek, N. Herzog, Z. Golob

Povzetek: S histološkimi tehnikami smo proučili kožo na glavi med regeneracijo peres (goljenjem) pri papigi skobčevki (*Melopsittacus undulatus*). V poskusu je bilo 16 živali, od tega smo 8 dajali tiroksin, ki pospeši goljenje, 8 živali pa je predstavljalo kontrolno skupino. Vzorce kože na glavi smo fiksirali v raztopini Bouin, zalili v parafin, tkivne rezine pa nato barvali s hematoksilinom in eozinom, touloidinskim modrilom in s trikromnim barvanjem po Goldnerju. S svetlobno mikroskopijo smo ugotavljali položaj tkivnih bazofilcev v koži in z računalniško podprtim sistemom za analizo slike analizirali njihovo številčnost. Ugotovili smo, da so tkivni bazofilci redna sestavina kože pri papigi skobčevki. V naši študiji so bili predvsem na epidermalno-dermalnem stiku, pa tudi v vezivu usnjice v bližini krvnih žil. Čeprav je znano, da se med golitvijo poveča izločanje ščitničnih hormonov, v prejšnji raziskavi pa smo ugotovili tudi pogoste limfocitne inflitracije v koži, je bilo pri s tiroksinom tretiranih živalih število tkivnih bazofilcev statistično značilno ($P < 0,01$) večje. Vse živali v poskusu so bile klinično zdrave, vendar smo z mikroskopsko preiskavo pri 37 % živali ugotovili invadiranost z zajedavci iz rodu *Cnemidocoptes*. Tudi te živali (tako iz tretirane kot kontrolne skupine) so imele povečano število tkivnih bazofilcev, kar lahko povezujemo z imunskim odzivom.

Ključne besede: tkivni bazofilci; papiga skobčevka; koža; golitev; parazitoza

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Original Research Paper

A META-ANALYSIS AND SYSTEMATIC REVIEW OF THE EFFICACY OF ENROFLOXACIN – INFECTIONS WITH THE BACTERIUM *ESCHERICHIA COLI* IN PIGS

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Summary: A meta-analysis is a method of surveying and combining the results of several independent clinical trials. Besides quantitative integration, a meta-analysis vastly improves the potential for uncovering and studying any differences in available scientific material and provides a basis for plausible explanations of them. It can also lead to new discoveries and allows the aggregation of knowledge in the field of interest. This analytical method is of particular importance when assessing the efficacy of a therapy when the sample sizes of individual studies are too small to cover all aspects of a particular subject or provide a quantitative evaluation of the treatment's effect as well as test a null hypothesis. Prior to meta-analysis, the traditional method was a narrative discourse on previous findings, which, however, could be misleading and subjective.

Systematic reviews are concise summaries of the best available evidence that address sharply defined questions; they seek to assemble and examine all the high quality evidence on any given subject.

Enrofloxacin is a fluoroquinolone chemotherapeutic that was developed exclusively for use in veterinary medicine. Shortly after administration, low concentrations have a bactericidal effect against most Gram-negative and Gram-positive bacteria and will also act against mycoplasmata.

Twenty-four studies were included in this survey, of which seven were included in a meta-analysis while others were used to build a susceptibility profile of bacteria to enrofloxacin. Eight studies dealt with economic aspects of using enrofloxacin in pig rearing. The total number of animals included in the meta-analysis was 1,296, of which 655 were from the enrofloxacin-treated test groups of the different studies and 641 from their control or alternative treatment groups. In total, there were 19,235 strains of *E. coli* examined for their susceptibility to enrofloxacin.

The results of the meta-analysis are presented graphically. The odds ratio (OR) was used as a measure of the effect size and the homogeneity and/or heterogeneity values (Q) were calculated for the graphs. Additionally, a funnel plot was used to check the dispersion of the studies included in the meta-analysis. The graphs and the calculations ($P = 0.43$) show that the studies were homogeneous.

We also combined the *in vitro* susceptibility of individual microbes to enrofloxacin and evaluated their MIC values. Each study supported the enrofloxacin treatment; although there were three results that were statistically significant ($P < 0.05$), the overall result clearly indicates the high efficacy of enrofloxacin ($P < 0.01$) in reducing mortality caused by *E. coli* infections. Of the 19,235 strains surveyed, there were only 3.4 % that were resistant and the MIC values ranged between 0.015 and 0.25 µg/ml. The studies that dealt with the economic aspects of using enrofloxacin to combat *E. coli* infections, showed that it also has important additional benefits in pig rearing, especially in regards to daily weight gain and feed conversion. Moreover, enrofloxacin reduced the level of haemolytic *E. coli* excretions and sick animals recovered faster than those in the control or alternative treatment groups did. Our results have confirmed the high efficacy of enrofloxacin usage in *E. coli* infections, which makes it economically and professionally justifiable for treating coli infections in pigs.

Key words: enrofloxacin; *E. coli;* meta-analysis; susceptibility; pigs

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Introduction

A meta-analysis is a method of surveying and combining results of several independent clinical trials. The term meta-analysis was first used by Glass in 1976, who, with his associate Mary Lee Smith, statistically compiled the results of 375 studies dealing with the efficacy of psychotherapy.

There are many definitions of the term meta-analysis. The most frequently used definition is the one by Huque: meta-analysis refers to a statistical analysis that combines or integrates the results of several independent clinical trials considered by the analyst to be combinable (1). Besides quantitative integration, a meta-analysis vastly improves the potential for uncovering and studying any differences in the available scientific material and provides a basis for plausible explanations of them. It can also lead to new discoveries.

A brief summary of the procedures involved in a meta-analysis would comprise the following logical steps:

1. the definition of the problem and the inclusion criteria for the studies;
2. positioning, classifying and coding the characteristics of individual studies and the quantitative measurement of their characteristics (scale);
3. the integration of the results and a comparison with the characteristics of the studies (analysis and explication of results); and
4. the reporting of the results. (2)

Systematic reviews are concise summaries of the best available evidence that address sharply defined questions; they seek to assemble and examine all the high quality evidence on any given subject. This includes a comprehensive search of all potentially relevant articles and the use of explicit, reproducible criteria in the selection of articles for review. When the results of primary studies are summarized but not statistically combined, the review may be called a qualitative systematic review. A quantitative systematic review, or meta-analysis, is a systematic review that uses statistical methods to combine the results of two or more studies (3).

Investigating the efficacy of certain drugs can be done in several ways: by *in vitro* susceptibility profiling of certain bacteria, by clinical trials or by using meta-analyses or systematic reviews.

Immediately after farrowing, colibacillosis diseases threaten the lives of both the farrowing sow (MMA syndrome) and the newborn piglets. *Escherichia* are considered to be part of the normal intestinal bacterial flora. Pathogenic subspecies of *Escherichia* adhere to intestinal mucous membranes with special organelles, adhesins, which they differ from. *Escherichia* subspecies produce at least one exotoxin, e.g. the one that causes diarrhoea in suckling piglets and in weaners. Other neurotoxin- and/or verotoxin-producing subspecies cause oedematous diseases in pigs. Bacteria are not usually invasive; therefore, they are effective substances that act specifically in the intestines. This contrasts with the treatment of *E. coli* infections in poultry where infections are of a systemic nature (4, 5).

Enrofloxacin is a fluoroquinolone chemotherapeutic that was developed exclusively for use in veterinary medicine. Shortly after its administration, extremely low concentrations have a bactericidal effect against most Gram-negative and Gram-positive bacteria and will also act against mycoplasmata. It acts against both bacteria in the multiplication phase and dormant microorganisms. It is effective in the presence of oxygen and, owing to this phenomenon, it does not damage beneficial anaerobic intestinal microflora.

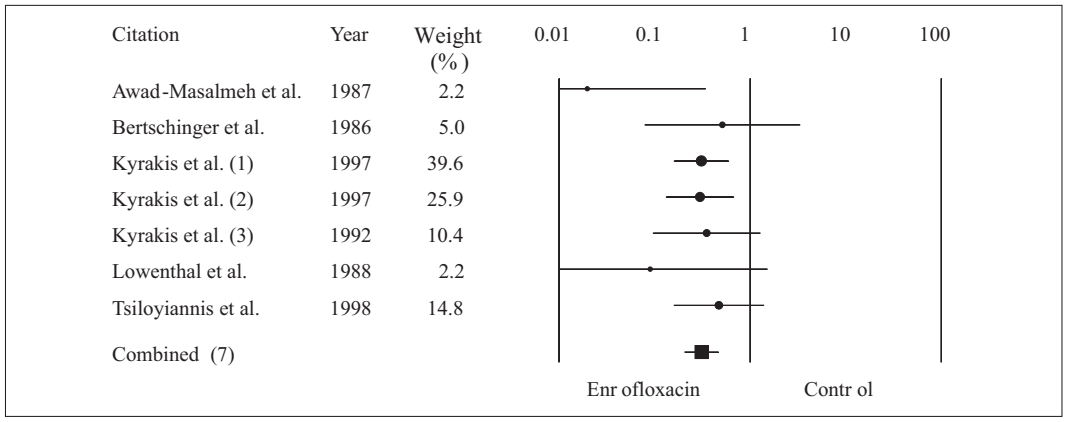
The efficacy of fluoroquinolones is related to both the maximum concentration and the time above their MIC value. *In vitro* pharmacokinetic models have shown that maximum concentrations of active substances, 8 times in excess of their MIC, have been able to reduce the number of bacteria by up to 99 % and inhibit their growth for up to 24 hours. The intensity of exposure may be quantified as the ratio between the area under the time-concentration curve (AUC) and the minimum inhibitory concentrations for the causative pathogens (MIC); a short term for this ratio is AIUC ? area under the inhibitory plasma concentration curve. For example, if in an enrofloxacin therapy the AIUC is higher than 125, the probability of a clinical and microbiological cure is above 80 %, otherwise it is only 42 % or 26 % in respect of a microbiological cure. Resistance to fluoroquinolones is also reduced to a minimum if these parameters are taken into consideration – C_{max}/MIC ratio is at least 8-10 and AUC/MIC at least 100-125 (6).

Table 1: Short survey of the studies included in the meta-analysis and the systematic review

Author, year of publication, number of animals in the study, (reference)	Meta-analysis and systematic review parameters Comparative control group
1. Lein et al., 1996; n = 86 (7)	- efficacy of enrofloxacin on the excretion of haemolytic *E. coli* - economic parameters (average daily gain, conversion, etc.) - negative control group
2. Lowenthal et al., 1988; n = 246 (8)	- clinical (intestinal bacterial infections) - mortality - gentamicin (1.0 mg/kg body weight) - trimethoprim (6.37 mg/kg body weight) and sulphadiazine (31.85 mg/kg body weight) - negative control group
3. Tsiloyiannis et al., 1998; n = 240 (9)	- mortality - economic parameters (average daily gain, conversion, feed intake) - negative control group - citric acid (1.5 %) and lactic acid (1.6 %) in feed
4. Kyrakis et al., 1997; n = 240 (10)	- mortality - economic parameters (average daily gain, conversion, feed intake) - negative control group
5. Kyrakis et al., 1997; n = 336 (11)	- mortality, clinical signs (diarrhoea) - economic parameters (average daily gain, conversion, feed intake) - negative control group
6. Kyrakis et al., 1992; n = 120 (12)	- mortality, clinical signs (diarrhoea) - economic parameters (average daily gain) - negative control group - apramycin (100 ppm)
7. Jae-Gil et al., 1992; n = 36 (13)	- economic parameters (average daily gain) - excretion of haemolytic *E. coli* - negative control group
8. Awald-Masalmeh and Willinger, 1987; n = 114 (14)	- mortality, clinical signs (diarrhoea) - economic parameters (average daily gain) - *in vitro* susceptibility testing of *E. coli* to enrofloxacin (% susceptibility) - negative control group
9. Bertschinger and Murdzinski, 1986; n = 48 (15)	- mortality, clinical signs (diarrhoea) - economic parameters (average daily gain) - ampicillin (40 mg/kg body weight) - control group (placebo)
10. Kołodziejczyk and Arh, 1999 (16)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
11. Melin et al., 1996 (17)	- *in vitro* susceptibility testing of *E. coli* (MIC values and % susceptibility)
12. Mateu and Martín, 2000 (18)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility 1997-1999)
13. Ala-Risku et al., 1997 (19)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
14. Bole-Hribovšek and Zdovc, 2002 (20)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
15. Bada et al., 1995 (21)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
16. Habrun et al., 1997 (22)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
17. Scheer et al., 1996 (23)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
18. Salmon et al., 1995 (24)	- *in vitro* susceptibility testing of *E. coli* (MIC values)
19. Aarestrup et al., 2000 (25)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility 1993-1998)
20. Trolldenier, 1996 (26)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility 1991-1994)
21. Deprez et al., 1986 (27)	- *in vitro* efficacy of enrofloxacin on the excretion of haemolytic *E. coli*
22. Awald-Masalmeh et al., 1992 (28)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
23. Belloc et al., 2002 (29)	- *in vitro* susceptibility testing of *E. coli* (% susceptibility)
24. Semjen and Wright, 1991 (30)	- *in vitro* susceptibility testing of *E. coli* (resistance development after multiple passages)

Material and methods

Twenty-four studies were included in this survey, of which seven were included in the meta-analysis while others were used to build a susceptibility profile of bacteria to enrofloxacin. All the susceptibility studies were comparative (e.g. comparisons between different antimicrobials), except for the two conducted by Aarestrup et al. and Semjen and Wright. Eight studies dealt with economic aspects of using enrofloxacin in pig rearing. The total number of animals included in the meta-analysis was 1296, of which 655 were from the enrofloxacin-treated test groups of the differ-



Graph 1: Meta-analysis of efficacy of enrofloxacin in treating *E. coli* infections in pigs (mortality)
Homogeneity testing:A Q = 5.17; df = 6; P = 0.52
(where Q = heterogeneity value (χ^2 distribution); df = degree of freedom; P = probability)

Table 2: *In vitro* susceptibility of *E. coli* to enrofloxacin

MIC range (µg/ml)	Resistant strains (% of 19,235 tested strains)
0.015 – 0.25	3.4

ent studies and 641 from their control or alternative treatment groups. In total, there were 19,235 strains of *E. coli* examined for their susceptibility to enrofloxacin.

Statistical analysis

The Comprehensive Meta-Analysis (Borenstein, 2000) computer programme was used for the statistical analysis.

