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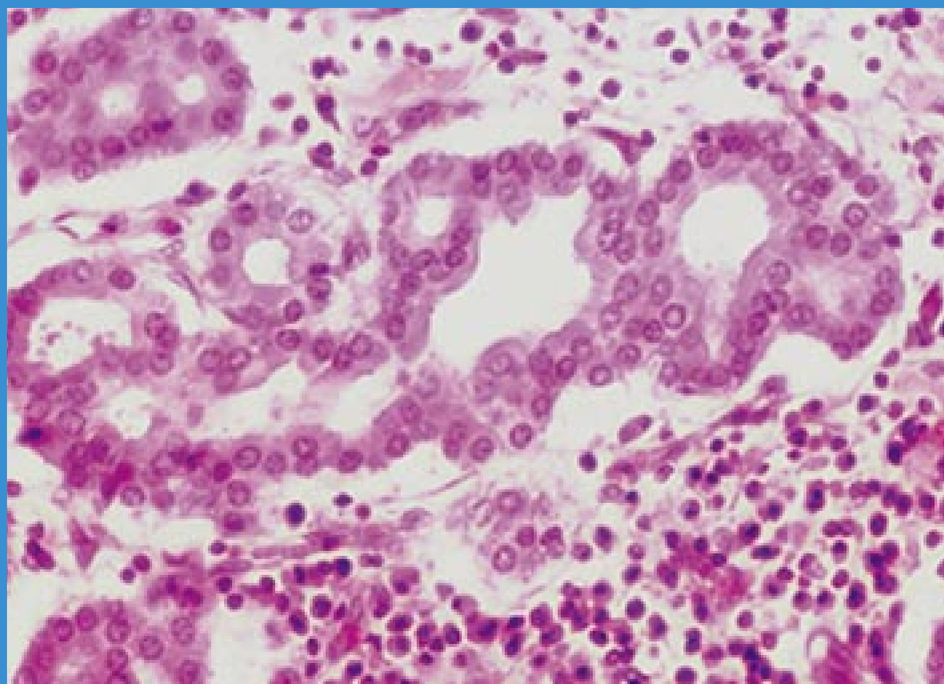

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SLOVENIAN VETERINARY RESEARCH

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UDC 619:626.211-006-07:636.3 **Review Paper**

ENZOOTIC NASAL ADENOCARCINOMA OF SHEEP

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Summary: Enzootic nasal adenocarcinoma (ENA) is a contagious viral disease of sheep and goats characterised by neoplastic growth of the ethmoidal mucosa in the nasal cavity. ENA is caused by retrovirus, which is closely related to the ovine pulmonary adenomatosis virus (JSRV). The disease is probably spread by respiratory route and it can be introduced into a flock by purchasing infected animals. In affected flocks, the prevalence of the disease is typically 0.5 – 2 %, although it can be as high as 15 %. The disease primarily affects young adult animals at the age of 2 to 4 years. It is usually clinically manifested by seromucous or purulent nasal discharge, dyspnoea, emaciation and less often by exophtalmus and skull deformations. A clinical diagnosis of ENA in a live animal can be confirmed by endoscopic or x-ray examination. There is no effective treatment and the majority of the animals die within 90 days after the onset of the first clinical signs. The tumour, which arises unilaterally or bilaterally from the ethmoidal area of the nasal cavity, may occludes both nasal cavities, grows into the pharynx, paranasal sinuses and skull cavity, but does not metastasise to the lymph nodes, brain or other organs. Pathoanatomic diagnosis of ENA must also be confirmed with the histopathological examination. In the latest classification of tumours, ENA is classified as a low-grade adenocarcinoma. Besides histopathological examination, Western blotting, immunohistochemical examination and electronic microscopy can be used for research purposes.

Key words: sheep diseases – epidemiology; nose neoplasms – pathology; adenocarcinoma – pathology – virology; diagnosis – methods; microscopy, electron; Slovenia

Introduction

Enzootic nasal adenocarcinoma (ENA) is a contagious viral disease of sheep and goats characterised by neoplastic growth of mucosal nasal glands (1, 2, 3, 4, 5). Synonyms for ENA are also enzootic nasal tumour, infectious adenopapillomatosis, infectious adenopapilloma and infectious nasal adenocarcinoma (1, 2, 4, 6).

In the past few years, some cases of ENA were also diagnosed in Slovenia (7). With this review article, we want to remind veterinarians, especially clinicians, to consider ENA as a possible diagnosis in the case of dyspnoea and chronic nasal discharge in sheep.

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Etiology

The disease is caused by enzootic nasal tumour virus (ENTV; also termed ovine nasal adenocarcinoma virus - ONAV). Although the first report of disease and the hypothesis of its viral etiology are a half century old, it was only in year 1978 that Yonemichi et al. (1978) for the first time demonstrated virus-like particles in the neoplastic tissue.

Owing to the progress in molecular biology, the ENT virus has been investigated comprehensively. The complete sequence of ENT virus has already been determined, and based on the sequence data the virus was classified as a type B/D retrovirus (8, 9). The ENT virus is closely related to the virus associated with the ovine pulmonary adenomatosis virus (JSRV), which causes neoplastic lesions in the lower respiratory system (9, 10).

Epidemiology

ENA occurs in many countries all over the world. The first case of the disease was described by Cohrs in 1953, and up till now the disease has been found in all countries with well developed sheep breeding, except in Australia, New Zealand (2) and Great Britain (6). In Slovenia, the first case of ENA was diagnosed in year 2001 (7).

The disease is probably spread horizontally, most likely by the respiratory route. ENA was successfully transmitted by experimental intranasal and intrasinusoidal inoculation of the homogenate of the neoplastic tissue of sheep (11) and concentrated nasal discharge of naturally infected goats (12).

The disease can be introduced into a flock by purchasing infected animals. In affected flocks, the prevalence of the disease is typically 0.5 – 2 %, although it can be as high as 15 % (6).

On the basis of the similar prevalence of ENA in many different sheep breeds, several authors concluded that sex and breed have no influence on the occurrence of ENA (2, 3, 13). Genetic predisposition to the ENA was also rejected (1), despite Duncan et al. (1967) reporting ENA in sheep and its offspring of the second generation (4).

Clinical signs

The ENA primarily affects young adult animals at the age of 2 to 4 years (6). The youngest sheep with ENA, described in the literature, were aged ninth months (2) and one year (4), while the oldest was nine years of age (2).

The disease is usually clinically manifested by seromucous or purulent nasal discharge, dyspnoea, like snoring (1, 2, 14), coughing, sneezing (1, 4), open mouth breathing (1, 4), and less often by exophthalmus (1, 2) and skull deformations (2, 3) (Figure 1). Depigmentation and alopecia occurs around the nostrils, as the consequence of chronic nasal discharge (6). Affected animals are anorexic, gradually lose weight (1, 2, 4, 14) and die within 90 days after the appearance of first clinical signs (3) due to pasteurellosis or other complications (1, 2, 3).

Pathology

Enzootic nasal tumour virus induces neoplastic growth of mucosal nasal glands and formation of the tumour (2, 5, 6), which arises unilaterally or bilaterally from the ethmoidal area in the nasal cavity

Figure 1: Head of sheep with enzootic nasal adenocarcinoma. Dense, mucous nasal discharge from nostrils of affected sheep. (The photo was kindly provided by Jože Starič from Clinic for ruminants)

(15). At the beginning the tumour appears as miliary protuberances and then becomes nodular and polyp-like and may occludes both nasal cavities, grows into the pharynx, paranasal sinuses (1, 4), skull cavity (4) and compresses the surrounding tissues (1, 2, 3, 13, 16) (Figure 2). The tumour surface may necrotise or exhibit secondary purulent inflammation (2). The ENA does not metastasise to the regional lymph nodes, brain or other organs (1, 2, 4, 6, 13, 14).

At the light microscopic level, the tumours are composed of the neoplastic epithelial cells arranged in tubular and/or papillary structures (Figure 3, 4). The neoplastic cells are mostly cuboidal and occasionally columnar. They have large round or oval nuclei with clumped chromatin pattern and they are located centrally or in the basal parts of the

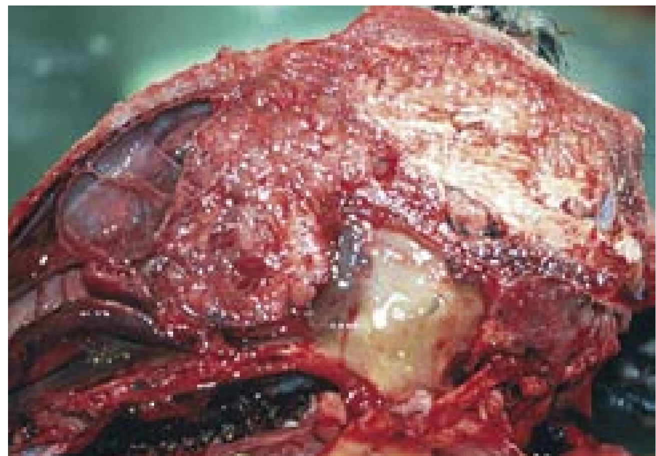

Figure 2: Sagittal section of the head of sheep with enzootic nasal adenocarcinoma. The tumour occupies the ethmoidal area of the nasal cavity

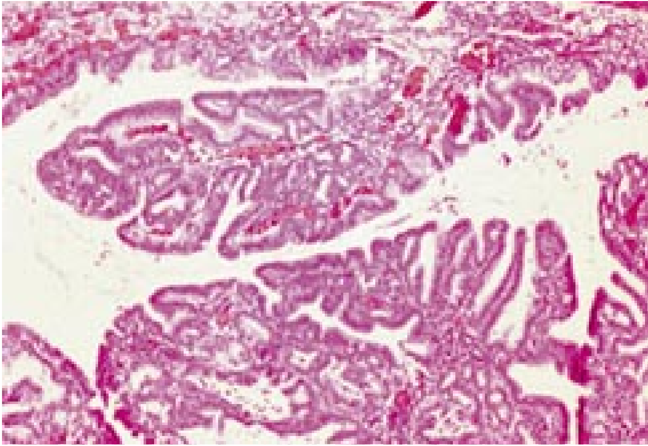

Figure 3: Enzootic nasal adenocarcinoma of sheep. Papillary type of growth. Stroma of papillary proliferation is densely infiltrated with lymphocytes and plasma cells and well vascularised. HE staining, x 100

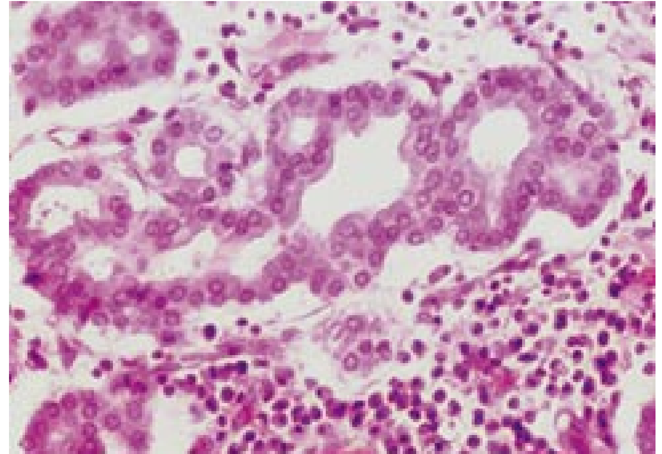

Figure 4: Enzootic nasal adenocarcinoma of sheep. A group of neoplastic tubules with cuboidal cells and large round nuclei with clumped chromatin and small nucleoli. Stroma is infiltrated with numerous plasma cells. HE staining, x 400

cells. The fibrous connective tissue stroma is frequently scanty, oedematous and densely infiltrated by numerous lymphocytes, plasma cells and macrophages. A fewer number of neutrophils and desquamated neoplastic epithelial cells can also be noticed in the lumina of several neoplastic tubules (1, 4).