Results

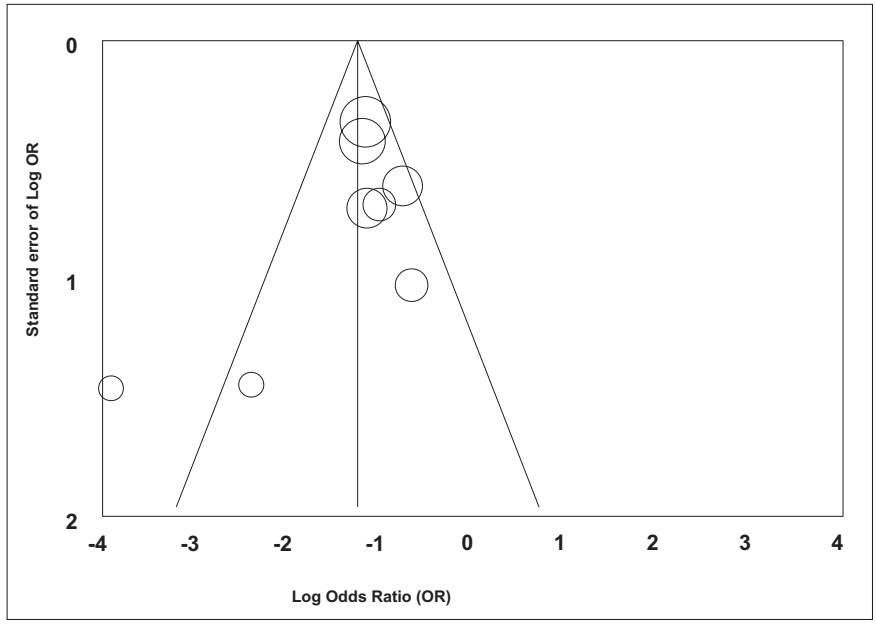
The results of meta-analyses are presented graphically. The graphs show the compiled data of individual studies (by author), the year that the study was conducted or its results published, a numerical comparison between the treatment and control groups and the mean values of effect size with 95 % confidence intervals.

The odds ratio (OR) was used as a measure of the effect size. An OR value of 1.0 means that a certain therapy has no effect, a value below 1.0 indicates that the therapy (in our case the use of enrofloxacin) is better than that of the control or alternate therapy. Values above 1.0 indicate that the control or alternate therapy was more effective. When interpreting the graph, if the mean value and the two lines either side of it, which indicate the confidence interval, do not intersect with the value of 1.0 then we speak about a statistical significance at different levels, e.g. with a 95 per cent confidence interval the P level of statistical significance equals 0.05; in 99 per cent intervals it equals 0.01, etc.

However, where a mean value, with its confidence intervals, intersects the 1.0 value line, we say that it has no statistical significance.

The homogeneity and/or heterogeneity (Q) values were calculated for the graphs. We must always form a null hypothesis that all studies pertain to the same population. The Q value is distributed to the Chi-square probability distribution with a k – 1 degree of freedom (df), where k represents the number of studies processed**.** However, statistically insignificant P values should not be considered as proof of homogeneity. Additional checks on the dispersion of the studies in the graph are obligatory. When we have evidence of heterogeneity, or a supporting calculation, we must check the graph and interpret the dispersion: do the majority of studies indicate that a treatment was effective while others indicate otherwise; does the effectiveness of the treatment vary greatly; or are there any extraordinary deviations. If a heterogeneity of $P < 0.05$ was calculated, then the total effect was always calculated using the random effects method as well.

The total effect size is always conditioned by the weight of individual studies, therefore, in a



Graph 2: Funnel plot showing standard error by effect size

meta-analysis it is referred to as the weighted total value, which, for individual studies, is represented by their mean values and confidence intervals. The weights of the individual studies are shown in the graph as full circles (•), studies with smaller weights have smaller circles and those with larger weights have larger circles. The total effect size is shown as a full square (■). Besides the graphical presentation of the meta-analysis results, a funnel graph was used to show the dispersion of the studies included in the meta-analysis. At first, funnel graphs were only used to monitor publication bias but we now know that they also indicate the heterogeneity of a study and the quality of its methodology. We also combined the *in vitro* susceptibility of individual microbes to enrofloxacin and evaluated their MIC values.

Discussion

Our survey included 24 studies, of which seven were included in a meta-analysis while others were used to build a susceptibility profile of bacteria to enrofloxacin. The studies included in the meta-analysis covered the period between 1986 and 2002.

The included studies were homogeneous, which is confirmed by the funnel graph where the only deviation (slight) was the study by Awald-Masalmeh et al. and this was probably due to its smaller sample size. As is evident from the meta-analysis, enrofloxacin is effective in the treatment of *E. coli* infections in pigs. Each study supported the enrofloxacin treatment; although there were three results that were statistically significant ($P < 0.05$) the overall result clearly indicates the high efficacy of enrofloxacin ($P < 0.01$) in reducing mortality caused by *E. coli* infections through neonatal septicaemia and diarrhoea, piglet scours, post-weaning diarrhoea and oedematous diseases.

Of the 19,235 strains surveyed, there were only 3.4 % that were resistant and the MIC values ranged between 0.015 and 0.25 µg/ml.

The studies that dealt with economically important aspects of using enrofloxacin to combat *E. coli* infections showed that it also has important additional benefits in pig rearing. In the majority of studies, groups that received enrofloxacin had statistically significantly better results than their control groups as well as better feed conversion rates and higher average daily weight gain. Moreover, enrofloxacin reduced the level of of haemolytic *E. coli* excretions and sick animals recovered faster than those in the control or alternative treatment group did.

The resistance of *E. coli* strains to enrofloxacin also is stable; however, to remain so it must be

used judiciously and in compliance with the results of scientific research, correct clinical judgement and dosage.

Our results have confirmed the high efficacy of enrofloxacin usage in the treatment of *E. coli* infections, which makes it economically justifiable and it is thus the right drug of choice in treating coli infections in pigs.

References

1. Huque MF. Experiences with meta-analysis in NDA submissions. In: Proceedings of the Biopharmac Section of the Am Stat Association. Rockville, 1988 (2): 28-33.

2. Sacks HS, Berrier J, Reitman D, Ancona-Berk VA, Chalmers TC. Meta-analyses of randomized controlled trials. New Engl Med 1987; 316: 450-5.

3. Mulrow C. Cook DC. Systematic reviews. Synthesis of best evidence for health care decisions. Philadelphia: American College of Physicians, 1998: 5-12.

4. Burch D. Commercially significant bacterial infections in the pig. In: Antibacterials: Products and markets. Animal Pharm Reports, 2000: 13-23.

5. Böhm O. Važnejše živalske bolezni v Sloveniji (1. del). Vet Nov, 2002; 28 (3): 115-28.

6. Walker RD. Fluoroquinolones. In: Prescott JF, Baggot JD, Walker RD, eds. Antimicrobial Therapy in Veterinary Medicine. 3rd ed. Ames: Iowa State University Press, 2000: 315-39.

7. Lein A, Verdonck M, De Kruif A. The efficacy of enrofloxacin in-feed medication (Baytril 2,5 medicated premix) on the excretion of haemolytic E. coli and post weaning diarrhoea. In: Proceedings of the 14th International Pig Veterinary Society Congress. Bologna, 1996: 267.

8. Lowenthal C, Dos Reis MT. A comparative test on the efficacy of enrofloxacin in intestinal bacterial infections in suckling pigs, from 3 different rearings, through individual oral medication, in comparison with gentamicin, and also with the association of trimethoprim + sulfadiazine. In: Proceedings of the 10th International Pig Veterinary Society. Rio de Janeiro, 1988: 109.

9. Tsiloyiannis VK, Kyrakis SC, Vlemmas J, Sarris K. The effect of organic acids on the control of post weaning oedema disease of piglets. In: Proceedings of the 15th International Pig Veterinary Society Congress. Birmingham, 1998: 152.

10. Kyrakis SC, Tsiloyianinis VK, Lekkas S, Petridou E, Vlemmas J, Sarris K. The efficacy of enrofloxacin in-feed medication, by applying different programmes for the control of post-weaning diarrhoea syndrome of piglets. J Vet Med B 1997; 44: 523-8.

11. Kyrakis SC, Tsiloyianinis VK, Lekkas S, Petridou E, Vlemmas J, Sarris K. The efficacy of enrofloxacin in-feed medication, by applying different programmes for the control of post-weaning oedema disease in piglets. J Vet Med B 1997; 44: 513-21.

12. Kyrakis SC, Sarris K, Papatsas JC. The effect of enrofloxacin (Baytril premix) in the feed for the control of post-weaning colibacillosis of piglets. In: Proceedings of the 12th International Pig Veterinary Society Congress. The Hague, 1992: 261.

13. Jae-Gil Y, Byeung-Gie K. Effect of Enrofloxacin on post-weaning diarrhoea in pigs. In: Proceedings of the 12th International Pig Veterinary Society Congress. The Hague, 1992: 259.

14. Awald-Masalmeh M, Willinger H. Untersuchungen über die Wirkung von Baytril gegen E. coli - Infektionen von Saug- und Absetzferkeln. Wien Tierärztl Monatsschr 1987; 64 (3): 105-8.

15. Bertschinger HU, Murdzinski. Experimental chemotherapy of porcine neonatal enteric colibacillosis with quinolone derivative (By Vp 2674, Baytril). In: Proceedings of the 9th International Pig Veterinary Society Congress. Gent, 1986; 142.

16. Kołodziejczyk P, Arh J. Analiza aktywności antybiotyków przeciwko Escherichia coli u prosiąt na fermie Ihan (Słowenia). Trzoda Chlewna, 1999; 7: 89-94.

17. Melin L, Franklin A, Horn af Rantzien M, Wallgren P. MIC values of faecal E. coli isolates from piglets in Sweden. In: Proceedings of the 14th International Pig Veterinary Society Congress. Bologna, 1996: 671.

18. Mateu E, Martín M. Antimicrobial resistance in enteric porcine Escherichia coli strains in Spain. Vet Rec 2000; 146: 703-5.

19. Ala-Risku V, Fossi M, Nyberg M. Vuosina 1992-95 sioista eristettyjen E. coli - bakteerikantojen mikrobilääkeherkkyys ja kahden eri herkkyysääritysmenetemän vertailu käyttäen oksitetrasykliiniä. Suomen Eläinlääkärilehti 1997; 103 (2): 67-70.

20. Bole-Hribovšek V, Zdovc I. Odpornost bakterij, ki se prenašajo z živali na ?loveka, v Sloveniji. Med Razgl 2002; 41 (Suppl 2): 7-24.

21. Bada R, Higgins R, Messier S. Sensibilité des isolates porcins de Pasteurella multocida, Escherichia coli, Actinobacillus pleuropneumoniae et Actinobacillus suis envers différents agents antibactériens. Méd Vét Québec 1995; 25 (3): 112-4.

22. Habrun B, Bilič V, Humski A. Bakterijske bolesti svinja u velikim aglomeracijama u Hrvatskoj tijekom 1996. In: Veterinarski dani '97: zbornik. Cavtat, 1997: 183-9.

23. Scheer M, Pirro F, Schmeer N. Baytril I.E.R. 2,5 - Eine neue Zubereitungsform für die orale Anwendung beim Schwein - antibakterielle Aktivität, Pharmakokinetik und Bioverfügbarkeit. Tierärztl Umschau 1996; 51: 489-97.

24. Salmon SA, Watts JL, Case CA, Hoffman LJ, Wegener HC, Yancey RJ. Comparison of MICs of ceftiofur and other antimicrobial agents against bacterial pathogens of swine from the United States, Canada, and Denmark. J Clin Microbiol 1995; 33 (9): 2435-44.

25. Aarestrup FM, Jensen NE, Jorsal SE, Nielsen TK. Emergence of resistance to fluoroquinolones among bacteria causing infections in food animals in Denmark. Vet Rec 2000; 146: 76-8.

26. Trolldenier H. Resistenzentwicklung von Infektionserregern landwirtschaftlicher Nutztiere in Deutschland (1990-1994): ein Überblick. Dtsch

Tierärztl Wschr 1996; 103: 237-84.

27. Deprez P, De Cupere F, Muylle E. Methaphylactic in feed medication with Baytril (enrofloxacin) against E. coli enterotoxemia. In: Proceedings of the 9th International Pig Veterinary Society Congress. Gent, 1986: 260.

28. Awald-Masalmeh M, Hinterdorfer F, El Hamaki-Jelinek H, Koefer J. Serotypes, K88 antigen, sensitivity of colicins and to chemotherapeutics of enteropathogenic E. coli isolated at two Austrian investigation centres between 1986 and 1990. In: Proceedings of the 12th International Pig Veterinary Society Congress. The Hague, 1992: 248.

29. Belloc C, Peron V, Scimia G, Goureau L, Pellerin JL, Laval A. Frequency of antimicrobial resistance in swine faecal Escherichia coli. In: Proceedings of the 17th International Pig Veterinary Society Congress. Ames, 2002: 179.

30. Semjen G, Wright P. In vitro development of resistance to nalidixic acid and enrofloxacin among Escherichia coli strains of porcine origin: Acta Vet Scand 1991; 32 (Suppl 87): 375-8.

METAANALIZA IN SISTEMATIČNI PRIKAZ UČINKOVITOSTI UPORABE ENROFLOKSACINA – OKUŽBE Z BAKTERIJO *ESCHERICHIA COLI* PRI PRAŠIČIH

L. Ščuka

Povzetek: Metaanaliza je metoda pregledovanja in kombiniranja rezultatov več neodvisnih kliničnih študij. Poleg kvantitativne integracije predstavlja tudi pomembno izboljšavo pri odkrivanju in preučevanju razlik v razpoložljivi znanstveni materiji in doseganju ali vsaj nudenju najverodostojnejših razlag ter celo pri odkrivanju novih spoznanj. Prispeva tudi h kopičenju znanja na določenem interesnem področju. Ta analitska metoda je še posebej pomembna pri ocenjevanju terapevtske učinkovitosti v primerih, ko posamezne študije ne zagotavljajo pregleda nad celotno obravnavano problematiko, po navadi imajo premajhne vzorce in zato ne morejo dati kvantitativne ocene učinka zdravljenja, in tudi ne preizkusiti ničelne hipoteze. Pred obdobjem metaanalize so pripovedno primerjali rezultate študij, kar pa je bilo lahko zavajajoče in subjektivno.

Sistematični prikazi so zgoščeni povzetki najboljših dostopnih dokazov, ki se nanašajo na jasno določena vprašanja; njihov namen je zbrati in preveriti vse visoko kakovostne dokaze o določenem specifičnem vprašanju.

Enrofloksacin je kemoterapevtik iz skupine fluorokinolonov, ki je bil razvit izključno za uporabo v veterinarski medicini. V zelo majhnih koncentracijah in hitro po dajanju deluje baktericidno na večino po Gramu negativnih in po Gramu pozitivnih bakterij, učinkuje pa tudi mikoplazmocidno.

V prikaz je bilo vključenih 24 študij, sedem od njih je bilo vključenih v samo metaanalizo, v drugih pa so opazovali profil občutljivosti bakterij za enrofloksacin. V osmih študijah so obravnavali ekonomske parametre prašičereje ob uporabi enrofloksacina. Skupno število živali iz različnih študij, vključenih v metaanalizo, je bilo 1296, od teh jih je bilo 655 razvrščenih v testne skupine, ki so prejemale enrofloksacin, ter 641 v kontrolne skupine, ki so prejemale primerjalno terapijo. Vse študije so vključevale skupno 19235 sevov *E. coli*, ki so jih preizkušali glede občutljivosti za enrofloksacin.

Rezultati metaanaliz so prikazani grafično. Kot velikost učinka smo izbrali razmerje obetov (odds ratio), izračunavali pa smo tudi vrednosti za homogenost oz. heterogenost (Q) študij. Z lijakastim grafičnim prikazom smo dodatno preverjali razpršitev študij v grafu. Iz grafov in izračunov ($P = 0.43$) je razvidna homogenost vseh vključenih študij. Pregledali smo tudi *in vitro* občutljivost posameznih mikrobov za enrofloksacin in ocenjevali vrednosti MIK.