Initially, ENA was histologically classified as a benign neoplasia. Terms used for ENA in the classifications were epithelioma (17), adenoma (13) and adenopapilloma (1, 11). Later, it was classified as a low-grade adenocarcinoma (2, 3, 5, 14). The most recent classification was based on the infiltrative growth of the neoplastic cells into the surrounding connective tissue and the absence of metastasation (2, 5).

Using electron microscopy, characteristic, round, membrane-coated secretory granules (2) of varied electron opacity and size from 0.2 to 1 μm (13) can be demonstrated in the cytoplasm of the neoplastic cells. Extracelullarly, close to the apical surfaces, and in the cytoplasmic vacuoles of the neoplastic epithelial cells, virus-like particles can also be found. The particles vary in diameter from 80 to 100 nm, and have characteristic eccentrically or centrally located electronic dense nucleoid with a diameter of 47 nm. The nucleoid is bounded by electron lucent zone and spiked unit membrane (2, 13). We successfully applied rapid reprocessing of paraffin-embedded tissue for diagnostic electron microscopy (7). Semi-thin sections were examined to confirm that

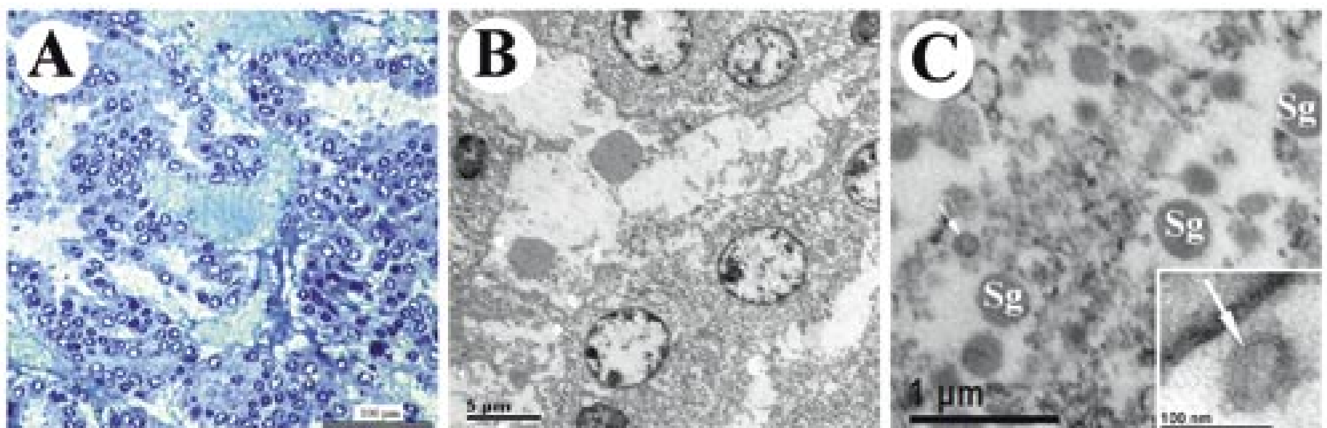

Figure 5: (A) Semi-thin section of the reprocessed tumor tissue displaying characteristic glandular morphology; toluidine blue. (B) Low-power electron micrograph of the neoplastic tissue showing cuboidal cells with distinctly euchromatic round or oval nuclei. (C) Portion of cell's cytoplasm; secretory granules (Sg) predominate in cytoplasm matrix. (D) Virus-like particle (white arrow) found in the cytoplasm and in intracytoplasmic vacuole (insert)

tumor regions displaying typical glandular morphology were successfully reprocessed (Fig. 5; panel A). Ultramorphological appearance of the cells and the location of spherical virus-like particles were in agreement with previous reports (3, 13). Briefly, neoplastic proliferations were predominantly composed of cuboidal cells. Their nuclei (8–14 nm in diameter) were round or oval and distinctly euchromatic (Fig. 5; panels B). The cells cytoplasm had an electron lucent matrix containing numerous secretory granules that ranged in size from of 220 to 540 nm (see Fig. 5; panel C). The presence of intracytoplasmic spherical virus-like particles of 70–90 nm in diameter is shown (Fig. 5C and insert).

Diagnostics of ENA

The above cited clinical signs are specific enough to to suspect ENA. At live animal, a diagnosis can be confirmed by demonstration of the neoplasia in the caudal parts of the nasal cavity, using fiberoptic endoscopy (14) or x-ray examination (13, 14). Currently, there is no laboratory test available to confirm the clinical diagnosis of ENA as the virus has rarely been detected in the blood and due to the lack immunological response in affected animals antibodies to ENT virus have not been detected in the sera of affected animals (18).

The easiest and most reliable way to confirm the diagnosis is the examination of dead or sacrificed animal. The neoplasia can be easily noticed by pathoanatomic examination of sagittal section of the head, and it is seen as a white- grey mass of soft to firm consistency, which occludes the caudal part of one or both nasal cavities. To confirm the pathoanatomic diagnosis of ENA histopathological examination must be performed (6).

There are also a few laboratory methods for the demonstration of ENT virus in the dissection samples. The virus has been detected in neoplastic parenchyma and nasal exudates of the affected animals by Western blotting (2, 15). Using primary antibodies raised against ovine pulmonary adenomatosis virus (JSRV), a positive immunohistochemical reaction was demonstrated in the apical parts of singular neoplastic cells (2). Characteristic viral particles with diameter from 80 do 100 nm, can be showed in neoplastic tissues by electronic microscopy (2, 3, 5, 13). ENT virus can not be grown in vitro conditions, so the isolation of the virus in cell culture is not applicable (8).

Control of the disease

At the moment, there is no efficient treatment nor vaccine against ENA. There are only two reports of unsuccessful attempts at treatment. Duncan et al. (1967) reported temporary improvement of clinical status in two sheep after irradiation of the nasal region (4). Rings and Robertson (1981) surgically removed a part of the neoplasia, but despite that, the sheep died 12 hours after (14).

Conclusion

ENA is a contagious, retroviral disease with fatal outcome. It always should be included in a list of the differential diagnoses, when dyspnoea and chronic nasal discharge are noticed in the sheep or goat. The suspicion of ENA can be easy confirmed by pathoanatomic examination of sagittal section of the head and histopathologic examination of the tumour. The only way to control ENA is the culling of the affected animals. Delay in the recognition of ENA will lead to delay in the control of the disease, to its further spreading and to an increase in deaths in the flock and consequently to financial damage for the farmer.

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ENZOOTSKI NOSNI ADENOKARCINOM OVC

T. Švara, M. Gombač, M. Vrecl, P. Juntes, M. Pogačnik

Povzetek: Enzootski nosni adenokarcinom (ENA) je nalezljiva virusna bolezen ovc in koz, pri kateri se v nosni votlini, na vohalni sluznici sitke, razvije novotvorba. ENA povzroča retrovirus, ki je soroden povzročitelju pljučne adenomatoze pri ovcah. Bolezen se širi horizontalno, v rejo se lahko vnese z nakupom nove živali. V okuženih čredah je prevalenca bolezni od 0,5 do 2 %, lahko pa doseže tudi 15 %. Učinkovitega zdravljenja bolezni zaenkrat ni, večina živali pogine v 90 dneh po pojavu prvih znakov bolezni. Zbolevajo predvsem mlade živali, v starosti med 2. in 4. letom. Najpogostejši klinični znaki so seromukozni ali gnojni nosni izcedek, oteženo dihanje, hujšanje, redkeje pa se pojavijo eksoftalmus in deformacije lobanje. Tumor se lahko razvije v eni ali v obeh nosnih votlinah, vrašča v žrelo, obnosne votline in v lobanjsko votlino, vendar v literaturi ni opisano, da bi metastazirala v bezgavke, možgane ali druge organe. Makroskopsko diagnozo potrdimo s patohistološko preiskavo. Po zadnji klasifikaciji tumorjev ENA uvrščajo med adenokarcinome nizke malignosti. V raziskovalne diagnostične namene pridejo v poštev tudi metoda western blot ter imunohistokemične in elektronskomikroskopske preiskave.

Ključne besede: ovce, bolezni – epidemiologija; nosne novotvorbe – patologija; adenokarcinom – patologija – virologija; diagnostika – metode; elektronska mikroskopija; Slovenija

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Review Paper

ELECTROGENE THERAPY IN CANCER TREATMENT

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Summary: Gene therapy offers the prospect of efficient and highly specific therapy of cancer. Vectors for introduction of therapeutic genes into target tissue can be broadly classified into viral and non-viral vectors. Viral vectors provide highly efficient gene delivery technique, but there are some major concerns regarding their safety for patients. Non-viral techniques involve delivery of naked plasmid DNA into tissue using physical methods, such as electroporation or gene gun technique or deliveries mediated by chemical carriers, for example cationic polymers or lipids. Non-viral methods provide safer, but less efficient alternative compared to viral DNA delivery.

Electroporation is method for delivery of various molecules into the cells by transiently increasing permeability of cell membrane using application of controlled external electrical field to the cells. Electroporation-based DNA delivery or electrogene therapy involves injection of plasmid DNA into target tissue, followed by application of controlled electric pulses. In electrogene therapy of cancer, therapeutic genes are usually transferred either intratumorally or intramusculary. Until now, electrogene therapy using a variety of therapeutic genes, mostly encoding cytokines, but also antiangiogenic factors, suicidal and apoptosis inducing genes has shown promising results for effective cancer therapy in preclinical studies.

Key words: neoplasms – therapy; electroporation; gene therapy – methods; drug delivery system

Introduction

Intensive scientific research in molecular biology in the last decades significantly increased growth of knowledge of the molecular basis of carcinogenesis and therefore led into improvements in cancer therapy. Despite considerable progress, which has been made, many types of cancer remain resistant to conventional therapy. Therefore new therapeutic approaches are being explored, among which immunotherapy and gene therapy hold great promise for cancer treatment.

The concept of gene therapy involves transfer of genetic material into target cells in order to overcome a genetic defect or to provide a protective or corrective function with the goal of curing a disease or improving clinical status of a patient. In case of genetic disease, caused by mutation in a specific gene, therapeutic effect of gene therapy is usually achieved by delivery of functional gene into a target cells or tissue. Exogenous gene delivery can also be a tool for treatment of non-genetic disorders by delivery of genes, which, for example, encode proteins to modulate immune response or other therapeutic proteins with specific function (1, 2).

Gene of interest can be inserted into target cells using different vectors, which can be broadly classified into two groups, viral and non-viral vectors. After the introduction of therapeutic gene, genetically altered cells start with production of RNA or protein, encoded by the transferred gene. The goal of this strategy is to achieve stable, preferably regulated expression of transgenes in the target tissue for required period of time without significant side effects (1, 2).

The first time gene therapy was employed in treatment of human patients was in 1990. Treated was a group of patients with genetic disease ada-SCID (i.e. severe combined immunodeficiency due to adenosine deaminase deficiency) (3). One year later the first clinical trial of gene therapy for can-

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cer was performed in patients with melanoma (4), and until now, over 1000 gene therapy clinical trials have been conducted around the world for different indications, vast majority of them (over 66 %) are in cancer treatment (Figure 1) (5).

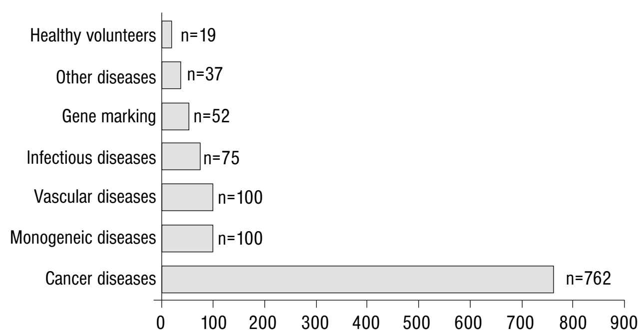

Figure 1: Distribution of ongoing gene therapy trials worldwide by indications (5)

Viral vectors are currently the most frequently used vectors in clinical trials of gene therapy worldwide (Figure 2) (5). Their main advantage is high

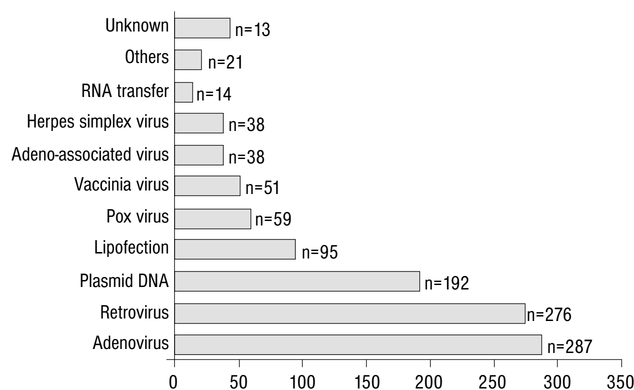

Figure 2: Distribution of ongoing gene therapy clinical trials worldwide, dependent on the vectors used (5)

Gene therapy offers the prospect of efficient and highly specific therapy of cancer, which created lots of excitement among investigators and clinicians and lead to intensive research in this field. Researchers developed several of different approaches to cancer gene therapy, which can be divided into three basic concepts (6, 7):

a.) Strategies to enhance immunological rejection of the tumor by the host;
b.) Strategies to repair the cell cycle defects caused by losses of tumor suppressor genes or inappropriate activation of oncogenes, and
c.) Suicide gene strategies.

DNA delivery systems in cancer gene therapy

The success of gene therapy largely depends on development of suitable vectors or vehicles for *in vivo* gene transfer. In order to eliminate potential risks of exogenous gene transfer, for example, evolution of new viral diseases in humans, induction of malignant transformation, systemic toxicity, etc, DNA vectors employed in gene therapy *in vivo* have to fulfill several conditions. Optimal DNA vector would have to enable high levels of stable and long-lasting exogenous gene expression without significant side effects for the patients undergoing gene therapy. In search for such vector, a number of viral- and nonviral- vector based delivery systems have been developed (1, 2, 6, 7, 8, 9).

transfection efficiency (6). Viral vectors are derived from naturally evolved viruses, which are capable of transferring their genetic material into the host cells. They are genetically modified by deleting genes, essential for viral replication, assembling or infection and replacing deleted genes with relevant therapeutic genes (1). Such viruses lose their ability to reproduce in target cells, and can be replicated only in cell lines, which provide the deleted function. This modification is necessary to prevent replication of recombinant viral vectors in the host organism in order to ensure safety of their clinical use.

Gene therapy vectors are being developed by genetic modification of retroviruses, lenitiviruses, adeno- and adeno-associated viruses, herpesviruses, poxviruses, and others. Among variety of different types of viral vectors, two of the most often used in gene therapy clinical trials are retro- and adenoviral vectors (5).

Retroviral vectors are one of the first constructed human gene therapy vectors, and have been used for DNA delivery since the early 1980s. Retroviruses are small RNA viruses, which use cellular transcription machinery to copy their own genome and integrate it into the genome of host cell. Ability for integration into host's genome is one of the most desirable features of retroviruses, since it allows long-term expression of transgenes. On the other hand, random integration is associated with risks of insertional mutagenesis. The other drawbacks for their use in clinical settings are inability to infect non-dividing

cells, problems with production of high titers and low capacity for exogenous DNA insertion (8).

Adenoviral vectors are considered among the most efficient DNA delivery methods, which are currently available for *in vivo* gene transfer to mammalian cells. They have large capacity for exogenous DNA insertion, can infect a large variety of cell types and are relatively easy to produce in high titers. However, their main limitations are the absence of expression of adenoviral receptors on certain types of cells and high prevalence of anti-adenoviral antibodies in humans, since naturally occurring adenoviruses are associated with common cold and other respiratory, intestinal and eye infections (9).

The major drawbacks, associated with DNA delivery using viral vectors, which raise concerns about their safe clinical use, are especially insertional mutagenesis, stimulation of the patient's immune system, which can preclude multiple administrations and cause adverse immune reactions, and toxicity with systemic application (2, 10). Therefore, as an alternative to overcome some of the major concerns and risks of viral vectors, different nonviral gene delivery techniques have been developed, for example naked DNA delivery using physical methods such as electroporation or gene gun technique or deliveries mediated by a chemical carriers, for example cationic polymers or lipids (11).

All of these modalities use naked plasmid DNA, which is, contrary to viral vectors, noninfectous and nonimmunogenic and has low toxicity profile. Compared to production of viral vectors, large amounts of endotoxine-free plasmid DNA are relatively easy and quick to produce (12, 13). But the major limitation of these techniques is low *in vivo* transfection efficiency, compared to viral methods (11).

Chemical methods for *in vivo* gene delivery employ synthetic vectors, which protect naked DNA from degradation and improve its admission intracellulary thus facilitating transfer of naked DNA into target cells. One of such approaches is use of cationic lipids, which interact with negatively charged DNA, forming DNA-lipid complexes, called lipoplexes. These complexes are positively charged, which allows them to bind to negatively charged cell surface. Entry of lipoplexes into the cell is achieved by endocytosis, followed by release of DNA into the cytoplasm. Cationic lipoplexes are suitable for clinical use, since they are noninfectious, nonimmunogenous, well tolerated, easy to produce and can be targeted to specific cells (14).

Another chemical method, which can be utilized for *in vivo* gene delivery, is use of cationic polymers. Positively charged polymers, for example DEAE-dextran, polybren, polylysin, polyethilenimine, spontaneously interact with DNA molecules to form complexes, called polyplexes. The potential for clinical use of polyplexes is in inhalation gene therapy, which is non-invasive and relatively effective gene transfer into respiratory tract, with permanent gene expression without adverse expression in other tissues (15).

One of the physical methods, which can dramatically enhance transfection efficiency of plasmid DNA application into tissue alone, is electroporation (13, 16). Electroporation is a method for delivery of various molecules into the cells by transiently increasing permeability of cell membrane using application of controlled external electric field to the cells (17, 18).

Electroporation is already well established as in vitro method for increasing delivery of various molecules (e.g. RNA, DNA, oligonucleotides, dyes, ions, chemotherapeutic drugs, etc) into different types of cells. *In vivo* it is gaining much interest as a tool for two prospective therapeutic modalities, electrochemotherapy (i.e. application of controlled electric pulses to tumor cells in order to increase uptake and cytotoxicity of chemotherapeutic drugs (19, 20, 21, 22) and electrogene therapy (i.e. enhancing transfection efficiency of plasmid DNA application into different tissues (13, 16, 23, 24).

Other physical method for introduction of plasmid DNA into cells is gene-gun technique or DNA-coated particle bombardment (25). This technique utilizes heavy metal (gold or tungsten) microparticles, covered with DNA, which are accelerated to the sufficient speed using compressed helium to penetrate the target cells. Clinical application of the technology remains limited because of relatively low efficiency of the method and the potential tissue damage created by impact of the particles.

One of the newest nonviral physical methods for gene delivery is use of ultrasound or so called sonoporation, which increases permeability of cell membrane to different macromolecules, including DNA (26, 27). The efficacy of this method can be improved by use of microbubbles, or ultrasound contrast agents. The use of ultrasound-enhanced gene delivery has potential for clinical use, because it allows safe and focused delivery of DNA to target tissue (26).

Another physical method is hydrodynamic delivery, which employs the force, generated by the rapid

injection of a large volume of DNA solution in the circulation to overcome the physical barriers of endothelium and cell membranes and enable gene delivery to parenchymal cells, e.g. liver or muscle cells (27, 28).

Electrogene therapy

Electroporation-based gene transfer *in vivo* or electrogene therapy involves injection of plasmid DNA into target tissue, followed by application of appropriate electric pulses, which facilitate transport of DNA molecules through the destabilized cell membrane into the cells (29).