Vse študije so govorile v prid zdravljenju z enrofloksacinom; le pri treh izmed njih so bili rezultati statistično pomembni ($P < 0.05$), skupni rezultat pa kaže na visoko učinkovitost enrofloksacina ($P < 0.01$) pri zmanjševanju smrtnosti zaradi okužb z *E. coli*. Skupni delež odpornih sevov ni presegal 3,4 % vseh v prikazu zajetih sevov in vrednost MIK se je gibala med 0,015 in 0,25 µg/ml. Študije, v katerih so raziskovali ekonomsko pomembne parametre rabe enrofloksacina pri okužbah z *E. coli*, so pokazale pomemben ugoden učinek enrofloksacina v prašičereji, še posebej na dnevno pridobivanje teže prašičev in izkoristek hrane. Enrofloksacin zmanjša tudi izločanje hemolitične *E. coli* in bolne živali si opomorejo hitreje kot tiste v kontrolni skupini, ki so vključene v primerjalno zdravljenje.

Naši rezultati potrjujejo, da je raba enrofloksacina pri zdravljenju okužb z *E. coli* zelo učinkovita, ekonomsko koristna in je zato pri okužbah s kolibakterijami pri prašičih uporaba enrofloksacin strokovno upravičena.

Ključne besede: enrofloksacin; *E. coli*; metaanaliza; občutljivost; prašiči

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Original Research Paper

INTRODUCTION AND MODIFICATION OF A MICROBIOLOGICAL METHOD FOR IDENTIFYING FLUMEQUINE IN MEAT

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Summary: In this study a bacteriological method for identifying flumequine residues in the meat of slaughtered animals was modified. We introduced a quantitative evaluation of the results by adding a lyophilization step to the existing bacteriological method, which made the method more sensitive. Our results proved that *E. coli* ATCC 25922 is susceptible to the antibiotic fluoroquinolone, which was already evident according to available literature. Due to the current regulations that prescribe maximum residue levels for antibiotic residues in food, we needed to modify our existing bacteriological method as the results it produced were not sensitive enough to identify the maximum residue levels in cattle and pig meat. We added lyophilization to the process, which we believe is the first time this has been attempted as no evidence of this procedure being used in this manner was found in the literature available. This modified method can be used for the quantitative as well as for the qualitative identification of flumequine.

Key words: methods; antibiotics; flumequine; residues; bacteriological technique; meat

Introduction

In both veterinary and human medicine, antibiotics have therapeutic, prophylactic, metaphylactic and/or nutritive applications (1, 21, 22). Flumequine is member of the fluoroquinolones class of antibiotics and is one of the most commonly prescribed antibiotics in veterinary medicine (2, 7, 8). Fluoroquinolones display bactericidal activity by inhibiting bacterial enzyme deoxyribonucleic acid (DNA) gyrase, which is vital for bacterial DNA duplication (10, 21, 23).

Fluoroquinolones are bactericidal against Gram-negative bacteria – *E. coli*, *Proteus, Shigella spp., Campylobacter jejuni, Pseudomonas aeruginosa*, Gram-positive bacteria – *Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis*, anaerobes – *Clostridium spp., Bacteroides fragilis* and against *M. tuberculosis and Mycoplasma pneumoniae* (19, 7, 8).

Flumequine is registered in Slovenia for use in veterinary medicine and its residues in food can be identified with microbiological, chemical and immunoenzyme methods (6, 11). At the Institute for Food Hygiene, we have used a bacteriological method for identifying antibiotic residues in food of animal origin since 1968. The method relies on the fact that antibiotic activity on certain bacterial strains produces inhibitory growth zones. It was used at the Institute to identify flumequine residues in food of animal origin until 11.5.2000, when the old regulation (17) that encapsulated a zero-tolerance policy towards antibiotics in food was replaced by EU directives (4, 5) that prescribe maximum residue levels (MRL) for antibiotic residues in food.

Therefore, we needed to quantify the levels of fluoroquinolone residues in food of animal origin in order to identify whether they were within the prescribed MRL. Hence, we needed to modify our bacteriological method.

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Materials and methods

The zones of inhibition found on the plates used as media to grow the different bacterial strains were measured. Two different bacterial test strains were used, one fully susceptible and the other resistant to the antibiotic being tested for. The width of the inhibition zone was entered in a calibration curve and the appropriate concentration of antibiotic was read off. A negative result on the plate seeded with the resistant bacterial strain was used to confirm the presence of the antibiotic.

Most laboratories use *Escherichia coli* to detect fluoroquinolone residues in food (3, 14, 15, 16, 9). In this study, we used *E. coli* ATCC 25922, which had been imported from the United States of America.

Preparation of bacterial strains

Lyophilized bacterial strains were rehydrated by adding enriched bujona and 0.9 % NaCl, multiplied by a bacteriological loop before being transferred onto slant agar and then put in a thermostat for 24 hours at 37 °C. The purity of the strain was confirmed by Gram staining.

Bacterial strains were made viable for 10-14 days at 2-8 °C by using this method.

Preparation of media

An antibiotic seed agar A1 (code 1075 Biolife, Lot 1E5601), of pH 6.5, was used as the basic media for the test plates and was put into inclined tubes to obtain slant agars, which were then seeded with the bacterial strains and stored in a refrigerator at 2-8 °C.

Preparation of the plates

Ten millilitres of the basic media, heated to approximately 50 °C, was poured onto Petri dishes and left to cool and solidify. Then 5 ml of a sterile saline solution was transferred by pipette into the slant agar to obtain a suspension, 4 ml of which was then poured onto the basic media after it had cooled and solidified. The plates were then stored in a refrigerator at 2-8°C and were valid for five days.

Growth density

The growth density on the plates seeded with the *E. coli* 25922 tested strain was evaluated at the beginning of the experiment.

Different concentrations of the bacterial strains were transferred onto the media and kept at 37 °C for 24 hours. After optimal growth, the plates were evaluated with reference standard flumequine (Sigma; catalogue number F-7016).

Calibration curve

A calibration curve was developed from standardized solutions of the antibiotic, which were made by weighing and dissolving standard measures after considering their purity and chemical form.

The basic standardized solutions of flumequine were made using 0.1 M of NaOH as a solvent and the working solutions of flumequine were made by further mixing the standardized solutions with a buffer solution with a pH of 6.

Working concentrations for testing the *E. coli* susceptibility to flumequine ranged from 200 mg/l to 1200 mg/l to correspond with the limits set for flumequine in beef and pork (200 mg/l), poultry (400 mg/kg) and fish (600 mg/kg) (4, 5).

Evaluation of results

The results were evaluated in both a qualitative and quantitative manner. The quantitative assessments were made by using the calibration curve. Working solutions of five different decreasing concentrations – 1000, 800, 600, 400, 300 µg/kg (a down to e), were prepared. The 600-µg/kg (c) concentration was used as the reference. The different concentrations were made using a buffer solution with a pH of 6.

Each concentration was poured onto the 9-welled plates containing the bacterial culture. This was done nine times, except for the reference solution, which was poured 36 times.

The different antibiotic solutions, as well as the reference solution, were added to each plate three times and three parallels were made, which means that three plates were used for each concentration.

The plates were placed in an incubator set at 37 °C for 18-24 hours and then the width of the inhibition zone of the bacterial strain for each concentration was measured using an electronic

Table 1: The width of the inhibition zones in the presence of different concentrations of flumequine (in mm)

N	600µg/kg (c)	300µg/kg (e)
1	12.3	10.3
2	11.7	10.4
3	12.3	10.3
4	12.4	10.4
5	12.4	10.2
6	12.4	10.3
7	12.4	10.3
8	12.5	10.1
9	12.4	10.2
average	12.3	10.3

N	600µg/kg (c)	400µg/kg (d)
1	12.4	11.0
2	12.5	11.5
3	12.4	10.8
4	12.4	11.0
5	12.4	11.0
6	11.9	10.9
7	12.3	10.9
8	12.3	10.9
9	12.3	11.3
average	12.3	11.0

N	600µg/kg (c)	800µg/kg (b)
1	12.6	14.3
2	12.4	14.4
3	12.3	14.5
4	12.4	14.3
5	12.5	13.9
6	11.7	14.2
7	11.9	14.2
8	12.3	14.1
9	12.1	14.3
average	12.2	14.2

N	600µg/kg (c)	1000µg/kg (a)
1	12.3	15.2
2	12.6	15.6
3	12.2	15.1
4	11.9	15.1
5	12.1	15.1
6	12.4	15.2
7	12.3	15.2
8	11.9	15.1
9	12.1	15.2
average	12.2	15.2

N = number of measurements
average = average value

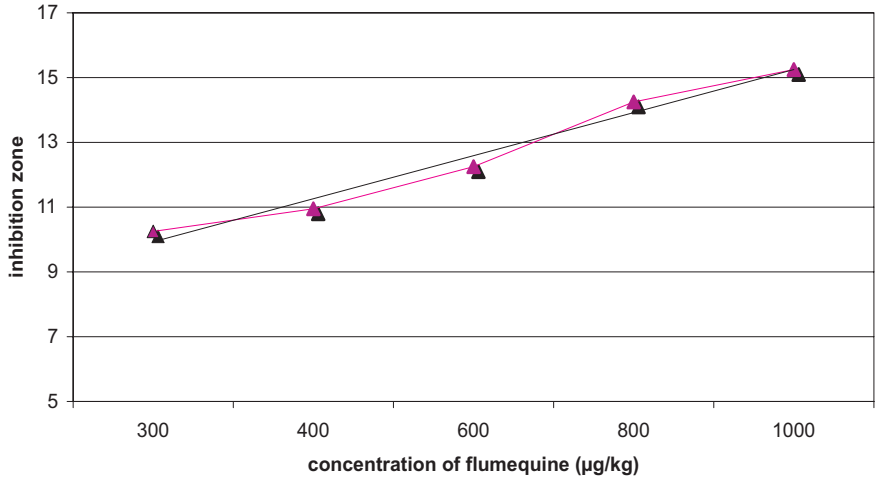

Figure 1: Calibration curve for flumequine
Axis x = concentration of flumequine (µg/kg)
Axis y = inhibition zone (mm)

movable tape (Stainless Hardened). The average values were calculated and the average value of the reference concentration was used to determine standard deviation. For each concentration of antibiotic, nine measurements were obtained and the average width of the zone was calculated for each concentration (Table 1).

The high point (HP) was calculated from the calibration curve (Equation 1):

Equation 1: $$HP \quad \frac{3a \quad 2b \quad c \quad e}{5}$$

In the same manner, the low point (LP) was also calculated from the calibration curve (Equation 2).

Equation 2: $$LP \quad \frac{3e \quad 2d \quad c \quad a}{5}$$

The calculations established the HP as being 15.25 mm and the LP as 9.95 mm.

The data was recorded and a trend curve was developed.

The trend curve is linear and the concentration of the tested antibiotic can be determined by the width of the inhibition zone.

Correction factor

A correction factor was used to modify the results. As microorganisms are living organisms their growth is never uniform; therefore, the size of the inhibition zones of the reference concentrations on each of the plates was never the same. With a correction factor we were able to get more accurate results taking into account the differences between each plate. The correction factor was determined using Equation 3:

Equation 3: $$X \quad \frac{C_{meas}}{C_{ref}} \quad 100$$

X = correction factor
C meas = measured antibiotic concentration
C ref = reference antibiotic concentration

The correction factor gives the percentage of the reference concentration that is obtained by testing each of the plates with the addition of the reference concentration of the same antibiotic. It is used when the results of food-sample analyses for the presence of antibiotic residues are interpreted in a qualitative manner.

The results can also be interpreted quantitatively, as follows:

The width of the inhibition zone is measured and the appropriate concentration of the antibiotic is read off the calibration curve. Before results are interpreted they must be massaged by the correction factor.

Example:

A correction factor of 80 means that when the reference concentration of a tested antibiotic is poured onto the media only 80 % of the poured antibiotic is identified.

$$Ca \quad \frac{C_{od} \quad 100}{X}$$

X = correction factor (for example 80)
C od = concentration read off the calibration curve
C a = antibiotic concentration in sample

Analysis of the samples

Meat from cattle, pigs, poultry and fish with known concentrations of the inoculated flumequine were analysed. Twenty grams of 0.5-cm chunks of meat were put into sterilized bags and then three times that amount of buffer, with a pH of 6, was added to assist in the flumequine identification.

A working solution of the antibiotic concentration was then added and mixed in a stomacher for 3 minutes. The mixture was then transferred into test tubes and heated for 5 minutes at 80 °C to inactivate any naturally occurring inhibitory substances and bacteria in the meat and exclude non-specific reactions on the plates. The mixture was then cooled and centrifuged for 10 minutes at 2500-3000 revolution per minute.

The upper layer was transferred into a clean test tube and a pipette was used to transfer 100 ml into an 8-mm cylinder. The plates were then put in an incubator for 18-24 hours at 37 °C.

Modifying the bacteriological method for identifying flumequine

The results we obtained using the bacteriological method to establish MRL values were not satisfactory, as our limit for identifying the flumequine residues was higher than the MRL.

As a result we modified the method with the addition of lyophilization. Samples with known concentrations of fluoroquinolone were lyophilized and rehydrated with a small amount of water. In this way higher concentrations of fluoroquinolone in the matrix were obtained.

Preparation of samples for lyophilization

Samples of muscle tissue were weighed and the prescribed concentrations of antibiotic were added. The samples were then homogenized for three minutes before being transferred into test tubes and centrifuged for 10 minutes at 2500-3000 rpm. Then approximately 2 ml of each sample was transferred into a rubber-sealed glass flask, closed and put into a lyophilizer.

Lyophilization

A DW6 freeze-dryer from Heto was used for the lyophilization, which took 24 hours and reduced

Table 2: Identification of predetermined concentrations of flumequine in beef and pork

	Concentration (µg/kg)							
N = 15	200	250	300	350	400	450	500	600
Beef Number of positive samples (%)	0 (0)	0 (0)	3 (20)	6 (40)	11 (73.3)	14 (93.3)	15 (100)	15 (100)
Pork Number of positive samples (%)	0 (0)	0 (0)	2 (13.3)	5 (33.3)	11 (73.3)	12 (80)	15 (100)	15 (100)

N = number of samples examined from each source

Table 3: Identification of predetermined concentrations of flumequine in poultry

	Concentration (µg/kg)							
N = 15	250	300	350	400	450	500	550	600
Number of positive samples (%)	0 (0)	6 (40)	8 (53.3)	12 (80)	14 (93.3)	15 (100)	15 (100)	15 (100)

N = number of samples examined

Table 4: Identification of predetermined concentrations of flumequine in fish

	Concentration (µg/kg)							
N = 15	400	450	500	550	600	650	700	800
Number of positive samples (%)	11 (73.3)	12 (80)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)

N = number of samples examined

the samples to 10-15 percent of their original mass on average.

Rehydration

The freeze-dried samples were then weighed and the weight difference was used to calculate the amount of water that needed to be added to obtain higher concentrations of the antibiotic.

Results

Examination of the samples

The samples of cattle, pig, poultry and fish meat with known concentrations of flumequine were analysed and the influence of the matrix on the sensitivity of the method was also examined.

We tested for the presence of flumequine residues that ranged from 1 to 3 times the MRL for beef and pork (200-600 µg/kg), from 0.625 to 1.5 times the MRL for poultry (250-600 µg/kg) and from 0.66 to 1.33 times the MRL for fish (400-800 µg/kg).

All the samples examined were positive (100 %) at concentrations of 500 µg/kg (Tables 2, 3 and 4), which is too high as the MRL for flumequine in beef and pork is only 200 µg/kg and 400 µg/kg in poultry. However, it is satisfactory for identifying flumequine residues in fish as the MRL for flumequine in fish is 600 µg/kg.

Modification of the microbiological method

The microbiological method was modified by lyophilizing the samples, which were then rehydrated to the appropriate concentration levels. This allowed the actual concentration of flumequine to be multiplied as many times as was needed to get the concentration of 800 µg/kg. Samples were then transferred onto the plates.

Table 5: Susceptibility of the bacterial strains after lyophilization

Lyophilized to 800 µg/kg	Concentration (µg/kg) in samples				
N = 10	100	150	200	250	300
Beef Number of positive samples (%)	8 (80)	10 (100)	10 (100)	10 (100)	10 (100)
Pork Number of positive samples (%)	8 (80)	10 (100)	10 (100)	10 (100)	10 (100)
Poultry (muscle tissue) Number of positive samples (%)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)

N = number of samples examined

The procedures used to prepare, lyophilize, rehydrate and transfer the samples to the plates are described in the Materials and methods section.

Discussion

In this study, we used the reference strain *E. coli* ATCC 25922 and observed its growth under different concentrations of flumequine in media. When an appropriate dilution was found we continued with the procedure. Using the same antibiotic seed agar medium as was used in other studies (3, 12, 15, 16), we identified 10^7 colonies per plate, which is comparable to results already described in literature (3, 12, 15). The method used to prepare the plates, the pH of the medium and the incubation procedures were the same as those described by Okerman (16).

We had some difficulties obtaining the MRL values for flumequine residues in pork and beef (200 µg/kg) and also, to some extent, with poultry (400 µg/kg). Even though we were able to determine the MRL values for flumequine residues in poultry, we were only partially successful (80 %). However, we identified all (100 %) of the flumequine residues in fish where the MRL value is 600 µg/kg.

The calibration curve for flumequine was developed using the same method as is used for other antibiotics. The concentrations of flumequine residues in the matrix were calculated from the calibration curve, which included a correction factor, and the growth-inhibition zones of the bacterial strains.

As we were unable to obtain the MRL values for flumequine residues in pork and beef, we modified the microbiological method by adding a lyophilization step. We were unable to find any reference to lyophilization as a step in the identification methods for lower concentrations of antibiotics in any available literature.

Our results were within MRL limits; therefore, our lyophilization-enhanced microbiological method is suitable for use in everyday practice.

References

1. Adams HR. Veterinary pharmacology and therapeutics 7th ed. Ames: Iowa State University Press, 1995: 263-8.

2. Brown SA. Fluoroquinolones in animal health. J Vet Pharmacol Ther 1996; 19: 1-14.

3. Choi J, Yee AJ, Thompson D, Samoluk J, Mitchell M, Black WD. Determination of fluoroquinolone residues in animal tissues using Escherichia coli as indicator organism. J Ass Off Anal Chem Int 1999; 82: 1407-12.

4. Commission Regulation (EC) No 2728/1998 amending Annex I to Council Regulation (EEC) 2377/90 (1998). Off J Eur Commun L 1998; 343: 8-10.

5. Commission Regulation (EC) No 2528/1999 amending Annex I to Council Regulation (EEC) 2377/90 (1999). Off J Eur Commun L 1998; 315: 26-8.

6. Ellerbroek L. Zum mikrobiologischen Nachweis der Chinolon carbosaeurederivate Enrofloxacin, Ciprofloxacin und Flumequin. Fleischwirtschaft 1991; 71: 187-9.

7. FAO/WHO. Residues of some veterinary drugs in animals and foods. No. 41/13
Rome: FAO, 2000: 43-53.

8. FAO/WHO. Residues of some veterinary drugs in

animals and foods. No 41/7
Rome: Food and Agriculture Organization of the United Nations, 1995: 31-55.

9. Fuchs PC, Barry AL, Brown SD. Is Escherichia coli ATCC 25922 useful for monitoring broth microdilution tests of fluoroquinolones? J Antimicrob Chemother 1997; 39: 549-50.

10. Hooper DC. Mode of action of fluoroquinolones. Drugs 1999; 58 (Suppl 2): 6-10.

11. Jackman R. Antimicrobials: A strategy for linking screening and confirmatory methods. In: Proceedings of the Euroresidue III conference. Veldhoven, 1996: 917-21.

12. Myllyniemi AL, Rannikko R, Lindfors E, Niemi A, Backman C. Microbiological and chemical detection of incurred penicillin G, oxytetracycline, enrofloxacin and ciprofloxacin residues in bovine and porcine tissues. Food Addit Contam 2000; 17: 991-1000.

13. Navodila za uporabo Flubactin. Novo mesto 1999; Krka: 1.

14. Okerman L, Croubels S, De Baere S, Van Hoof J, De Backer P, De Brabander H. Inhibition tests for detection and presumptive identification of tetracyclines, beta-lactam antibiotics and quinolones in poultry meat. Food Addit Contam 2001; 18: 385-93.

15. Okerman L, De Wasch K, Van Hoof J. Detection of antibiotics in muscle tissue with microbiological inhibition tests: effects of the matrix. Analyst 1998; 123 (a): 2361-5.

16. Okerman L, Van Hoof J, Debeuckelaere W. Evaluation of the European four-plate test as a tool for screening antibiotic residues in meat samples from retail outlets. J AOAC Int 1998; 81 (b): 51-6.

17. Pravilnik o koli?inah pesticidov in drugih strupenih snovi, hormonov, antibiotikov in mikotoksinov, ki smejo biti v živilih. Ur. list SFRJ 1983; 39 (59): 1634-51.

18. Pravilnik o spremembi in dopolnitvah pravilnika o veterinarsko-sanitarnem nadzoru živilskih obratov, veterinarsko-sanitarnih pregledih, ter o pogojih zdravstvenega varstva živil in surovin živalskega izvora. Ur. list RS 2000; 38: 4534-5.

19. Rattan A. Mechanisms of resistance to fluoroquinolones. Natl Med J India 1999; 12: 162-4.

20. Van Schothorst M, van Leusden FM, Nouws JF. Antibiotic residues: regulations, tolerances, and detection in the European Economic Community. J Assoc Off Anal Chem 1978; 61: 1209-13.

21. Vancutsem PM, Babish JG, Schwark WS. The fluoroquinolone antimicrobials: structure, antimicrobial activity, pharmacokinetics, clinical use in domestic animals and toxicity. Cornell Vet 1990; 80: 173-86.

22. Wolfson JS, Hooper DC. Fluoroquinolone antimicrobial agents. Clin Microbiol Rev 1989; 2: 378-424.

UVEDBA IN MODIFIKACIJA MIKROBIOLOŠKE METODE ZA UGOTAVLJANJE FLUMEKVINA V MESU

A. Kirbiš, J. Marinšek

Povzetek: Z raziskavo smo želeli razširiti analitiko ostankov antibiotikov v mišičnem tkivu klavnih živali. V ta namen smo uvedli in modificirali bakteriološko metodo za ugotavljanje flumekvina.Metodo smo modificirali na ta način, da smo kvalitativnemu vrednotenju dodali še kvantitativno vrednotenje rezultatov, in sicer liofilizacijo vzorcev. Na ta način smo bistveno izboljšali občutljivost metode.Rezultati so potrdili dejstvo, ki smo ga povzeli po literaturi, da je bakterijski sev E. coli ATCC 25922 dejansko občutljiv za predstavnike fluorokinolonov. Ta občutljivost pa glede na to, da obstajajo najvišje dopustne meje vsebnosti ostankov antibiotikov v živilih (MRL, maximum residue level), ni bila povsod zadostna. Naleteli smo na težave pri doseganju MRL v mišičnih tkivih prašičev in goveda. To je tudi bil razlog, da smo metodo modificirali. Modifikacijo metode z uvedbo liofilizacije ekstraktov vzorcev prištevamo k originalnosti raziskave, saj v literaturi nismo zasledili podobnih prijemov za izboljšanje občutljivosti metode. Zato tako modificirano metodo lahko uporabljamo tako za kvali- kakor tudi za kvantitativno ugotavljanje flumekvina.

Ključne besede: metode; antibiotiki; flumekvin; zdravila; bakteriološka tehnika; meso

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Original Research Paper

IN VIVO EVALUATION OF THE INFLUENCE OF VARIOUS DRUG CARRIERS WITH INCORPORATED HYPERAEMIC DRUG TO CHANGES IN THE PARTIAL PRESSURE OF OXYGEN IN RAT ORAL MUCOSA USING EPR OXIMETRY

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Summary: The purpose of this study was to select the best drug carrier among different types of liposomes for the topical treatment of oral mucosa lesions. A hyperaemic drug, benzyl nicotinate (BN), which increases perfusion and consequently also tissue oxygenation, was used as the active ingredient. Electron paramagnetic resonance (EPR) oximetry, using a lithium phthalocyanine paramagnetic probe, was used in vivo to measure the effects of the benzyl nicotinate which had been incorporated into liposomes of varying lamellarity and composition. The liposomes were made from either hydrogenated or non-hydrogenated soy lecithin. We used polymethyl methacrylate (PMMA) as the ointment for preparing the drug for application to the oral mucosa because of its good mucoadhesive properties.
EPR oximetry was used to measure the partial pressure of oxygen (pO_2) in the oral mucosa before and after the application of the liposomes. It was found that the most pronounced changes of pO_2 in oral mucosa and also the longest action of the drug occurred after the topical application of BN in multilamellar liposomes made from hydrogenated soy lecithin ($p < 0.0001$). Therefore, these liposomes proved to be the most appropriate for local drug delivery to oral mucosa.

Key words: mouth diseases; drug therapy; liposomes; *in vivo* EPR oximetry; oral mucosa; rat

Introduction

One of the characteristics of oral mucosa is its selective permeability, which provides a barrier to most chemicals. However, drugs can be used for local or systemic delivery (1, 2, 3, 4, 5, 6, 7). Local delivery of drugs to tissues of the oral cavity has numerous applications such as the treatment of aphthous stomatitis, lichen planus, bacterial and fungal infections and periodontal diseases (6, 8, 9). The success of a topical treatment of mucosal lesions via the application of a drug onto intact oral mucosa depends on the selection of a suitable active ingredient and an appropriate carrier, the rate of penetration through the mucosa and the residence time of the active ingredient in oral mucosa. Additionally, a major part of a successful application of a local drug delivery system into the oral cavity is the selection of an appropriate vehicle. For example, ointments that act as vehicles for local drug delivery to the oral mucosa need to have excellent mucoadhesive properties (10).

Topical treatments of ulcerative inflammatory diseases are associated with several general disadvantages such as the high permeability of the oral mucosa for drugs, which could result in the uncontrolled release of the drugs into the blood causing unwanted side effects (2, 10). Liposomal formulations have been used to regulate the release and localize the effect of incorporated drugs (11) and studies have shown that treatments with liposomal formulations result in an increase in the local and a decrease in the systemic concentration of a drug (11, 12).

The aim of this study was to investigate the effects of a liposome's composition and size on

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the delivery of the hyperaemic drug, benzyl nicotinate (BN), to the oral mucosa using in vivo EPR oximetry.

Materials and methods

The study protocol was submitted to and approved by the Veterinary Administration of the Republic of Slovenia (N° 323-02-76/01).

Animals, anaesthesia and implantation of the paramagnetic probe

Adult female Wistar rats, weighing 200-250 g and 7-9 weeks old were supplied by the Pathology Laboratory of Ljubljana's Faculty of Medicine, Slovenia. Anaesthesia was induced using an intraperitoneal injection of a mixture containing xylazine hydrochloride, 10 mg/kg (Rompun, Bayer, Leverkusen, Germany) and ketamine hydrochloride, 75 mg/kg (Ketanest 50, Parke-Davis, Berlin, Germany) and when indicated, anaesthesia was prolonged by the administration of a further amount of each drug at half the initial dosage. Twenty-four hours prior to starting electron paramagnetic resonance (EPR) measurements, the rats were anaesthetized and crystalline particles of lithium phthalocyanine (LiPc), the paramagnetic probe, were implanted beneath the buccal mucosal epithelium through a 23 gauge injection needle (Microlance, Becton Dickinson, Fraga, Spain). The approximate volume of the crystals (a generous gift from the EPR Centre for Viable Tissues, Dartmouth College of Medicine, Hanover, New Hampshire, USA) was 0.5 μm^3. The needle tip was filled with approximately 0.1 mm^3 of LiPc microcrystals. The needle was inserted 2 mm laterally from the injection site and the microcrystals deposited about 1 mm below the mucosa surface. Measurements began 24 hours after the implantation of the paramagnetic probe, permitting time for oxygen concentrations to balance between the paramagnetic crystals and the surrounding tissue and to minimize the risk of the results being affected by the initial stress and tissue injury from the implantation of the probe. The delay also permitted the injection site to start healing, reducing the likelihood that the hyperaemic drug – benzyl nicotinate (BN) – would permeate directly through the wound caused by the implantation of the LiPc microcrystals. Each rat was anaesthetised and the EPR spectra measured for 15 minutes to ensure that both the equipment and the implant were functioning correctly and to establish the baseline value for the partial pressure of oxygen (pO_2) in the tissue before proceeding further.

Anaesthesia interferes with temperature homeostasis and changes in temperature have been reported to significantly influence the linewidth of the EPR spectra (15, 16), so a preliminary study was performed to develop a method to counter such effects.

Benzyl nicotinate

Benzyl nicotinate indirectly increases local blood flow through the release of nicotinic acid, which is followed by the formation of prostaglandin D2 (13, 14). The increased blood flow leads to an elevation of tissue oxygenation, pO_2, which can be measured by EPR oximetry. The gradual increase in pO_2 after the application of the formulations containing BN was measured and the overall effectiveness of the incorporated drug in different types of liposomes was determined.

Liposomes with benzyl nicotinate

Liposomes were prepared using the thin-film method from cholesterol and either hydrogenated or non-hydrogenated soy lecithin (HSL or NSL) in a weight ratio of 3:7. The lipophilic phase containing phospholipid, together with cholesterol and the benzyl nicotinate (Lek; Ljubljana, Slovenia), was dissolved in dichloromethane for the NSL or in chloroform:methanol (1:1) for the HSL. The solvent was removed in a rotary evaporator, which left a thin film clinging to the evaporator's wall. Any remaining solvent was removed completely under vacuum (10 to 15 minutes at 40 °C and pressure 100 Pa). The dry film was then hydrated with distilled water at approximately 80 °C for the HSL (i.e. above its phase transition temperature) and at room temperature (22 °C) for the NSL. The flask was shaken until the film was completely removed from its walls. The dispersion was then stabilized by stirring it for 2 hours on a magnetic stirrer (300 rpm) at room temperature. A 1-mL sample of the dispersion contained 25 mg of lipids and 12.5 mg of BN. A higher concentration of BN would not permit the liposomes to form.