In vivo gene delivery using electroporation was first performed in the 1990's (30) and since then a number of different types of tissue have been successfully transfected using this approach, for instance tumors (13, 24), skeletal muscle (16), skin (31, 32) and liver (33). Transfection efficiency of this method is still low compared to viral vectors (34); yet its advantages, mostly lack of pathogenicity and immunogenicity, make it promising new gene therapy technique which can in the future become well established in clinical work.

Potential for use of electrogene therapy in treatment of several different diseases, including muscle disorders, blood disorders, arthritis and cancer, was demonstrated in number of preclinical studies (23, 35).

Types of tissue, targeted for transfection with therapeutic genes in cancer electrogene therapy

Gene therapy in cancer patients can be instituted using two different approaches. The first one is *ex vivo* gene therapy, where cells are removed from patient, transfected *in vitro* with the plasmid or viral vector, selected, amplified, and then reinjected back into the patient. The other approach is *in vivo* gene therapy, where exogenous DNA is delivered directly into host's target tissue (e.g. tumor, peritumorally or into skeletal muscle) (11).

Among variety of tissues, which have already been successfully transfected using electrically-assisted plasmid DNA delivery, the most interesting target tissues for electrogene therapy in cancer patients, are tumor tissue and skeletal muscle.

Electrically-assisted gene delivery into tumors

Electrically-assisted delivery of therapeutic genes into tumors facilitates local intratumoral production of high concentrations of encoded proteins, which enables sufficient therapeutic concentrations without the need for systemic delivery of high concentrations of therapeutic genes or proteins. This is especially important in case of cytokines, where high systemic concentration is associated with severe toxicity (36). This approach can be used as a single therapy or in combination with other modalities for cancer treatment, for example electrochemotherapy.

The first evaluation of intratumoral electrogene therapy for cancer treatment was performed on murine melanoma tumor model in 1999 by Niu et al (37). Since then, a variety of therapeutic genes, mostly encoding cytokines, but also antiangiogenic genes, p53 gene, HSV-TK gene, etc, have been introduced to a number of animal tumor models, e.g. melanoma, squamous cell carcinoma and hepatocellular carcinoma (35).

Results of preclinical studies indicate, that electrically-assisted intratumoral delivery of therapeutic genes enables efficient transgene expression with sufficient production of therapeutic proteins, which can lead to pronounced antitumor effect on treated tumor (for example suppression of tumor growth, partial or complete reduction of tumor nodule) and even induces long-term antitumor immunity in treated animals (38, 39, 40).

Electrically-assisted gene delivery into skeletal muscle

Skeletal muscle is an attractive target tissue for delivery of therapeutic genes, since it is usually large mass of well vascularized and easily accessible tissue with high capacity for synthesis of proteins, which can be secreted either locally or systemically (41). Electrically-assisted gene delivery into skeletal muscle can be applied for therapy of different muscle diseases, for local secretion of angiogenic or neurotrophic factors or for systemic secretion of different therapeutic proteins, such as erythropoetin, coagulation factors, cytokines, monoclonal antibodies, etc. (16, 34, 42, 43).

The transfection efficiency of electrically-assisted gene delivery is the highest in skeletal muscle, compared to all other types of tissue (41). Electroporation significantly enhances expression of plasmid DNA, even up to 2000-times, and reduces variability of gene expression compared to application of plasmid DNA into skeletal muscle without electroporation (16, 44). Owing to the postmitotic status and slow

turnover of skeletal muscle fibers, which ensures that transfected DNA isn't readily lost, it is possible to achieve long-term expression of exogenous DNA, which can last up to 1 year (16, 41). This is due to the dynamics of naked DNA transfer, since plasmid does not integrate into genome of transfected cell and thus duration of exogenous DNA expression in part depends on lack of cell division. In contrast to muscle cells, in tissues, where cell turnover is much higher, plasmid DNA is rapidly lost from the cells (41).

It was established in different studies, that electrically-assisted gene delivery into skeletal muscle enables sufficient systemic expression of transgene products to ensure antitumor therapeutic effect. Therapeutic genes, which manifested encouraging antitumor effect after electrically-assisted delivery into skeletal muscle, are for example genes, encoding interleukin-12 (45), interleukin-24 (46), interferon-α (47) and different antiangiogenic factors (48).

Therapeutic genes used in electrogene therapy of cancer

A number of different therapeutic genes were employed in successful electroporation-mediated gene therapy of cancer in preclinical studies.

One of the major classes of genes of interest are immunostimulatory genes. The concept of stimulation of host's immune system to attack tumor cells has long been investigated as an alternative to conventional cancer therapies, since specificity of the immune system could provide means to target tumor cells while leaving normal cells intact (49). Unfortunately, many early attempts to employ immunotherapy for cancer treatment showed only modest benefits or were even highly toxic. Recently, gene therapy offered new possibilities to develop clinically applicable immunotherapy of cancer.

Some of the most significant clinical responses in cancer immunotherapy to date have been achieved with employment of active nonspecific immunotherapy, i.e. use of cytokines. However, wide-spread use of recombinant cytokines in clinical work is limited by short half-life of recombinant cytokine proteins. In order to obtain sufficient therapeutic effect, repetitive systemic applications of high dosages of cytokine proteins are required, which can lead to severe side effects (36, 49). Therefore new application strategies have been developed to improve therapeutic efficiency and alleviate side effects. One of such alternatives to application of recombinant cytokine proteins is immunogene therapy - transfer of genes, which encode production of different cytokines into target tissue, e.g. tumor or muscle. Electrically-assisted therapeutic gene delivery into tumor nodule has a direct therapeutic effect on tumor cells, since high concentrations of encoded proteins are produced locally in the tumor tissue. This local approach has an obvious disadvantage that it can not be employed for nodules, which are not easily accessible (e.g. nodules in internal organs) or are not visible (e.g. microresidues of tumor tissue after surgical removal or micrometastases). Delivery of therapeutic genes into skeletal muscle cells will have therapeutic effects on distal tissue targets, which can be both primary tumor nodules and metastases, via secretion of transgene products in the systemic circulation.

Electrogene therapy with genes, encoding different cytokines, has already shown promising results in preclinical trials on different animal tumor models. Cytokine genes, which showed the most potential for cancer therapy, are interleukin (IL)-2, IL-12, IL-18, interferon (IFN) α, and GM-CSF (23).

Currently, one of the hot topics in cancer immunotherapy is use of IL-12 (49), which plays important role in the induction of cellular immune response through stimulation of T-lymphocyte differentiation and production of IFN-γ and activation of natural killer cells (50). Antitumor effect of electrically-assisted delivery of gene, encoding IL-12, has already been established on various tumor models, e.g. melanoma, lymphoma, squamous cell carcinoma, urinary bladder carcinoma, mammary adenocarcinoma and hepatocellular carcinoma (45, 51, 52, 53, 54). Results of these preclinical studies show that beside regression of tumor at primary and distant sites, electrogene therapy with IL-12 also promotes induction of long-term antitumor memory and therapeutic immunity, suppresses metastatic spread and increases survival time of experimental animals (40, 45, 51, 52, 53, 54, 55).

Electrically-assisted gene delivery was also employed in suicide gene therapy of cancer. The concept of suicide gene therapy is intratumoral transfer of a prodrug-activating gene, which selectively (intratumorally) activates otherwise non-toxic drugs (6). The most often used strategy in suicide gene therapy is the delivery of gene, encoding herpes simplex virus thymidine kinase (HSV-TK) and prodrug ganciclovir (GCV) (56, 57). HSV-TK activates GCV, which blocks extensions of DNA strands, leading to cell death by apoptosis (56). Results of several studies show that electroporation-based HSV-TK/GCV gene therapy

may provide potentially effective gene therapy for cancer (57, 58, 59).

Another approach to antitumor therapy, which is currently being widely investigated, is based on inhibition of angiogenesis of tumor nodules. The basic concept of antiangiogenic gene therapy is transfection of cells with genes, encoding inhibitors of tumor angiogenesis, which prevent formation of new tumor vessels within growing tumor and thus block tumor growth or even lead to regression of tumors. Electrically-assisted delivery of genes, encoding antiangiogenic factors (angiostatin and endostanin) was demonstrated to be effective in inhibition of tumor growth and metastatic spread of different tumors (39, 60, 61).

Other gene therapy strategies, based on *in vivo* electroporation, which show potential for effective cancer treatment, include introduction of apoptosis inducing genes (62) and p53 gene (63).

Conclusions

The use of naked plasmid DNA as a DNA delivery system for *in vivo* gene therapy is an attractive alternative to viral gene delivery techniques due to its safety and simplicity. Relative poor efficiency of this gene transfer approach can be dramatically increased using *in vivo* electroporation.

Safety and efficiency of electroporation-based DNA delivery in treatment of cancer is already well established on preclinical level in numerous studies on different tumor models, which have made the potential of electrogene therapy in cancer quite clear. Even though research on this topic is still relatively new, the amount of gained knowledge already allowed electrically-assisted delivery of plasmid DNA intratumorally to reach clinical level. If future research continues to produce encouraging results, electrogene therapy will probably become promising alternative to other strategies of *in vivo* gene therapy for successful treatment of cancer patients.

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ELEKTROGENSKA TERAPIJA PRI ZDRAVLJENJU RAKA

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Povzetek: Genska terapija je nova oblika zdravljenja, ki je v zadnjih nekaj desetletjih deležna velike pozornosti raziskovalcev na področju onkologije, saj obljublja možnost učinkovite in visoko specifične terapije rakavih obolenj. Vektorje, s pomočjo katerih se v ciljno tkivo vnašajo terapevtski geni, delimo v dve glavni skupini, na virusne in nevirusne vektorske sisteme. Glavna prednost virusnih vektorjev je učinkovit vnos genskega materiala v celice, vendar pa je lahko njihova uporaba povezana s hudimi stranskimi učinki, kar vzbuja pomisleke glede varne klinične uporabe. Med nevirusne metode vnosa DNK v celice prištevamo vnos gole plazmidne DNK s pomočjo fizikalnih metod, kot sta na primer elektroporacija in genska puška, ter kemijski načini vnosa, na primer uporaba kationskih polimerov in lipidov. Te metode omogočajo varnejši, vendar manj učinkovit način vnosa DNK v primerjavi z virusnimi vektorji.
Elektroporacija je postopek, pri katerem z uporabo zunanjega električnega polja začasno povečamo prepustnost celične membrane in omogočimo vnos različnih vrst molekul v celice. Uporaba elektroporacije za izboljšanje prehoda DNK preko celične membrane intracelularno se imanuje električno posredovani vnos DNK ali elektrogenska terapija. Izvede se z injiciranjem plazmidne DNK v ciljno tkivo, ki mu sledi aplikacija ustreznih električnih pulzov. Pri elektrogenski terapiji raka se na ta način najpogosteje vnašajo terapevtski geni v tumorsko tkivo ali v skeletno mišičnino. Do sedaj je bila v številnih predkliničnih raziskavah ugotovljena protitumorska učinkovitost takega načina vnosa različnih terapevtskih genov, zlasti genov, ki nosijo zapis za citokine, pa tudi antiangiogene faktorje in gene, ki izzovejo apoptozo celic.

Ključne besede: novotvorbe – zdravljenje; elektroporacija; genska terapija – metode; zdravilo, sproščanje, sistemi

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Review Paper

VETERINARY PARASITICIDES – ARE THEY POSING AN ENVIRONMENTAL RISK?

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Summary: The environmental risks of pharmaceuticals have been studied less frequently in comparison to other chemicals such as pesticides and biocides. Nevertheless, during the last few years, veterinary and human medicinal products gained increasingly more attention.
Medicinal products for use in veterinary medicine include various groups of chemicals, used for a wide range of purposes for companion and farm animals. The parasiticides and antibiotics are two of the most important groups and as such used fairly often in animal treatment.
There are different entry routes of veterinary drugs into the environment. Manure of treated farm animals may contain significant amounts of the active ingredients or metabolites. They can be excreted from treated animals in agricultural soils directly (pasture) or with the application of manure as a fertiliser. The aquatic environment can also be one of exposure compartments.
In this review we will focus on certain veterinary parasiticides and give a few examples how they can be excreted into environment and what is their environmental persistency and toxicity to some aquatic and terrestrial organisms.

Key words: Environmental pollutants – toxicity; antiparasitic agents – pharmacokinetics; biodegradation; feces – chemistry; animals, domestic

Introduction

A general term, parasiticide, is used to describe a medicinal product that is effective in killing of different forms of parasites. It does not mean that the drug kills all parasite species, merely that it will kill at least one species of parasites. Most antiparasitic drugs are usually effective in killing several related species of parasites. Others, on the other hand, may have broad spectrum properties and they are effective against a wider range of parasites. In the early years of drug development, the compounds discovered were usually effective only against some of the parasites' species in one of the major groups such as the helminths. In this case, the drugs were collectively called antihelmintics. Similarly, those compounds active only against insects were called insecticides and those, effective only against ticks and mites (the acarina), were called acaricides.

More recently the avermectins and the milbemycins (collectively called the macrocyclic lactones) have been marketed as broad spectrum parasiticides with most of them having activity against nematodes, insects, mites and ticks. Therefore they can be classified as anthelmintics, insecticides and acaricides.

Veterinary parasiticides can be divided into a number of main classes, namely the ectoparasiticides, the endectocides, and the endoparasiticides (including anthelmintics and antiprotozoals). Generally speaking the ectoparasticides are antiparasitic agents used to control external parasites; endectocides are antiparasitic agents used to treat both internal and external parasites, whereas endoparasiticides are used to control internal parasites including gastrointestinal nematodes and lungworms. In human medicine prevalence of

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parasitic invasions is not as high as in veterinary medicine. It is known, that classes like the two major groups within the anthelmintics – avermectins and benzimidazoles were developed initially for veterinary use only, and since they are the most frequently used parsiticides they will be the main focus of this review.

The discovery of macrolide endectocides (avermectins e.g. ivermectin, abamectin, doramectin, milbemxcin, eprinomectin, selamectin) revolutionized the treatment and prevention of parasitic diseases. They are widely used because of their broad spectrum of activity against ecto- and endo-parasites, high efficiency and high safety margin. The most frequently used avermectins are ivermectin (introduced in mid-1980s as probably the most broad-spectrum anti-parasite medication ever), abamectin and doramectin.

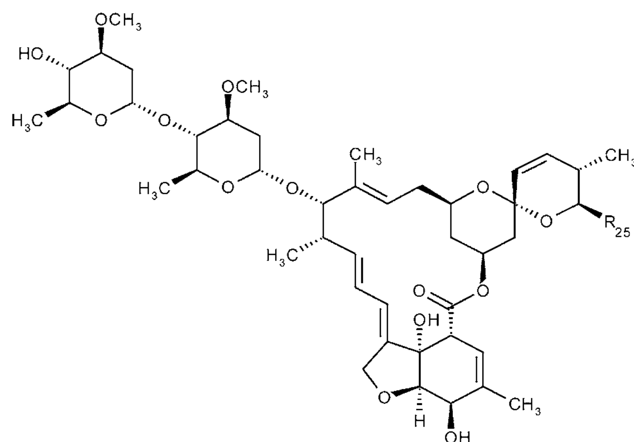
Benzimidazoles constitute one of the main groups of antihelmintics used clinically and they are the largest chemical family used to treat endoparasitic diseases in domestic animals (1). Those of current interest are mebendazole, fenbendazole, oxfendazole, oxibendazole, albendazole and triclabendazole (2). All compounds in this group have also a broad spectrum of activity, a wide safety margin and are often effective against adults, larvae, and eggs.

Pharmacological properties

Avermectins

Avermectins are insecticidal or anthelmintic compounds derived from the soil microorganism *Streptomyces avermitilis*. They belong to a group of chemicals called macrolactones (3). Chemical structure of avermectins is presented in Figure 1. Their mode of action includes strong chloride influx into nerve cells, which results in disruption of nerve impulses, blocks the channel causing nerve hyperexcitation and decreases nerve transmission. They are potent agonists at the $GABA_A$ (gama amino butyric acid) receptor but they also interact with GluCl (glutamate-gated chloride) channels in the nervous system of a parasite (e.g. arthropod, nematode). Visible activity, such as feeding and egg laying in parasites, stops shortly after exposure, though death may not occur for several days (4).

In veterinary medicine avermectins are frequently used as anthelmintics against internal and external parasites of cattle, pigs, and horses, sheep and goats as well as cats and dogs. The recommended dose of avermectins for all domestic animals is 200 µg/kg b.w. applied in injectable or oral form and 500 µg/kg b.w. in topical form of the drug (5-7). They are excreted mainly through faeces, with up to 98 % being excreted as the non-metabolised drug (8,9). Although, drug formulation, dosage and route of administration are the most important factors in determination of the elimination profile and persistence of faecal residues of avermectins, the majority of the administrated dose is usually excreted in first 10 days after application (10,11). Avermectins are highly insoluble in water and have a strong tendency to bind to faeces and soil particles. Faecal residues or metabolites of avermectin drugs might be highly toxic for non-target organisms living in soil (9). The disturbances that macrocylic lactones can produce on non-targeted invertebrates and on their associated participation in dung degradation and soil element recycling are unpredictable and can negatively affect biodiversity and the agricultural ecosystem sustainability (12). The combination of their physical/chemical properties (non-volatile, low water solubility, strong affinity for lipids and strong sorption to organic matter, soil and sediment) with the high excretion rate of the parent compound from treated animals has raised concerns that toxic levels of avermectins are entering and persisting in various environmental compartments. Consequently they may pose an ecotoxicological risk, especially during periods of their frequent use when large number of animals is treated on a limited area.



Abamectin: R_{25} = $CH(CH_3)CH_2CH_3$ and $CH(CH_3)_2$

Doramectin: R_{25} = Cyclohexyl

Ivermectin: R_{25} = $CH(CH_3)CH_2CH_3$ and $CH(CH_3)_2$; bond C-22 and C-23 not saturated

Figure 1: Chemical structure of avermectins

Benzimidazoles

The benzimidazoles bind to free β-tubulin, inhibiting its polymerisation and thus interfering with microtubule-dependent glucose uptake (13-15). Binding of benzimidazoles to β-tubulin is reversible and saturable. The depolymerization of microtubules damages the integrity and transport functions of cells within the parasite and thereby disturbs the parasitic energy metabolism. The antiparasitic effect is a lethal, but relatively tardy process. Because benzimidazoles progressively deplete energy reserves and inhibit excretion of waste products and protective factors from parasite cells, an important factor in efficacy of the benzimidazoles is prolongation of contact time between drug and parasite (1).

All benzimidazoles share the same central structure with 1, 2-diaminobenzene – e.g. thiabendazole. Other members of this group – albendazole, fenbenfazole, oxfendazole - have a substitution on carbon 5 of the benzene ring. They are slightly soluble in water (from to 40 10 ng/g). Chemical structure of benzimidazoles (thiabendazole) is presented in Figure 2.

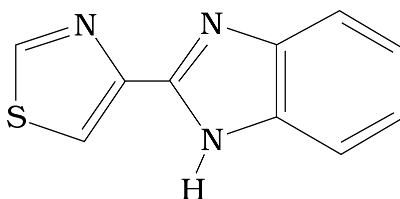


Figure 2: Chemical structure of benzimidazoles (thiabendazole)

The most effective benzimidazoles are less readily metabolized to inactive soluble products than earlier compounds, i.e., the kinetics of elimination is slower. After administration, anthelmintics are usually absorbed into the bloodstream and transported to different parts of the body, including the liver, where they are metabolized and eventually excreted in the faeces and urine. Following single oral administration benzimidazoles have relatively short time of elimination. The very low maximal concentrations in faeces were detected 36 h (thiabendazole), 96 h (albendazole) or 168 h (oxfendazole, febendazole) after treatment.

Environmental fate

Chemicals that come in contact with natural ecosystems will be distributed into different environmental compartments. To understand their potential environmental fate, it is first necessary to assess their probable concentration in these compartments. For pharmaceuticals, environmental concentrations depend initially on the route of drug administration, drug formulation and pharmacokinetics, the dose applied and the frequency of treatment as well as on the number and category of treated animals. Furthermore, the environmental risk assessment requires reliable information on their physical/chemical properties e.g. solubility, adsorption as well as information on their behaviour and persistence in the environment and ecotoxicology. Knowledge of all above mentioned points could enable us to evaluate environmental risk of pharmaceuticals use as well as to predict possible danger for animal and human health. In case of veterinary pharmaceuticals, pasture ecosystems have been of greatest concern.

While talking about the fate of pharmaceuticals in the environment a few very important terms have to be introduced. The K_{ow} or n-octanol - water partition coefficient of chemicals, is simply a measure of the hydrophobicity of an organic compound and it is commonly used as a good estimate of the potential bioaccumulation. Water solubility itself is of great importance for understanding the soil mobility of organic chemicals. On the other hand, the lipophilicity may be of great importance for potential accumulation of chemicals in living organisms (16).