A portion of the multilamellar liposome (MLV) was extruded with a Liposofast extruder (Avestin, Ottawa, Canada) using polycarbonate membranes with defined pore diameters (Nucleopore Corporation, Pleasanton, CA, USA), which started at 800 nm and decreased in diameter to 100 nm. The liposomes were extruded at temperatures slightly above their phase transition temperature.

Characterization of liposomes

The size of the liposomes and their polydispersity index (PI) rating were determined by photon correlation spectroscopy (PCS; Zetasizer 3000, Malvern, Malvern, UK) at a fixed angle of 90ş. The samples were diluted in dust-free water to give the recommended scattering intensity of 100,000 counts s^{-1}. The diameter was calculated from the autocorrelation function of the intensity of light scattered from the particles, assuming that the particles were spherical in form. For mean size calculation, the cumulant algorithm, which took into account only one population of particles was used. The PI is a measure of a dispersion's homogeneity, which ranges from 0 (homogeneous dispersion) to 1 (high heterogeneity). In our study, the mean diameters of the NSL and the HSL were respectively 400 ± 40 and 1,000 ± 100 with PI's close to 1; after extrusion it was 250 nm ± 20 with a PI of 0.7 ± 0.2 and 250 ± 10 with a PI of 0.3 ± 0.1.

Formulation for application

A mucoadhesive ointment, polymethyl methacrylate (PMMA) (a neutralized co-polymer of methacrylic acid and methyl methacrylate) was used in the application of the liposomal formulation to the tissues of the oral cavity. The PMMA (Sigma-Aldrich, Steinheim, Germany) was prepared as described elsewhere (7, 8). The liposomes encapsulating BN were mixed with PMMA in a weight ratio of 2:3, resulting in a final concentration of BN in the PMMA formulation of 0.5 wt %.

For a negative control, a PMMA without BN was used.

EPR measurements

The measurements taken in the oral cavity were conducted by in vivo EPR oximetry, which has been described in detail elsewhere (16). Briefly, 0.05 ml of the prepared liposomal formulation was applied to the surface of the buccal mucosa over the site where the LiPc had been implanted using a syringe (Plastipak, Becton Dickinson, Fraga, Spain).

The surface coil of an extended loop resonator, which was 11 mm in diameter, was placed over the implanted area and the EPR spectra were recorded on a Varian E-9 EPR spectrometer with a custom-made low-frequency microwave bridge (designed by Dr. T. Walczak, Dartmouth College of Medicine, Hanover, NH, USA), operating at 1.1 GHz. The spectra were recorded under the following conditions: magnetic field density 44-45 mT, modulation amplitude 2.5×10^{-3} mT and the microwave power 20 mW. The linewidth of the EPR spectra, which is proportional to local pO_2 changes in the tissue, was measured and converted to mucosal pO_2 according to the calibration curve for the LiPc (17).

Each experimental formulation, including the negative control, was tested in 8 or 9 rats. In order to obtain the basal pO_2 of the oral mucosa, five EPR spectra were recorded before the application of the test formulation and the mean value was taken as the basal pO_2. The local pO_2 changes were then measured over 90 minutes (at 2 to 5 minute intervals) following the application of the test material. As the basal pO_2 varied from animal to animal the difference in pO_2 with respect to the basal value was measured. Time points and pO_2 measurements, which represent the efficiency of the drug's absorption and action, were evaluated for each type of liposome as follows: onset of increasing tissue oxygenation (lag time, t_{lag}), the maximal pO_2 (ΔpO_{2max}), the time when pO_{2max} was reached (t_{max}), the return to the basal pO_2 levels (t_{end}) and the area under the curve (AUC).

Statistical analysis

The hypothesis of average equality in different groups was tested with one-way single factor ANOVA. The GLM procedure for unbalanced data was used for the analysis of data. If the analysis of variance test was significant, a post-test analysis using a Duncan test was used to find the specific difference and P values of less than 0.05 were accepted as statistically significant.

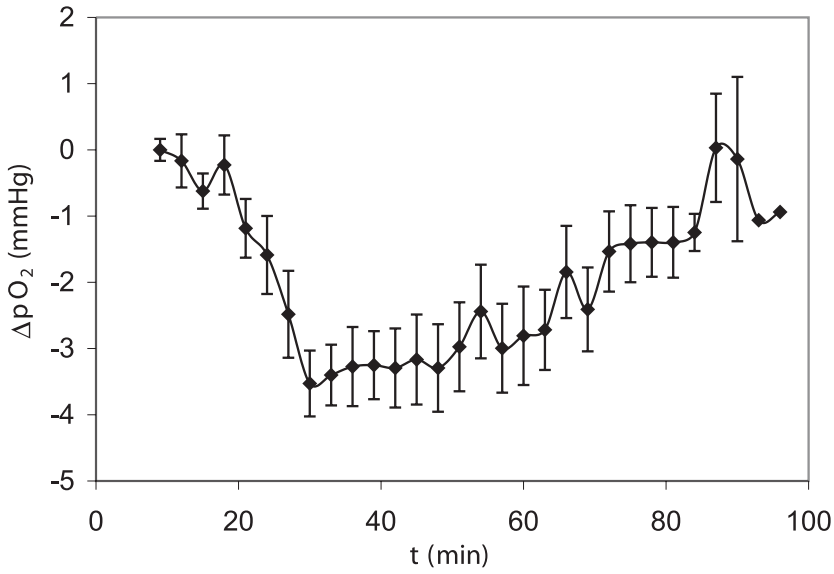

Figure 1: Dependence of partial pressure of oxygen (pO_2) on anaesthesia in rat oral mucosa. For the first 30 minutes after the application of the anaesthetic and sedative, the pO_2 in the oral mucosa decreased, then remained fairly steady for the next 30 minutes and then started to rise after one hour - when the animal began to wake up

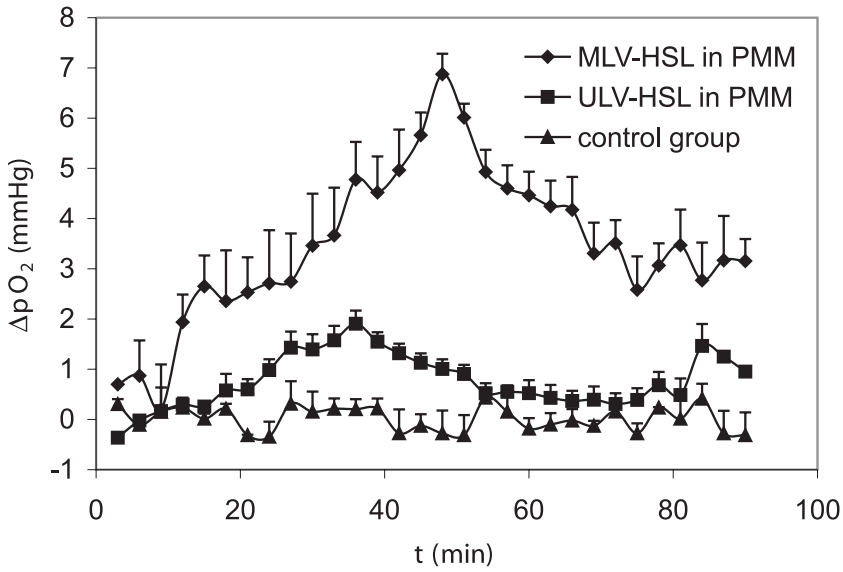

Figure 2: The time-course of oxygen level variation (ΔpO_2) in rat oral mucosa after the application of benzyl nicotinate in HSL liposomes of different sizes: (◆) multilamellar HSL liposomes, (■) unilamellar HSL liposomes and (▲) control group. Each point represents mean value ± SD of 8-9 measurements

Results

Effect of body temperature and anaesthesia on pO2 in oral mucosa

Preliminary EPR measurements were performed without the application of medications to investigate the effect of changes in body temperature and duration of anaesthesia on the oxygenation of the oral mucosal. It was found that the tissue oxygenation in oral mucosa is influenced by changes in body temperature. The pO_2 of oral mucosa decreased, as expected, with the reduction of body temperatures below that considered normal (16). Minimal changes were observed when temperature homeostasis was maintained at 36.5 ± 0.5 °C throughout anaesthesia by a flow of hot air; this was measured rectally with a thermocouple inserted into a glass capillary. This method was therefore used during the liposome evaluation. Measuring the temperature of oral mucosa is invasive and interferes with its pO_2, therefore, it was not performed.

Furthermore, tissue oxygenation is also influenced by anaesthesia. After the application of the anaesthetic combination, the pO_2 in the oral mucosa decreased before stabilizing after

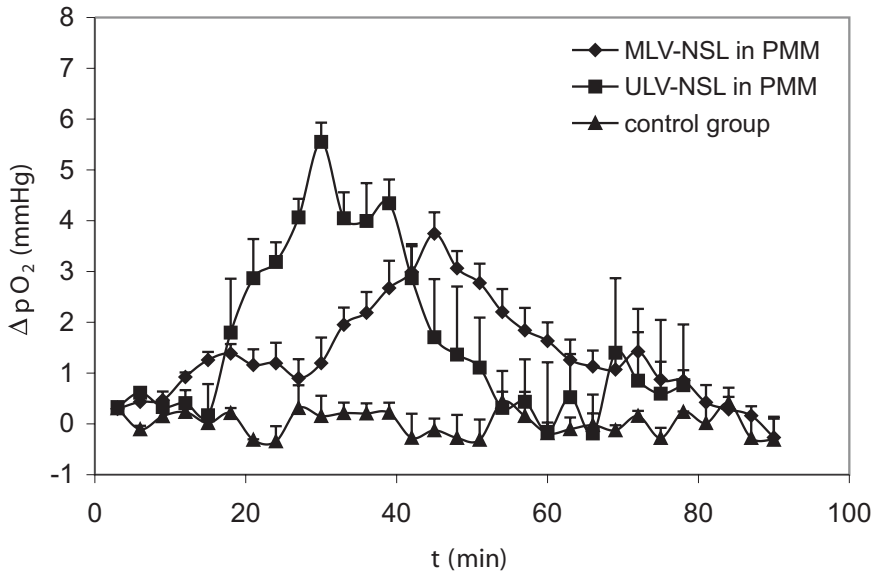

Figure 3: The time-course of oxygen level variation (ΔpO_2) in rat oral mucosa after the application of benzyl nicotinate in NSL liposomes of different sizes: (◆) multilamellar MLV liposomes, (■) extruded MLV liposomes and (▲) control group. Each point represents mean value ± SD of 8-9 measurements

about 30 minutes. After one hour, as the anaesthesia lightened, the pO_2 started to rise again (Figure 1.). As the measurements of the local pO_2 changes after the application of the liposomal formulations were performed over 90 minutes, an additional half of dose of the anaesthetic combination was administered (without the animal being moved) one hour after the initial dose. This was found to maintain the anaesthesia adequately and to stabilize the pO_2 over a longer period of time. With respect to the initial influence of anaesthesia, the formulations were not applied to the mucosa until 30 minutes after anaesthesia – when the basal pO_2 levels had been confirmed as having stabilized.

Effect of liposome composition

In the control experiments it was determined that PMMA alone has no influence on the oxygenation of oral mucosa (Figures 2 and 3). For the test substances, the lag time (t_{lag}), the maximal relative increase of pO_2 after the application of the liposomes and the time when it was reached (ΔpO_{2max}, t_{max}), the area under the curve (AUC), and the time when BN stopped acting (t_{end}) were determined from the individual pO_2 curves. The influence on the oxygenation of the oral mucosa of BN incorporated in the HSL or NSL liposomes is shown in Figures 2 and 3. The influence of the different carriers investigated was significant. The major changes to the pO_2 of the oral mucosa occurred after the application of the multilamellar liposomes made from hydrogenated soy lecithin (MLV-HSL) ($p<0.0001$), which is expressed in maximal changes of pO_2 as well as in the AUC. The drug also had the longest lasting effects (the average exceeded the measurement time) for this type of liposome ($p<0.0001$). The least effective were the extruded liposomes from hydrogenated soy lecithin (ULV-HSL), this being lower than for liposomes from non-hydrogenated soy lecithin. When BN was incorporated in liposomes made from non-hydrogenated soy lecithin, their effectiveness was more pronounced when incorporated in multilamellar (MLV-NSL) rather than the extruded (ULV-NSL) liposomes, although the differences were much less marked than with the HSL liposomes. Maximal pO_2 is greater in extruded liposomes while the time when maximal increase in pO_2 was achieved as well as the duration of the effect was longer when using the non-extruded liposomes.

The analysis of variance test for the unbalanced data showed significant differences ($F=54.37$, $p<0.0001$) in the average values of the maximal increase of pO_2 (ΔpO_{2max}) for all four carriers (Table 1). Statistically significant differences ($F=4.47$, $p=0.01$) in the values of t_{max} were observed between the extruded and non-extruded liposomes of both types (NSL and HSL). The maximal change in pO_2 in the oral mucosa was achieved sooner in extruded rather than non-extruded NSL and HSL liposomes, while the differences in t_{lag} between the different carriers were

Table 1: The effect of topical applications of benzyl nicotinate (BN) incorporated in liposomes of varying lamellarity and composition on the oxygenation of rat oral mucosa

Liposomes	N	$\Delta pO_{2\ max}$ (mmHg)	t_{max} (min)	t_{lag} (min)	AUC (mmHg x min)	t_{end} (min)
MLV-HSL	8	7 ± 0.8^{a}	48 ± 11^{e}	14 ± 7.6	$270 \pm 103^{*}$	**
ULV-HSL	9	2 ± 0.6^{b}	36 ± 7^{f}	12 ± 4.50	54 ± 37	50 ± 23
MLV-NSL	9	4 ± 1.0^{c}	45 ± 10	16 ± 8.4	110 ± 49	57 ± 15
ULV-NSL	8	6 ± 1.0^{d}	30 ± 6^{f}	12 ± 7.4	90 ± 8.0	40 ± 10.4

Each value represents the mean ± SD of measurements.
Key:
N - Number of measurements
ΔpO_{2max} - the maximal relative increase of pO_2 after application of the liposomes
tmax - the time when pO_{2max} was reached
tlag - the time when the BN starts to act (lag time)
AUC - area under the curve
tend - the time when BN stops acting (after the application of BN incorporated in different carriers)
a,b,c,d - statistically significant difference between different carriers (Analysis of variance, Duncan's test; $p < 0.0001$)
** - statistically significant difference between different carriers (Analysis of variance, Duncan's test; $p < 0.0001$)*
e,f - statistically significant difference (Analysis of variance, Duncan's test; $p<0.01$)
*** - the effect lasted longer than the time of measurements*

not significant. However, the most pronounced effect was observed for MLV-HSL liposomes, where the pO_2 remained above the baseline longer, exceeding the time of measurement. The effectiveness of BN, expressed as AUC, was most pronounced when applied in MLV-HSL ($F=17.65$, $p<0.0001$) (Table 1), while in other formulations the differences in AUC were not significant.