A compound with a high K_{ow} is therefore considered relatively hydrophobic and would tend to have low water solubility, a large soil/sediment adsorption coefficient and a large bioconcentration factor. The K_{ow} of a compound can also be used to find the distribution coefficient (K_d) of a particular contaminant. It is a ratio between contaminant concentration in the solid phase (soil or sediment) and contaminant concentration in the liquid phase (pore water). Distribution coefficient is therefore a direct expression of the partitioning of substance between the aqueous and solid (soil or sediment) phase. For many soils and chemicals, the distribution or partition coefficient can be estimated using as the ratio between soil organic matter (mass of organic carbon per mass of soil). It is called organic carbon normalized sorption coefficient (K_{oc}). It is an indicator of mobility of pharmaceuticals in the environment. Substances with K_{oc}'s >1000 likely have low mobility. Values for macrocyclic lactones range from 3231 (eprinomectin) to 86900 (doramectin) and from 31500 to 50000 for fenbendazole (a ben-

zimidazole). There is no data available for imidazothiazoles, tetrahydropyrimidines, silicylanilides, or other benzimidazoles (17). The fact that the soil organic carbon content seems to be mainly responsible for the adsorption of at least non-polar organic chemicals led to the assumption that soil sorption processes are partitioning processes between water and lipophilic soil phase (16).

Physicochemical properties of some avermectins and some benzimidazoles are presented in Table 1.

Table 1: Physicochemical properties of some avermectins and some benzimidazoles

Pharmaceutical	Solubility in water	Koc	Kd	Kow	Ref
Abamectin	7.8 µg/l	5300-15700		9900	ARS Pesticide Properties Database (18,19,20)
Doramectin	25 µg/l	7520 silty loam 13330 clay loam 86900 silty clay loam	70.8 silty loam 23.4 clay loam 562 silty clay loam	9700	(21)
Ivermectin	4 mg/l	12600-15700		1651	(22)
Albendazole	0.53-0.59 mg/l	1862		1380	SRC PhysProp database, (23)
Mebendazole	71.3 mg/l	n.a.		239	SRC PhysProp database, (24)
Fenbendazole	10-30 ng/l	12022	630-1000	7079	SRC PhysProp database, (24)

Table 1 summarizes studies done investigating the sorption behaviour of some avermectins and benzimidazoles in soils. The available data indicate that compounds are highly sorbed to soils with organic carbon normalised sorption coefficients (K_{oc}) from 1862 to 86900. The fact that all values are greater than 1000 indicates that the substances are not particularly mobile in the environment. High K_{ow} values cause limited aqueous solubility of presented compounds. Therefore, when drug residues reach the environment they tend to be adsorbed on soil or sediment particles. Their degradation to less toxic and more water soluble degradation products are known. Degradation products of avermectins are more likely to leach from dung and, therefore, pose less risk to dung-dwelling organisms. But on the other hand, degradation products leached from dung and soil into surface and ground waters may pose greater risk to aquatic organisms (17). Avermectins e.g. ivermectin undergo rapid photodegradation as a thin, dry film on a glass with half-life (DT_{50}) of 3 h (25). The half-life for ivermectin photodegradation in the surface water is 12 h in summer and 39 h in winter (25). Halley et al. reported also that the degradation half-life of ivermectin in soil or faeces-soil mixture was in the range of 91 to 217 days in the winter and 7 to 14 days in the summer and that the degradation products are less toxic than ivermectin. A soil column leaching experiment performed by Halley et al. was proved that ivermectin is rather immobile in soil and not readily leached through soil into ground water (25). The consequence of that could be its accumulation in soil. McKellar also reported (26) that no apparent degradation of ivermectin residues in faeces of cattle treated with pour-on or subcutaneous preparations over a 45-day time period was observed. Similar results were obtained by Sommer and Steffansen in Danish and Tanzanian weather conditions where photodegradation had minimal effect (27). Fisher and Mrozik (28) also reported that the half-life of abamectin degradation in sandy loam, clay and sand soil ranged from 20 - 47 days and 13 degradates were identified. No degradation was observed in sterile soil which indicates that soil organisms are responsible for degradation. Taylor (29) reported the same behaviour in soil for doramectin as it is known for ivermectin and abamectin. Sorption properties have been examined in three natural soil types with a variety of proportions of sand and clay. Depending on soil type, the aerobic degradation of doramectin was observed. The half-life was 61 - 79 days in the dark at 22 °C (29). Kolar et al. established DT_{50} value of 23 and 22 days for dissipation of abamectin and doramectin from sheep faeces under the field conditions in the pasture, respectively (11).

Benzimodazoles are slightly soluble in water and as avermectins they have high tendency to bind soil

and organic matter. There are limited data on the degradation of benzimidazoles. Nevertheless, some investigations on the biodegradability of febendazole indicate that it is degraded slowly (30). Persistence in soil or faeces is not known.

Ecotoxicology

Ecotoxicology is the study of harmful effects of chemicals on ecosystems (31). The main theme of (eco)toxicology is the relationship between the quantity of chemical to which an organism is exposed and the nature and degree of consequent harmful and toxic effects. The toxicity is usually evaluated using dose-response relationships and also enables basis for assessment of hazards and risks posed by environmental contaminants. The usual parameters used while assessing ecotoxicity of certain chemical are lethal concentration (LC_{50}) and effective concentration (EC_{50}). Lethal toxicity testing represents the median lethal concentration, whereas effective concentration investigates adverse response other than death. Both measurements are carried out on 50% of the population. Ecotoxicological studies are very important and essential also for the assessment of environmental effects of veterinary drugs. There are several organisms involved in toxicity testing: terrestrial organisms (invertebrates; soil dwelling organisms), vertebrates (birds, mammals, reptiles, amphibians), aquatic organisms (water fleas; fish), plants (algae).

Also for avermectins, it is very important to predict the environmental risk of their possible non-controlled and irregular use. Accordingly, an increase in knowledge about the elimination profile from treated animals, the rate of degradation and the distribution of avermectins in the environment, especially in pastures, is needed. Their residues in faeces of treated animals and in soil have toxic effects on some dung-associated insects, especially their larval forms (32-34), beetles (34-36), faeces- and soil-invertebrates (37) and some other decomposer organisms in temperate climate pasture (12, 18, 38, 39). They show effects on reproduction, biological function and survival of non-target aquatic and terrestrial organisms which have an important role in the food web (40, 41). Interruption of the food web may affect the diversity of a system or may influence the relative importance of one species assemblage over another (40). In this case, avermectins might influence the food web, due to their known effects on the species involved in faeces decomposition which are also a part of the food chain (41). Avermectins are toxic also to avians e.g. abamectin dietary LC_{50} values for bobwhite quail and mallard duck of 3102 mg/kg and 383 mg/kg was established, respectively (18, 20). The most sensitive organisms to avermectins are some freshwater organisms, such as *Daphnia magna* and fish (e.g. rainbow trout) (18).

There are not that much data available for benzimidazoles, a few are mentioned in the Table 2, which gives a brief overview of the data listed in the literature. They are presented systematically, including results of toxicity testing of dung-dwelling organisms, which are one of the major concerns especially in regard of using avermectins.

The toxicity data obtained by the testing procedures are eventually used to make assessments of hazard (the potential to cause harm) and risk (the probability that harm will occur). To asses risk, you must know the toxicity of the compound in question (expressed as LC_{50}, EC_{50}, or NOEC (non observed effective concentration) values) and the anticipated exposure of the organism to the toxic compound (31). The predicted environmental concentration (PEC) and the predicted environmental no-effect concentration (PNEC) can be calculated and the risk is expressed as a risk quotient: PEC/PNEC = risk quotient. In the case of PEC, calculations are based on known rates of release and dilution factors in the environment. For the environmental release scenarios the important measures in regard of PEC are – the use and consumption, interval of medicinal treatment, the metabolic rate, the agricultural practise when collecting, storing and applying manure/slurry on the field as well as exposed area. The PEC in manure for instance, is ratio between total dose administered (mg/animal/day) multiplied by number of treatment days and divided by the total amount of manure produced during manure production period. Such examples serve mainly for studies of environmental fate and are especially important for pharmaceuticals which are excreted in urine or manure (57).

For example reported predicted environmental concentration (PEC) for doramectin at worst-case scenario (one treatment of a feedlot bovine animal, all dose excreted in first 14 days via faeces, no degradation, runoff is one-third of rainfall) ranging from 0.011 μg/L in surface runoff to 18 μg/L in wet feedlot waste (45). Reported concentrations could not pose high harmful effects on terrestrial organisms comparing to toxicity data presented in Table 2. On the other hand the risk of avermectins is higher for aquatic and dung-dwelling organisms responsible

Table 2: Toxicity data of some avermectins and some bezimidazoles to different non-target organisms (only some examples are shown)

Test organism	Species	Pharmaceutical	Toxicity data		Ref.
Aquatic organisms					
Fish	*Salmo gairdneri* (rainbow trout)	Ivermectin	LC_{50} = 3.0 mg/l	96 hours	(28)
	Oncorhynchus mykiss (rainbow trout)	Abamectin	LC_{50} = 3.2 µg/l LC_{50} = 1.5 µg/l	96 hours 96 hours	(20) (42)
	Salmo gairdneri	Abamectin	LC_{50} = 3.2 µg/l	48 hours	(18)
	Salmo gairdneri	Fenbendazole	LC_{50} = 40 µg/l	96 hours	(30)
	Lepomis macrochines (bluegill sunfish)	Ivermectin	LC_{50} = 4.8 mg/l NOEC = 0.9 mg/l	96 hours	(26)
	Lepomis macrochines (bluegill sunfish)	Abamectin	LC_{50} = 9.6 µg/l	96 hours	(20)
	Lepomis macrochines	Abamectin	LC_{50} = 9.6 µg/l	48 hours	(18)
	Cyprinodon variegatus (sheepshead minnow)	Abamectin	LC_{50} = 15 µg/l	96 hours	(20)
	Ictalurus punctatus (channel catfish)	Abamectin	LC_{50} = 24 µg/l	96 hours	(20)
	Cyprinus carpio (carp)	Abamectin	LC_{50} = 24 µg/l	96 hours	(20)
	Cyprinus sp. (carp)	Abamectin	LC_{50} = 42 µg/l	96 hours	(43)
	Lepomis macrochirus	Oxfendazole	LC_{50} > 2.7 mg/l	96 hours, 2.7 mg/l was the highest tested conc.	(44)
Crustaceans					
	Daphnia magna (water flea)	Ivermectin	EC_{50} = 0.025 ng/g NOEL = 0.01 ng/g	48 hours	(18)
	Daphnia magna	Abamectin	EC_{50} = 0.34 µg/l	48 hours	(18)
	Daphnia magna	Doramectin	EC_{50} = 0.001 mg/l	48 hours	(45)
	Gemmarus duebeni and G. zaddachi (amphipoda)	Ivermectin	LC_{50} = 0.033 µg/l	96 hours	(43)
	Daphnia magna	Fenbendazole	LC_{50} = 12 µg/l	48 hours	(30)
	Daphnia magna	Oxfendazole	LC_{50} = 52 µg/l	48 hours	(46)
Others					
	Panaeus duorarum (pink shrimp)	Abamectin	LC_{50} = 1.6 µg/l	96 hours	(20)
	Msyidopsis bahia (mysid shrimp)	Abamectin	LC_{50} = 0.022 µg/l	96 hours	(20)
	Crassostrea virginica (eastern oysters)	Abamectin	LC_{50} = 430 µg/l	96 hours was observed at the embryo-larval stage of the life	(20)
	Callinectes sapidus (blue carb)	Abamectin	LC_{50} = 153 µg/l	96 hours	(20)
Soil-dwelling organisms					
Bacteria	8 different genera of Eubacteria	Oxfendazole	No effect found on replication or growth at maximum solubility, 9 ng/g		(46)