Discussion

Oral mucosa is a stratified squamous epithelium, whose intercellular spaces are filled with lipids extruded from the membrane coating granules (MCG). The lipids may be organised into lamellae and they constitute the principal barrier against molecular diffusion through the mucosa. Keratinized areas in the oral cavity are generally more permeable than the skin because the intercellular lipids are less well structured. They exist mainly in discrete lamellar domains and there are fewer structural contributions from lipids (ceramides) covalently bound to the corneocyte surface (18, 19, 20). There are considerable differences in the permeability of different oral mucosae (9). In non-keratinized regions (e.g. cheek, floor of the mouth and lips) the chemical nature of the intercellular material is less well defined and the barrier is less efficient than that in the keratinized epithelia (19). In general, the permeability of oral mucosae decreases in the order of sublingual is greater than buccal, which in turn is greater than palatal (6). The permeability of the oral mucosa is estimated to be 4 to 4,000 times greater than that of the skin (3, 6, 9).

The oral epithelia of a number of experimental animals are entirely keratinized (6), and the rat has a buccal mucosa with a very thick, keratinized surface layer (9). Human oral mucosa is thin and non-keratinized. From the point of view of human mucosal drug delivery, the carriers tested in rats are expected to be even more effective in the thin non-keratinized human buccal mucosa. An inflammatory infiltrate in connective tissue increases epithelial permeability (2), and an ulcerated surface, i.e. without the epithelial barrier, provides an easier entry as well as an easier exit for the drug (11).

The continuous flow of saliva and the mechanical movements of the tongue may prevent the long-term adhesion of carriers to oral mucosa. Among the different hydrophilic polymers that have been investigated, PMMA has been found to be the most appropriate mucoadhesive ointment for local liposome applications in the oral cavity. They are most stable in this polymer and the penetration of the incorporated

substance into the oral mucosa or gingiva is greatest when PMMA was used (8). Therefore we chose PMMA as the vehicle for the liposomes with the entrapped BN. We found that multilamellar liposomes made from hydrogenated soy lecithin were the most effective carriers of the liposomes investigated in this study. Several studies have shown that a liposome's composition and, to a lesser extent, its size influences the rate of transport and effectiveness of a drug's action in skin (21, 22, 23, 24). The effect of free BN in PMMA has been evaluated before, the drug's effect increasing linearly with a BN concentration up to 3 %; higher concentrations having no greater effect indicating that the saturation level had been achieved (16). If the concentration of the hyperaemic drug was less than 1 % then no local changes of pO_2 were observed (16). As we achieved significant effects with each of the four liposomal formulations in our study when the concentration of BN in the formulation was 0.5 % we can conclude that the encapsulation of a drug into liposomes enhances its delivery into oral mucosa.

Multilamellar liposomes made from HSL were the most effective carriers for the hyperaemic drug used in this study and also produced the greatest duration of action (Table 1). The HSL liposomes cause a greater effect than NSL liposomes. These results are in agreement with in vitro and in vivo results previously obtained, which show that a hydrophilic probe, when applied entrapped in a NSL liposome, does not penetrate deeper than 100 μm, while the HSL liposomes enable penetration into the deeper layers of the skin (22, 24). We studied the influence of a lipophilic substance that can penetrate into the skin even if it is not entrapped in a liposome. Therefore, the enhanced effect of all four formulations is not surprising. However, multilamellar liposomes from HSL caused the most pronounced increase in pO_2 and prolonged the effect of the BN action in accordance with previous findings, which show that liposomes allow a controlled and continuous release of a drug over a longer period of time (25, 26). The pO_2 was still above the baseline when we stopped taking measurements after 90 minutes. The populations of multilamellar liposomes are very heterogeneous in size and lamellarity; therefore, they release the entrapped substance more evenly over a prolonged period while penetrating the oral mucosa. In contrast, after an initial increase in pO_2 a decrease was observed after 35 minutes with the extruded liposomes. We suggest that the extruded liposomes, which are smaller, more homogeneous in size and with less layers in their structure, release a drug more uniformly and rapidly, therefore the effect is shorter. We assume that most unilamellar liposomes break down on the surface of the oral mucosa and the released free BN then penetrates the mucosa as if it had been applied directly.

The drop in pO_2 after the administration of the anaesthetic's agents is likely to be related to the depressed respiration rate and altered circulation induced by the anaesthetics. Anaesthesia affects the tissue pO_2 directly through its effect on the respiratory centre and indirectly due to peripheral vasoconstriction (33). Xylazine hydrochloride is a sedative and muscle relaxant commonly used in veterinary medicine (34). Being an agonist for α2-adrenoceptors, xylazine hydrochloride decreases the heart rate, causes a biphasic change in mean blood pressure (transient hypertension followed by hypotension), decreases venous cerebral blood volume and intracranial pressure, and depresses the central nervous system (33, 35, 36). During the initial stage of hypertension there is peripheral vasoconstriction, which reduces the blood flow through the capillaries. This varies with the type of α2-adrenoceptors present in the tissue, the dose and the route of administration. This initial stage is followed by a lowering of blood pressure, circulation and the peripheral resistance of blood vessels. The reduced peripheral blood flow, together with the decreased arterial oxygen content, account for the remarkable reduction in the oxygenation of the oral mucosa in ketamine-xylazine hydrochloride anaesthetised rats. This explains the decrease of pO_2 that was observed after the application of the anaesthetic and sedative. It is therefore impossible to avoid a certain influence of the anaesthesia during in vivo measurements. There was also fluctuation of pO_2 around the basic value of pO_2, which was obtained as an average of the EPR spectra linewidths taken before the application of the ointment.

Conclusions

In this study we have proven that in the rat, a topical application of a liposome preparation

facilitates the penetration of BN through the oral mucosa in vivo, the drug increasing the oxygenation of the oral mucosa. The liposome's composition and size plays an important role in the penetration of this lipophilic drug through the oral mucosa. The present study has indicated that multilamellar liposomes made from hydrogenated soy lecithin are much more effective carriers for BN in oral mucosa than are the non-hydrogenated liposomes or the extruded liposomes of the same type. EPR oximetry in vivo as a non-invasive method can be used to precisely monitor the penetration of BN into oral mucosa by following the physiological response of the body to the drug's action. In addition, our results indicate that pO_2 in the oral mucosa is altered by body temperature and anaesthetics.

Acknowledgement

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References

1. Hicks DC. The buccal absorption of some adrenoceptor blocking drugs. Br J Pharmacol 1973; 47: 680-1.

2. Squier CA, Johnson NW. Permeability of oral mucosa. Br Med Bull 1975; 31: 169-75.

3. Galey WR, Lonsdale HK, Nacht S. The in vitro permeability of skin and buccal mucosa to selected drugs and tritiated water. J Invest Dermatol 1976; 67: 713-7.

4. Pimlott SJ, Addy M. A study into the mucosal absorption of isosorbide dinitrate at different intraoral sites. Oral Surg Oral Med Oral Pathol 1985; 59: 145-8.

5. Barsuhn CL, Olanoff LS, Gleason DD, Adkins EL, Ho NF. Human buccal absorption of flurbiprofen. Clin Pharmacol Ther 1988; 44: 225-31.

6. Harris D, Robinson JR. Drug delivery via the mucous membranes of the oral cavity. J Pharm Sci 1992; 81: 1-10.

7. Sveinsson SJ, Holbrook WP. Oral mucosal adhesive ointment containing liposomal corticosteroid. Int J Pharm 1993; 95: 105-9.

8. Petelin M, Sentjurc M, Stolic Z, Skaleric U. EPR study of mucoadhesive ointments for delivery of liposomes into the oral mucosa. Int J Pharm 1998; 173: 193-202.

9. Shojaei AH. Buccal mucosa as a route for systemic drug delivery: a review. J Pharm Sci 1998; 1 (1): 15-30.

10. Bremecker KD, Strempel H, Klein G. Novel concept for a mucosal adhesive ointment. J Pharm Sci 1984; 73: 548-52.

11. Harsayi BB, Hilchie JC, Mazei M. Liposomes as drug carriers for oral ulcers. J Dent Res 1986; 65: 1133-41.

12. Mazei M, Gulasekharam V. Liposomes: a selective drug delivery system for the topical route of administration. Gel dosage form. J Pharm Pharmacol 1982; 34: 473-4.

13. Wilkin JK, Fortner G, Reinhardt LA, Flowers OV, Kilpatrick SJ, Steeter WC. Prostaglandins and nicotinate-provoked increase in cutaneous blood flow. Clin. Pharmacol Ther 1985; 38: 273-7.

14. Morrow JD, Adwad JA, Oates JA, Roberts LJ. Identification of skin as a major site of prostaglandin D2 release following oral administration of niacin in humans. J Invest Dermatol 1992; 98: 812-5.

15. Šentjurc M, Kristl J, Abramovic Z. Transport of liposome entrapped substances into skin as measured by electronic paramagnetic resonance oximetry in vivo. Methods Enzymol. (2002) in press.

16. Petelin M, Pavlica Z, Bizimoska S, Sentjurc M. In vivo study of different ointments for drug delivery into oral mucosa by EPR oximetry. Int J Pharm 2004; 270: 83-91.

17. Sentjurc M, Krzic M, Kristl J, Grinberg O, Swartz HM. EPR Oximetry in vivo in mouse skin. Polish J Med Phys Eng 2001; 7: 165-74.

18. Wertz PW, Squier CA. Cellular and molecular basis of barrier function in oral epithelium. Crit Rev Ther Drug Carrier Syst 1991; 8 (3): 237-69.

19. Wertz PW, Swartzendruber DC, Squier CA. Regional variation in the structure and permeability of oral mucosa and skin. Adv Drug Dell Rev 1993; 12: 1-12.

20. Squier CA, Wertz PW. Structure and function of the oral mucosa and implications for drug delivery. In: Rathbone MJ, ed. Oral mucosa drug delivery. New York: Marcel Dekker, 1996: 1-25.

21. Kirjavainen M, Monkkonen J, Saukkosaari M, Valjakka-Koskela R, Kiesvaara J, Urtti A. Phospholipids affect stratum corneum lipid bilayer fluidity and drug partitioning into the bilayers. J Controll Rel 1999; 58 (2): 207-14.

22. Šentjurc M, Vrhovnik K, Kristl J. Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method. J Controll Rel 1999; 59 (1): 87-97.

23. Coderch L, Fonollosa J, De Pera M, Estelrich J, De La Maza A, Parra JL. Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. J Controll Rel 2000; 68 (1): 85-95.

24. Honzak L, Sentjurc M, Swartz HM. In vivo EPR of topical delivery of a hydrophilic substance encapsulated in multilamellar liposomes applied to the skin of hairless and normal mice. J Controll Rel 2000; 66: 221-8.

25. Šentjurc M, Gabrijel?i? V. Transport of liposome-entrapped molecules into the skin as studied by electron paramagnetic resonance imaging methods. In: Lasic DD, ed. Handbook of non-medical applications of liposomes. Vol. 4. Boca Raton: CRC Press, 1996: 91-114.

26. Kržic M, Šentjurc M, Kristl J. Improved skin oxygenation after benzyl nicotinate application in dif-

ferent carriers as measured by EPR oximetry in vivo. J Controll Rel 2001; 70: 203-11.

27. Swartz HM, Boyer S, Gast P, et al. Measurements of pertinent concentrations of oxygen in vivo. Magn Reson Med 1991; 20 (2): 333-9.

28. Norby SW, Swartz HM, Clarkson RB. Electron and light microscopy studies on particulate EPR spin probes lithium phthalocyanine, fusinite and synthetic chars. J Microsc 1998; 192 (2): 172-85.

29. Liu KJ, Gast P, Moussavi M, et al. Lithium phthalocyanine: a probe for electron paramagnetic resonance oximetry in viable biological systems. Proc Natl Acad Sci USA 1993; 90 (12): 5438-42.

30. Smirnov AI, Norby SW, Clarkson RB, Walczak T, Swartz HM. Simultaneous multi-site EPR spectroscopy in vivo. Magn Reson Med 1993; 30 (2): 213-20.

31. Smirnov AI, Norby SW, Weyhenmeyer JA, Clarkson RB. The effect of temperature on the respiration of cultured neural cells as studied by a novel electron paramagnetic resonance technique. Biochim Biophys Acta 1994; 1200 (2): 205-14.

32. Smirnov AI, Norby SW, Walczak T, Liu KJ, Swartz HM. Physical and instrumental considerations in the use of lithium phthalocyanine for measurements of the concentration of oxygen. Magn Reson 1994; B103 (2): 95-102.

33. Liu KJ, Bacic G, Hoopes PJ, et al. Assessment of cerebral pO2 by EPR oximetry in rodents: effects of anesthesia, ischemia, and breathing gas. Brain Res 1995; 685: 91-8.

34. Greene SA, Thurmon JC. Xylazine – a review of its pharmacology and use in veterinary medicine. J Vet Pharmacol Ther 1988; 11: 295-313.

35. Hsu WH. Xylazine-induced depression and its antagonism by alpha adrenergic blocking agents. J Pharmacol Exp Ther 1981; 218: 188-92.

36. McCormick JM, McCormick PW, Zabramski JM, Spetzler RF. Intracranial pressure reduction by a central alpha-2 adrenoreceptor agonist after subarachnoid hemorrhage. Neurosurgery 1993; 32: 974-9.

SPREMLJANJE VPLIVA RAZLIČNIH NOSILCEV Z VGRAJENIM HIPEREMIKOM NA PARCIALNI TLAK KISIKA V PODGANJI USTNI SLUZNICI Z ELEKTRONSKO PARAMAGNETNO RESONANČNO OKSIMETRIJO

V. Erjavec, Z. Pavlica, M. Šentjurc, M. Petelin

Povzetek: Namen študije je bil izbrati med različnimi liposomi najboljši nosilec zdravilne učinkovine za lokalno zdravljenje bolezenskih sprememb na ustni sluznici. Kot zdravilno učinkovino smo uporabili hiperemik benzil nikotinat (BN), ki poveča prekrvitev in s tem oksigenacijo tkiva. Z elektronsko paramagnetno resonančno (EPR) oksimetrijo smo ob uporabi paramagnetne snovi (litijev ftalocianin) spremljali učinek benzil nikotinata, ki je bil vgrajen v liposome različnih oblik in sestave. Liposomi so bili pripravljeni bodisi iz hidrogeniranega bodisi iz nehidrogeniranega sojinega lecitina. Kot podlago za pripravo zdravila, ki smo ga nanašali na ustno sluznico, smo uporabljali polimetilmetakrilat (PMM), ki se na ustno sluznico dobro lepi. Z EPR smo merili parcialni tlak kisika (pO_2) v ustni sluznici pred nanosom liposomov in po njem. Ugotovili smo, da pride do največjega povečanja pO_2 v ustni sluznici po lokalnem nanosu benzil nikotinata, vključenega v večplastne liposome iz hidrogeniranega sojinega lecitina ($p<0.0001$), zato so ti liposomi najprimernejši za lokalno dovajanje zdravilnih učinkovin na ustno sluznico.

Ključne besede: ustne bolezni; zdravljenje z zdravili; liposomi; in vivo EPR oksimetrija; ustna sluznica; podgana

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Case Report

BRONCHOCUTANEOUS FISTULA IN A DOG

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Summary: A case of chronic bronchocutaneous fistula due to pulmonary foreign body in the French pointer breed Epagneul Breton is described. Identification of the origin of fistula with fistulography and removal of plant foreign body enabled complete remission after median sternotomy, cranial right pulmonary lobectomy. Related data from current literature are listed in the present article finally.