Test organism	Species	Pharmaceutical	Toxicity data		Ref.
Fungi	5 different genera of Fungi	Oxfendazole	No effect found on growth at maximum solubility, 9 ng/g		(46)
Springtails	*Folsomia fimetaria*	Ivermectin	NOEC = 0.3 mg/kg EC_{10} = 0.26 mg/kg EC_{50} = 1,7 mg/kg LC_{50} = 8.4 mg/kg		(47)
Worms	*Enchytraeus crypticus* (potworm)	Ivermectin	NOEC = 3mg/g EC_{10} = 14 mg/kg EC_{50} = 36 mg/kg		(47)
	Esienia foetida	Ivermectin	LC_{50} = 315 ng/g	28 days	(43)
	Lumbricus terrestris	Ivermectin	No effect on survival and growth	24 weeks	(43)
	Earthworm	Ivermectin	LC_{50} = 15.7 mg/kg NOEC (repro) = 4.7 mg/kg LC_{50} = 18-100 mg/kg	 28 days	(47) (28)
	Earthworm	Fenbendazole	NOEC = 56 mg/kg LOEC = 120 mg/kg LC_{50} = 180 mg/kg	28 days	(30)
	Earthworm	Oxfendazole	No effect found at highest experiment concentration, 971 mg/kg soil	28 days	(46)
Dung-dwelling organisms					
Dung beetles	*Onthophagus binodis*	Abamectin	Not affected in dung of treated cattle		(48)
	Onthophagus binodis	Fenbendazole	NOEC = 770 ng/g LC_{50} >770 ng/g	7 days	(30)
	Onthophagus gazella (immature)	Doramectin	LC_{50} =0.0125 mg/kg LC_{90} = 0.0382 mg/kg NOEC =>0.25 mg/kg	Effect endpoint used: number of brood balls	(45)
	Onthophagus gazella (immature)	Fenbendazole	LC_{50}= > 770 µg/g NOEC = > 770 µg/g	Amounts in spiked dung used as diet, 7 d study	(30)
	Onthophagus gazella	Ivermectin	17 days 21 days	Sensitivity of coleopteran larvae, indicated by days post-treatment until adult emergence from dung equalled that of control	(49) (50)
	Onthophagus taurus	Ivermectin	15 days	% dung pat dispersal, number of beetles/pat; reductions on days 7 and 10 after treatment	(43)
Flies	*Musca vetuistissima* (bushfly)	Avermectin B1	No bush flies survived from eggs to adult following cattle injection of 200 µg/kg		(33)
	Musca domestica (house fly)	Ivermectin	30 days	Increased mortality for 20 days	(43)

Test organism	Species	Pharmaceutical	Toxicity data		Ref.
	Haematobia irritans (horn fly)	Ivermectin	LC_{50} = 0.032 – 0.061 μg/g LC_{50} = 0.0032 – 0.0066 μg/g	Amounts in blood, 48h mortality 88 h mortality	(51) (52)
	Haematobia irritans	Doramectin	LC90(larvae) = 0.003 mg/kg NOEC = 0.0024 mg/kg	Amounts in spiked cattle dung, effects on larvae development/emergence	(45)
	Musca autumnalis (autumn house-fly)	Ivermectin	14 days	Sensitivity of dipteran larvae, indicated by days post-treatment until adult emergence from dung equalled that of control	(53)
	Neomyia cornicina (dung fly)	Ivermectin	32 days 17 days	Sensitivity of dipteran larvae, indicated by days post-treatment until adult emergence from dung equalled that of control	(54) (55)
	Stomoxys calcitrans (stable fly)	Ivermectin	14 days	Sensitivity of dipteran larvae, indicated by days post-treatment until adult emergence from dung equalled that of control	(56)
	Scatophaga scercoraria (yellow dung fly)	Ivermectin	EC_{50} = 0.051 μg/g EC_{50} = 0.036 μg/g EC_{50} = 0.015 μg/g EC_{50} = 0.001 μg/g	24 h mortality 48 h mortality Pupariation prevented Emergence prevented (cm) all amounts in spiked cattle dung	(33)
	Scatophaga stercoraria	Ivermectin	EC_{50} = 0.051 μg/g	24 hours mortality	(43)

for dung degradation. Nevertheless, they could still pose a risk to aquatic as well as terrestrial environment, especially during periods of their frequent use in large number of animals. Climate conditions and type of soil have to be considered also.

Studies on benzimidazoles are limited, but suggest that these class of compounds are generally not toxic even to dung-dwelling organisms (17).

Results from our studies on avermectins

Although there are several reports on the environmental effects and fate of avermectins, disagreement between scientists still exists about their possible environmental impact (58, 59, 60). McKellar (26) summarized that the contributory factors to the environmental impacts of avermectin residues are the activity of excreted avermectins or their metabolites on non-target fauna, the amount and temporal nature of excretion and the stability and persistence of avermectin residues in the environment as well as environmental influences on the processes of physical degradation of excreta (e.g. sunlight, temperature, rainfall and mechanical disruption).

The aim of our work was to evaluate the possible risk of avermectin (abamectin, doramectin) use in pastured sheep. First we developed a sensitive and selective analytical tool for determination of

avermectins in sheep faeces and in soil (61), that enabled us to determine time profile of elimination of both avermectins via faeces after sheep treatment with a single subcutaneous dose of 200 μg/kg b.w. The maximal abamectin concentration in sheep faeces (1277 ng/g dry faeces) was detected on day 3 after treatment, while maximal concentration of doramectin was detected on days 2 and 5 after treatment (2186 and 1780 ng/g dry faeces, respectively). Both avermectins were excreted approximately at the same rate (k was 0.23 d^{-1} for abamectin and 0.19 d^{-1} for doramectin). The majority of both avermectins was excreted in 10 days after treatment (11).

In addition, some experiments were also performed on sheep pasture. We studied degradation time profile of both avermectins in sheep faeces and in soil under environmental conditions. Environmentally important parameters – e.g. samples moisture, temperature and weather conditions were recorded during the experiments. A rapid loss of abamectin and doramectin from sheep faeces was observed during the first 32 days. After that, concentrations of abamectin and doramectin remained constant at approximately 77 ng/g and 300 ng/g, respectively. The DT_{50} for abamectin and doramectin dissipation from sheep faeces were 23 and 22 days, respectively (16). Dissipation of both avermectins was strongly correlated with moisture content in faeces. Due to low contamination of soils, dissipation of avermectins in soil was not significant (62).

We have studied dissipation also under laboratory conditions, where results showed that abamectin and doramectin in homogenized, contaminated sheep faeces were evidently degraded under the UV light at the wavelength of 370 nm; DT_{50} of less than one day was established for abamectin and 4 days for doramectin (63).

For evaluation of possible toxicity of both avermectins to aquatic and soil-dwelling organisms some experiments were also performed. Namely, Halley et al. (18) reported high toxicity of avermectins to some freshwater organisms, such as *Daphnia magna* and fish (e.g. rainbow trout). Our investigations on toxicity of avermectins to the same and some other water organisms (*Daphnia magna*, rainbow trout, zebrafish, green unicellular algae and bacteria) and some soil-dwelling organisms (*Folsomia candida*, *Enchytraeus crypticus* and *Porcelio scaber*) confirmed high toxicity of both avermectins. Toxic effect was observed in all investigated water organisms (concentration ranges in ng/kg and μg/kg) (unpublished data) and in soil-dwelling organisms (concentration ranges in mg/kg) (unpublished data). Results show extremely high toxicity of abamectin to daphnids since the concentration 0.0094 μg/l still caused mortality and inhibited the reproduction of daphnids. The NOEC was detected at 0.0047 μg/l of abamectin and the LOEC was 0.0094 μg/L (unpublished data).

Based on gained experimental results we partially assessed possible environmental risk related to the use of avermectins in sheep grazing in Slovenian Karst. We estimated PEC of abamectin and doramectin according to the experimental data obtained in our experiments with time profile of excretion and degradation of avermectins in sheep faeces after single subcutaneous administration of 200 μg/kg body weight for both substances (unpublished data). The worst case scenario was used for calculations (30 sheep kept at limited area of 800 m^2 for 9 days; excretion of the total dose given during that time; no degradation of avermectins in faeces; entire average monthly rainfall occurring on day 9; runoff is half of the rainfall). Calculated PECs as well as experimental data were compared to toxic concentrations of both substances for tested aquatic and soil-dwelling organisms. From results we may conclude that the detrimental environmental effect of tested avermectins for soil-dwelling and aquatic organisms after their single administration to sheep is unlikely to occur (unpublished data). But additional experiments are needed for environmental assessment after repeated applications in sheep grazing in karst region.

Conclusions

The fate and behaviour of pharmaceuticals in the environment have been studied for several decades (64, 65, 66). More recently several reviews on use, emission, fate, occurrences and effects of pharmaceuticals have been published (16, 67-72). The environmental risk of the use of medicinal products is currently assessed at their registration procedure, but the methodology has not been finalised yet (73-75). In the thesis of Montforts from 2005 (76), an in-depth study was made about European legislation and guidance documents for the risk assessment and this work is a very good starting point for more precise insights in the field of pharmaceuticals in the environment.

There are still a lot of opened issues related manly on frequent and repeated applications of veterinary drugs. In addition different environmental conditions (e.g. climate conditions, soil type) should

be considered as well. Large-scale, long-term and multidisciplinary field studies are needed to monitor the effects of fecal residues on dung degradation and pasture productivity. Systematic studies would enable us to develop modelling approach, mainly focused on prediction.

In future we will continue with our studies which in order to understand how certain veterinary medicinal products reach the environment – determination of time profile of elimination and process afterwards – degradation pathways, persistence and toxicity to non-target species in the environment.