Key words: bronchocutaneous fistula; contrast radiography; pulmonary foreign body; lung lobectomy; dog

Introduction

Current literature does not offer many information regarding bronchocutaneous fistula (BCF) which can originate at different levels from the major airways to the peripheral lung.

Fistula is any abnormal tubelike passage or communication within body tissue, usually between two internal organs, or leading from organ to the surface of the body. Fistula may also be defined as unnatural narrow channel leading from some natural cavity, such as duct of the mammary gland, or the interior of the rectum or anal sac, to the surface. Some fistulae are created surgically, for diagnostic or therapeutic purposes; others occur as a result of injury or as congenital abnormalities (1, 2).

Some reports on fistulae related to presented case are listed below:

A gastrobronchial fistula is reported (3) in a dog with clinical signs conzisted to laryngeal paralysis because of gagging and coughing of 1- month duration. The dog was pyretic and because of gagging, suspicion of aspiration, and poor response to antibiotic therapy, a swallowing disorder and esophageal dysfunction were differential diagnoses. On the survey thoracic radiographs alveolar infiltrates were evident in the ventral portions of all right lung lobes. Bronchi in the affected lobes were dilated and irregular, and cranial displacement of the pylorus and right cardiac displacement was observed. Esophagogram (using pure barium paste per os) was normal, during evaluation of gastric contraction, contrast medium appeared in the right caudal lung lobe. On subsequent radiographs, a fistula from the cranial margin of the displaced pylorus to the right caudal lobe bronchus was seen. During surgery no evidence of foreign body was found. A complete recovery is reported.

A bronchoesophageal fistula and transient megaesophagus in a dog with chronic cough of 2 year duration is another clinical case to mention (4, 5). The dog was depressed and emaciated. A mineral-dense foreign body within the midthoracic portion of the esophagus was observed on survey thoracic radiograph. The lumen of thoracic portion of the esophagus was large and air-filled. The cranioventral aspect of the right cranial lung lobe was consolidated. A flat piece of bone at the level of the heart, adhered to the ventral mucosa was removed by esophagoscopy. Four weeks later despite the therapy with antibiotics the dog returned with clinical signs of dyspnea and coughing. Contrast radiography using liquid barium sulphate, revealed a fistula between esophagus and the bronchi of the right cranial and middle lung lobes, with secondary reflux into the right cranial lung lobe. The dog improved rapidly after the sur-

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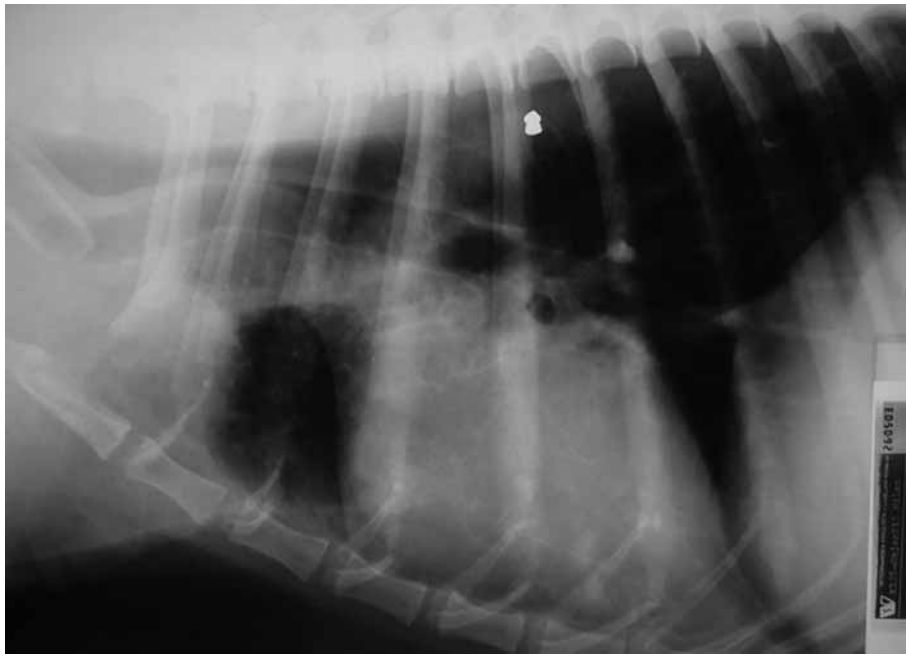


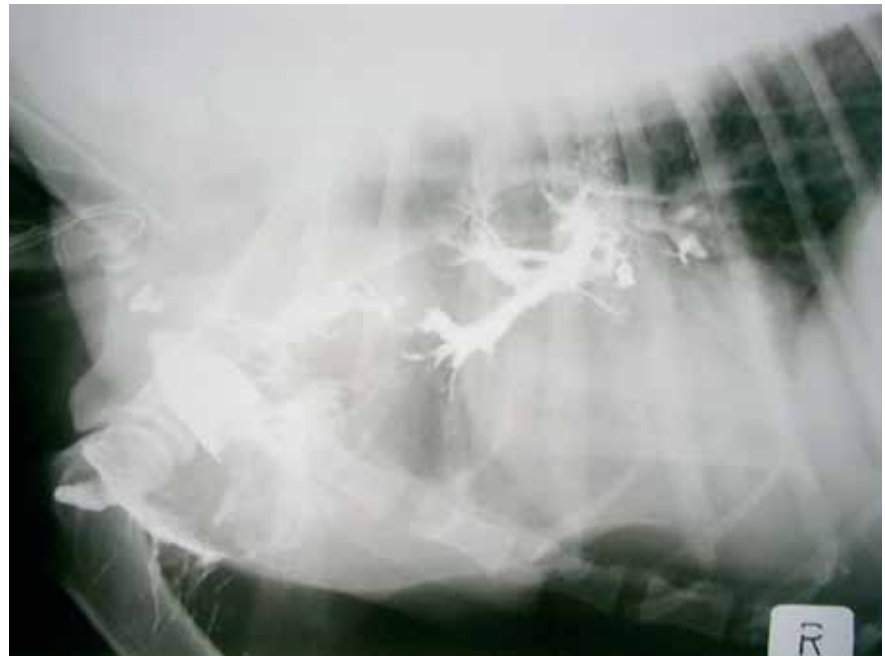
Figure 1: Survey right lateral thoracic radiograph: a marked localized interstitial infiltrate obscuring the pulmonary vessels in the right cranial lung lobe; borders are classified as alveolar in nature; radiopaque airgun projectile was found; nonradiopaque foreign body still suspected because of the infiltrate which develos around the affected bronchus

gery and no evidence of megaesophagus was found. Bronchoesophageal fistulae are rare in dogs and have not been associated with megaesophagus. In small-breed dogs with bronchoesophageal fistula, the clinical signs were pulmonary (bronchial, alveolar, interstitial changes); esophageal diverticula as a prominent feature related to foreign bodies in 9 of 10 cases (4, 5). The right caudal lung lobe was usually involved. If the localized esophagitis assocated with the foreign body-fistula could cause the diffuse neuromuscular esophageal dysfunction is unknown (4, 5).

Cutaneopulmonary fistula in a dog caused by migration of a toothpick has been reported lately (6). The 6-year-old, mixed-breed dog had a chronic, nonhealing, sinus tract located over the left cranial thoracic wall and intermittent, moist cough had been noted by owner. The dog had had an abscess over that region approximately 1 year prior to presentation. The abscess was incised, explored, and drained twice with no resolution. The resultant wound from the second surgery formed a draining tract and had been treated with antibiotics. On initial physical examination, a sinus tract associated with the left thoracic wall was visualized. Moderate, generalized lymphadenopathy was also noted. The drainage from the wound was described grossly as purulent exudate. The remainder of the examination was unremarkable. Survey radiographs of the thorax revealed a mild alveolar pattern associated with caudal aspect of the left cranial lung lobe. No evidence of pleural effusion was noted. A fistulogram was performed to determine the extent of draining tract and to visualize a suspected foreign body within the tract. Foley catheter was placed 3 cm into the fistula, and low osmolar, water-solubile, nonionic iodinated contrast medium was injected. Right lateral and ventrodorsal thoracic radiographs revealed contrast material within the bronchial tree of the left cranial and left caudal lung lobes. The contrast medium extended from the cutaneous surface through the thoracic wall, which confirmed a cutaneous-pulmonary fistula with a possible foreign body. The contrast material was disseminated within the bronchial tree. Surgical exploration of the fistula was indicated. A fibrous structure was found at the level of the costochondral junction within the thoracic cavity. This structure was excised, and a tooth-pick was found. The dog recovered without complication (6).

Clinical Report

An 11- year-old male Epagneul Breton was examined of intermittent draining tracts from the ventral neck for two years duration. The dog showed hyperthermia at exacerbations of disease and periods of remissions usually following antibiotic courses. As foreign body was suspected, tracts were unsuccessfully explored surgically twice at a private clinic. Fistulography had been



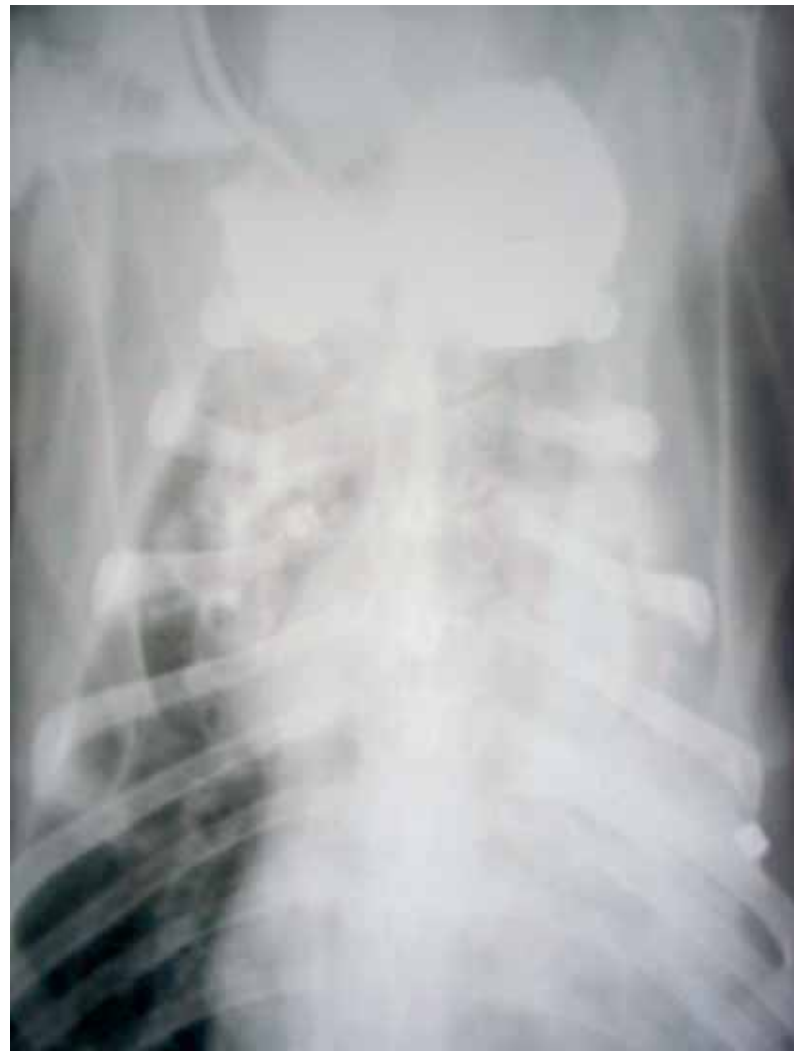


Figure 2 and 3: Right lateral and ventrodorsal thoracic radiograph (immediately after the injection of contrast material): contrast passed through the fistulous tract into the thoracic cavity and spread through bronchus in the right cranial lung lobe; bronchi in the affected lobe are dilated and irregular; no periostal reaction of the adjacent ribs found. Bronchocutaneous fistula was diagnosed

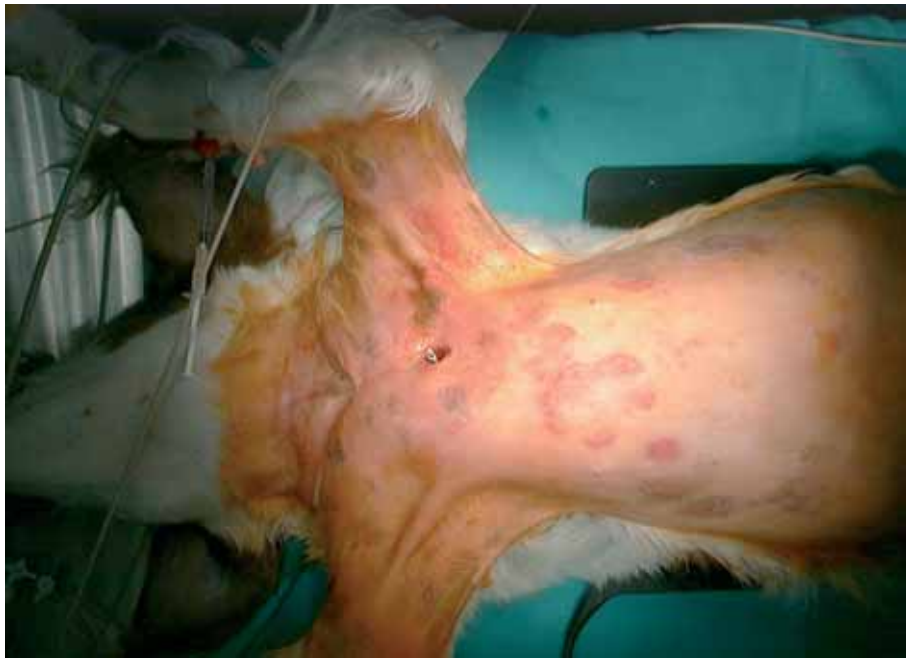


Figure 4: Patient prepared for surgery (fistulous tracts)

performed, but no foreign body was found. Three months later the dog got all again and was presented to our institution with multiple sinus tracts, purulent discharge and severely inflammed skin of the ventral neck.

The survey right lateral and ventrodorsal thoracic radiographs were taken prior to fistulography monitored under general anaesthesia . An airgun projectile as a foreign body was found. A marked localized interstitial infiltrate obscured the pulmonary vessels in the right cranial lung lobe.; borders were classified as alveolar in nature. Nonradiopaque foreign body was still suspected because of the infiltrate which develops around the affected bronchus. The larger of two fistulous tracts with diameter of about 3 mm was cannulated with Foley cathether (14 FR/CH, Kendall, Curity) and baloon was filled with sterile water which prevented catheter removal and sealed the tract. The smaller opening was closed with manual pressure. Low osmolar, water solubile, ionic iodinated contrast material (Urografin 76%, Schering) was injected under controlled pressure (8, 9, 10, 11).

Right lateral and ventrodorsal thoracic radiographs were taken immediately after the injection of the contrast material. Contrast material passed through the fistulous tracts into the thoracic cavity and spreaded through the bronchus in the right cranial lobe of the lung. Bronchi in the affected lobe were dilated and irregular. There was no periostal reaction of the adjacent ribs. Adverse reaction to the contrast medium was not observed. Bronchocutaneous fistula was dagnosed.

Surgical procedure

Based on the imaging findings, the diagnosis was a bronchocutaneous fistula. Surgical procedure was suggested. The patient was premedicated with midazolam (Dormicum, Roche) and metadon (Heptanon, Pliva), induced with thiopental, (Nesdonal, Specia) and maintained during course of general anaesthesia with anaesthestic breathing mixture of oxygen, air and isoflurane (Forane, Abbott). Incremental doses of ketamin (Bioketan, Vetoquinol) and fentanyl (Fentanyl, Janssen) were used to improve intraoperative analgesia and to decrease vol.% of isoflurane. The patient was mechanically ventilated (IPPV), monitored with ECG, pulse oxymeter, capnometer, and blood pressure was measured directly. Median sternotomy was performed with oscilating saw, sternopericardial ligament was freed and Finochietto rib spreader used to expose both hemithoraces. The adhesion of right cranial pulmonary lobe to the craniolateral chest wall was found and the most part of the lobe was indurated. Adhesion was carefully freed with combina-

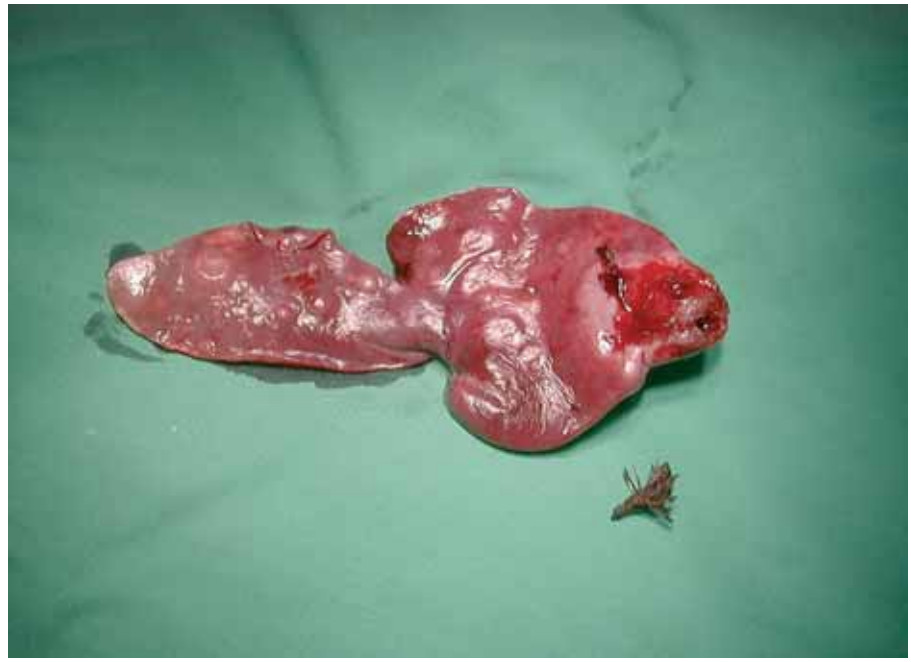


Figure 5: Resected lung lobe with foreign body (plant seed)

tion of sharp and blunt dissection and lobectomy performed with automatic stapler (Auto Suture). A canal leading from fibrotic mass to craniolateral chest wall was found. The stump was checked for air leakage, thorax copiously flushed with warm saline, chest tube was placed and sternotomy closed with stainless steel wire, lactomer and nylon. The incision site was infiltrated with bupivacain prior to skin closure.

A grass awn was found within the fibrotic mass. The dog remained at Intensive care unit for 24 hours, when chest tube was removed and remained hospitalised for another 48 hours when discharged to home care. The fistulous canal closed completely in 2 months and the dog does well at time of writing the paper as confirmed with telephone quiery.

Discussion

Fistulography are rather rare and sporadic diagnostic tool in small animals comparing to horses despite the fact that penetrating injuries involving foreign bodies are common problem, specially in active dogs. The presented clinical case is a good reason to stress the importance of the old fashion fistulography along with modern contrast enhanced diagnostic techniques. (7, 8, 9, 10, 12).

As it is well known that airgun projectiles, an 4, 5 mm lead bullet, are usually encapsulated and cause no major problems and reactions, but a nonradiopaque foreign body was still a most possible reason for connection between the fistula and the affected bronchus (10).

In buffalo a traumatic reticulo-bronchial fistula has been reported. In human the leading causes of acquired gastrobronchial fistulae were gastric ulcer and subphrenic abscess (3, 8). Other reported causes related to gastrointestinal tract are congenital, previous esophageal or gastroesophageal surgery, infection caused by a foreign body or neoplasia. Regardless of etiology, occurence of gastrobronchial fistula in humans or animals is rare. The prefered method of confirming a presumptive diagnosis of gastrobronchial fistula is upper gastrointestinal contrast study and more recently of course CT and MRI (3). Foreign body migration is not an uncommon syndrome as well as in dogs as in human. Clinical outcomes range from small, cutaneous, inflammatory reactions to severe inflammation and bacterial infection of body cavities, organs or both. Diagnosis and treatment of such conditions can be challenging. According to the literature data (7), no specific breed was overrepresented. Most of the patients had a history of soft tissue swelling, abscess, or draining tract of uncertain duration. The mean duration of the clinical signs was 9.8 months (in our case 2 years) and at least two surgical procedures had been performed before the

fistulography and further definitive diagnosis. Neck region remain the first among the others (head, paws, flank, inguinal region, gluteal region). The most common cause was the foreign body (7). Foreign body inhalation is quite common in pointer breeds, specially in Mediterranian region. Pointer dogs hunt with extended neck, highly elevated head and open mouth. Typical hunting pose and early hunting season when plants still bloom enable inhalation of grass awns (personal information, dr. Butinar). Early removal of inhaled foreign body with endoscopy after major complaint of cough, breed and history of hunting enables complete remission.

Demonstrating the presence of a foreing body can be difficult, and successful treatment usually hinges on the complete excision of the object and the majority of diseased tissue associated with it. In the dog, many different objects have been described as migrating foreign bodies, including wood fragments, grass awns, needles, and toothpicks. In the human literature, there have been multiple reports of toothpick migration and toothpick injuries. In veterinary literature only two cases of tooth-pick-related injuries could be found (7). In dogs, the most probable route of injury would be swallowing and/or digestion of the toothpick (7) with subsequent migration. It is believed that aspiration and subsequent migration was the course of the presented case although cutaneous migration can not be ruled out. Fistulae that have been previously reported in association with a migrating foreign object include esophagoaortic, esophagotracheal, and esophagobronchial, with the latter being the most common in dogs (7). When attempting to diagnose a migrating foreign body, many modalities can be used. If peritonitis or pyothorax is present, survey radiographs, blood work, abdomino/thoracocentesis, and surgical exploration may suffice. If a draining tract is present, one additional, simple diagnostic test that can be completed is contrast radiography (8, 9, 10). Fistulography (injection of iodinated contrast into a sinus or draining tract) or fistulography (when result indicates a cutaneocavitary communication) is a quick, inexpensive tool to help in identifying the presence of a foreign object. Historically, these types of radiographic studies have been used frequently in equine practice, but there has been sparse reporting of their use in small animal practice. In a previous study, sensitivity for fistulo/sinography diagnosing foreign bodies in small animals was reported as 87% (8). This study also reported that 44% of the animals examined, fistulography demonstrated that the extent, the position of the draining tract or both were different than expected on the basis of clinical signs and survey radiographs. This emphasizes that valuable informations can be obtained by the use of contrast radiography. Other modalities which have been used to detect foreign bodies include ultrasound, magnetic resonance imaging, and computed tomography. (8, 9, 10). These modalities can be very useful, athough they are not a convinient modality for initial evaluation because their requirements of anesthesia, expertise, or expensive instrumentation. Fistulography is an excellent diagnostic test available to any practicioner with radiographic capability and can provide valuable information that can help surgical planning.

A good quality survey radiograph of the tract and the surrounding tissue should be obtained before the contrast study. If no diagnosis is apparent then fistulography may be performed. The aim of the technique is to fill and distend the tract and any communicating cavity with the contrast material, injected by hand. During the injection some adjustment of the position of the catheter or tube may be necessary to obtain good filling. Initially there will be a little resistance to the flow of the contrast material but when tract is completely filled, the pressure required on the syringe plunger will increase. At this point the operator moves to a position safe from primary and scattered radation and a radiographic exposure is made. There must not be a long delay between the filling of the sinus and the radiographic exposure because this delay may allow the sinus to begin to empty. Radiographic exposure factors for the fistulography should be greater than those used in survey radiograph (8, 9, 10).

Although fistulography is a simple technique, attention to detail is important. Contamination of the hair coat with exudate, topical applications and contrast material must be avoided. A survey radiograph must always be taken before the contrast examination because fragments of opaque foreign material may be obscured by the positive contrast. Two perpendicular projections are normally required to locate the focus accurately. When contrast material leaves the area of interest rapidly, either through the tract opening or because it is rapidly absorbed, an infusion of con-

trast may be required for additional projection. The sinus must not be over distended because the tract may rupture and contaminated contrast material may spread along otherwise normal fascial planes (8, 9, 10).

It is important to note that low osmolar, water-soluble, nonionic contrast agents are the agents of choise especially in cases where the agent comes in direct contact with pulmonary tissue. Ionic agents are contraindicated in fistulograms that may enter the pulmonary parenchyma, because they can stimulate severe inflammation reaction in pulmonary tissue, which can result in fatalities (11). The biologic characteristics of the nonionic agents are similar to those of the nonionic monomers. General characteristic commonly shared are low molecular weight, low lipid solubility, inertness, and rapid glomerular filtration. Like ionic agents, extracellular space distribution occurs throughout the body, except in brain tissue. Nonionic contrast agents have a higher LD50 and a lower incidence of adverse clinical reactions than ionic compounds (11).

Filling defects are important radiographic findings. If sinus is associated with a stick the filling defect may be of similar dimensions on both views and have a regular or geometric outline. It is important to use two views to gain a three dimensional impression of the shape and location of any filling defect (8, 9, 10.11, 12).

The majority of draining tracts due to chronic foreign body result in complete resolution of clinical signs, when the diseased tissue is fully excised (13).

Conclusions

- bronchocutaneous fistula is a rare pathologic condition in a dog
- diagnostic imaging is crucial to localize the origin of the fistula
- choice of diagnostic imaging depends on equipment availability
- correctly performed fistulo/sinography is an easily performed, and relatively accurate method
- lobectomy of the affected lung lobe results in complete remission
- grass awn or similar foreign bodies can be found in resected lobe
- foreign body inhalation is not uncommon, specially in pointer dogs

References

1. Fistula. In: Dorland's illustrated medical dictionary, 29th ed. Philadelphia: W.B. Saunders Company, 2000.

2. Blood DC, Studdert VP. Saunders comprehensive veterinary dictionary, 2nd ed. London: W.B. Saunders, 1999.

3. Silverstone AM, Adams WM. Radiographic diagnosis-gastrobronchial fistula in a dog. Veterinary Radiology & Ultrasound 1999; 40:477-9.

4. Van Ee RT, Dodd VM, Pope ER, et al. Bronchoesophageal fistula and transient megaesophagus in a dog. Journal of the American Veterinary Medical Association 1986; 188:874-6.

5. Basher AW, Hogan PM, Hanna PE, et al. Surgical treatment of a congenital bronchoesophageal fistula in a dog. Journal of the American Veterinary Medical Association 1991; 199:479-82.

6. Jackson AH, Degner DA. Cutaneopulmonary fistula in a dog caused by migration of a toothpick. Journal of the American Animal Hospital Association 2002; 38:545-7.

7. Hittmair KM, Wanivenhaus G. Ultrasonographic detection of ingested toothpick perforating the spleen in a dog. Wien Tierartzl Monatsschr 2001; 88:252-6.

8. Lamb CR, White RN, McEvoy FJ. Sinography in the investigation of draining tracts in small animal: retrospective review of 25 cases. Veterinary Surgery 1994; 23:129-34.

9. McEvoy FJ, Lamb CR, White RN. An application of sinography in small animal practice. Veterinary Record 1993;132:183-5.

10. Yanofsky G, Bonneau NH, Breton L. Fistulography as an aid in the diagnosis of a nonradiopaque foreign body in a dog. Canadian Veterinary Journal 1986; 27:291-2.

11. Holland M. Contrast agents. Veterinary Clinics of North America: Small Animal Practice 1993; 23:269-77.

12. Yamagishi N, Yamada K, Ishikawa H et al. Bronchocutaneous fistula in a dog. Veterinary Radiology & Ultrasound 2000; 5:422-4.

13. Reich T, Tait J. Chronic bronchocutaneous fistula in the dog: a method for construction. Surgery 1971; 69:895-8.

BRONHOKUTANA FISTULA PRI PSU

T. Ivanuša, J. Butinar

Povzetek: Opisan je primer bronhokutane fistule pri psu, kot posledica inhaliranega tujka v pljučih. Kontrastna fistulografija je omogočila identifikacijo tujka, njegovo kasnejšo kirurško odstranitev (lobektomija dela desnega kranialnega lobusa pljuč) in uspešno ozdravitev oz. popolno sanacijo bolezenskega procesa. V članku je uporabljena sorodna novejša litertura.

Ključne besede: bronhokutana fistula, contrastna radiografija, tujek, lobektomija, psi

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AUTHOR INDEX VOLUME 41, 2004

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Contributions should be written in English and should not exceed 12 pages (27 lines per page, approx. 75 characters per line). They should be submitted electronically, written on any word processor for Windows, and accompanied by a hard copy. The text should be double-spaced and the lines should be numbered on the left-hand side. The margin on the left-hand side of the page should be 4 cm and the text should have no page breaks. Words should not be divided.

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Article in proceedings of a meeting or symposium: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica 1995: 83-6.

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Priloge so tabele, grafikoni, diagrami, slike ipd. Te avtorji priložijo posebej, lahko pa jih smiselno vključijo v datoteko z besedilom. Priloge morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. Na hrbtni strani prilog naj bodo napisani ime in priimek prvega avtorja, naslov članka ter besede in številke, ki priloge opredeljujejo. Če je potrebno, se označi tudi položaj (zgoraj, spodaj). Vsi navedki (reference), citirani v besedilu, se morajo nanašati na seznam literature. V besedilu jih je treba oštevilčiti po vrstnem redu, po katerem se pojavljajo, z arabskimi številkami v oklepaju. Prvi navedek v besedilu opredeli številko oziroma vrstni red ustreznega vira v seznamu literature. Če se avtor v besedilu ponovno sklicuje na že uporabljeni vir, navede tisto številko, ki jo je vir dobil pri prvem navedku. Citirana so lahko le dela, ki so tiskana ali kako drugače razmnožena in dostopna javnosti. Neobjavljeni podatki, neobjavljena predavanja, osebna sporočila in podobno naj bodo omenjeni v navedkih ali opombah na koncu tiste strani, kjer so navedeni. V seznamu literature so viri urejeni po vrstnem redu.

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Načini citiranja

Knjiga: Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

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Članek iz zbornika referatov: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica 1995: 83-6.

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