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PROTIZAJEDAVSKA ZDRAVILA ZA UPORABO V VETERINARSKI MEDICINI – ALI PREDSTAVLJAJO TVEGANJE ZA OKOLJE?

L. Kolar, N. Kožuh Eržen

Povzetek: Študije, ki bi preučevale, ali zdravila predstavljajo tveganje za okolje, so v primerjavi s tovrstnimi študijami pesticidov in biocidov redke. Počasi pa se to razmerje popravlja, tako da so v zadnjih nekaj letih začeli aktivno raziskovati tudi uporabo humanih in veterinarskih zdravil in njihovih možnih vplivov na okolje.

Zdravila za uporabo v veterinarski medicini predstavljajo zelo raznoliko skupino, ki se uporablja za različne namene, tako pri ljubiteljskih kot farmskih vrstah živalih. Protizajedavska zdravila in antibiotiki spadajo v najpomembnejši skupini in so tudi najpogosteje uporabljani pri zdravljenju živali.

Zdravila za uporabo v veterinarski medicini pridejo v okolje na različne načine. Eden od možnih načinov je z blatom, ki ga izločijo zdravljene živali neposredno na pašne površine ali posredno s sredstvi za gnojenje, kot je gnojevka. Tudi vodni ekosistemi so pogosto izpostavljeni tovrstnim vplivom in so pomemben pokazatelj kontaminacije.

V prispevku bomo predstavili nekatera protizajedavska zdravila in podali nekaj primerov, kako le-ta vstopajo v okolje, kako so v njem obstojna in kakšna je njihova toksičnost za nekatere vodne in zemeljske organizme.

Ključne besede: okolje, onesnaževalci – toksičnost; protiparazitarna sredstva – farmakokinetika; biodegradacija; zdravila, ostanki – analize; feces – analize; živali, domače

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Original Research Paper

EFFECTS OF PROCESSING PROCEDURES AFTER FLOW SORTING TO SEX BOVINE SPERMATOZOA AND CRYOPRESERVATION ON SPERM QUALITY AND FERTILITY

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Summary: The objectives of the study were to analyse the effects of semen processing after flow-cytometrical sorting and subsequent freezing in liquid nitrogen on the quality and fertilizing capacity of frozen/thawed spermatozoa. Quality of the frozen/thawed semen was evaluated by motility estimation, morphology analysis, membrane stability (6-CFDA/PI) test, capacitation test (FITC-PNA/PI staining with addition of L-α-Lysophosphatidylcholine) and fertility assessment in an insemination trial on a farm. Prolonged high dilution of flowcytometrically sorted spermatozoa before freezing had a significant negative effect on motility ($P<0.001$) and capacitation status ($P<0.05$). Positive effects on membrane stability were seen when glycerol was added at 5 °C shortly before straws were frozen compared to a system where glycerol was added before the equilibration process at room temperature ($P<0.001$). Independently of sperm processing after sorting, sexed spermatozoa had significantly more damaged acrosomes and morphological abnormalities ($P<0.001$). In addition sex sorted frozen/thawed spermatozoa (immediate centrifugation and glycerol addition at 5 °C) and unsorted frozen/thawed semen samples were submitted to a thermo-resistance test at 37 °C. Immediately after thawing no significant difference was seen in the percentage of motile spermatozoa between sorted and unsorted semen samples. However, after further incubation at 37 °C, motility of sorted spermatozoa was significantly lower than unsorted spermatozoa after 3 h ($P<0.001$) and 6 h ($P<0.05$). The pregnancy rates after insemination with sex-sorted and unsorted spermatozoa from the same ejaculates was tested in a field trial in heifers and cows with natural and synchronised oestrus cycles. In natural oestrus, more animals became pregnant after artificial insemination with unsorted than with sex-sorted spermatozoa (56.5 % vs. 17.6 %; $P<0.001$) No significant differences were observed between unsorted and sex-sorted frozen/thawed semen samples after artificial insemination of the animals with synchronised oestrus (36.4 % vs. 21.3 %; $P=0.076$). There was a significant effect of the bull on the pregnancy rates after artificial insemination with sex-sorted spermatozoa ($P<0.05$).

Key words: semen - analysis; spermatozoa - physiology - ultrastructure; quality control; semen preservation; insemination, artificial - veterinary - methods; pregnancy, animal; pregnancy rate; cattle - male

Introduction

Flowcytometrically sorted frozen/thawed spermatozoa have been successfully applied to artificial insemination in cattle (1). However, semen quality and pregnancy rates after artificial insemination were rather low compared to unsorted frozen/thawed semen (2-4). Main sources that may reduce the semen quality during flowcytomerical sorting are:

1. UV-Laser (5;6),
2. Pressure (7),
3. Dilution effects (8) and
4. Electric charge/electro-static field.

The dye itself at concentrations used for sorting does not have a significant impact on fertility of boar semen (9). Exposure to laser light accelerated Ca^{2+} transport into irradiated bull spermatozoa (10), enhanced Ca^{2+} binding to plasma membranes and

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inhibited Ca^{2+} uptake by mitochondria (5). Because of these insults the processing of sorted spermatozoa before freezing needs to be adjusted in order to obtain high quality and fertility rates after insemination.

Additionally, spermatozoa are highly diluted during sorting ($8x10^5$ spermatozoa/1 mL) by the sheath fluid to approximately one million spermatozoa/mL in the collection tube. High dilution was found to be detrimental for sperm motility and viability and could also affect the fertilizing capacity of spermatozoa (11, 12). For further processing, sexed sperm samples have to be centrifuged. Centrifugation however, increases the production of reactive oxygen species, which are detrimental for the sperm viability (13). As sorted spermatozoa are sensitive to membrane insults that may occur during centrifugation and storage at room temperatures, one objective of the present study was to test whether it is better to keep the highly diluted sorted spermatozoa at room temperature for a few hours and centrifuge all sorted samples together after 4 to 6 h or to perform centrifugation immediately after sorting, chilling and freeze each sample separately.

Centrifuged spermatozoa need to be diluted in a suitable extender, cooled to 5 °C and frozen in liquid nitrogen. The semen extender has to provide a suitable environment for survival and maintaining the viability of spermatozoa. The composition of semen extenders is mainly based on an energy resource (sugars such as glucose, fructose, lactose) and a buffer medium of different inorganic or organic salts. Additionally, the semen extenders contain components such as egg yolk, skim milk, specific amino acid, glycerol, detergent and antioxidants, which protect the spermatozoa (14). Egg yolk is an important component of semen extenders. The protective action of egg yolk is assumed to be due to membrane protection by low density lipoproteines (15), and the antioxidant ability of the phosphoprotein Phosvitin, which serves as an iron-carrier and protects spermatozoa against the Fenton reaction (16). Recently, egg-yolk of freezing extender for bull semen has been successfully replaced with soybean extract (17), but Tris egg-yolk freezing extenders are still preferred for freezing sorted bull semen (18). Glycerol is a cryoprotector, which is toxic to spermatozoa. Its toxicity is temperature dependent as seen especially in human semen (19). Some authors suggest the addition of glycerol after cooling the semen to 5 °C (14), whereas others prefer room temperature (20, 21). Therefore the second goal of our research was to determine the effect of glycerol addition at different temperatures.

Material and methods

Materials

L-α-Lysophosphatidylcholine (L 5004); Lectin, FITC labelled from Arachis hypogaea (peanut) (L7381); Propidium iodide (P4170) and Bisbenzemide H 33342 (B2261 were purchased from Sigma-Aldrich (Taufkirchen, Germany); all the other chemicals, if not specially stated, were purchased from Carl Roth GmbH + Co (Karlsruhe, Germany).

Composition of solutions

Tris-sample fluid: 200.0 mM Tris-hydroxymethyl-aminomethan, 64.7 mM citric acid monohydrate, 95.5mM D-fructose and 50mg Gentamicin sulfate diluted in double distilled water.

Hoechst 33342 working solution: 8.9 mM of 2-(-4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5-bi-1H-benzimidazole in double distilled water.

Tris-sheath fluid: 197.1 mM Tris-hydroxymethyl-aminomethane, 55.3 mM citric acid monohydrate, 47.5 mM D-fructose, 0.058 mg Penicillin G and 0.050 mg Streptomycin sulfate diluted in 5 L double distilled water.

Hancock solution: 2.784 g Tri-sodiumcitrate-dihydrate, 4 mL 37 % formaldehyde solution and double distilled water to 100 mL.

Tris egg-yolk freezing extender I: mixture of 67.2 mL stock solution (297.6 mM Tris-hydroxymethyl-aminomethan, 96.3 mM Citric acid monohydrate, 82.6 mM D-fructose, 0.606 g Penicillin and 1.480 g Streptomycin sulfate per 1 L double distilled water.), 20.0 mL egg-yolk and 12.8 mL double distilled water.

Tris egg-yolk freezing extender II: the same as extender I, except that 12.8 mL double distilled water was replaced with 12.8 mL of 87 % glycerol.

Processing of semen

Semen was collected twice per week from two fertility proven Holstein Friesian bulls with an artificial vagina and kept in a water bath at 27 °C. Motility was estimated under a phase contrast microscope at 37 °C, and only ejaculates with >70 % total motility were used in the experiment. Concentration of ejaculated spermatozoa was determined with a

Thoma counting Chamber (Thoma neu®, Hecht, sontheim, Germany). One part of the raw semen was diluted with Tris-sample fluid to $1x10^8$ spermatozoa per millilitre and divided into three subsamples. Diluted samples were labelled with 15, 20 and 25 µL of 8.12 mM Hoechst 33342 solution and incubated at 37 °C for 1.5 h. The labelled samples were pre-tested with a flowcytometer, and the concentration of Hoechst 33342 stain giving the best resolution of the two sperm populations was used for daily sort. Labelled and incubated semen samples were kept at 22 °C in the dark and sorted within 7 h after onset of incubation. The remaining unlabelled part of the ejaculates was frozen according to a standard protocol described by Thun et al. (17). All semen samples were gradually diluted (by dripping) the same amount of TRIS egg-yolk freezing extender I and II, giving 6.4% final concentration of glycerol. Extender II was added to the semen, depending on the experimental design, at room temperature or at 5 °C. Final sperm concentration was set to $13.2x10^6$ spermatozoa/ml or $3.3x10^6$ spermatozoa/straw. Diluted semen was then filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany), cooled to 5 °C within 2 h and frozen in liquid nitrogen. Freezing of the samples was performed in closed Styrofoam box (30 cm x 40 cm x 85 cm = high x bright x length). Briefly, straws were placed on metal holder in nitrogen vapour 3-5 cm above LN2 for 15 min. Frozen samples were than plunged into LN_2 and kept in the semen container (in LN_2) until used for analysis or artificial insemination.

Sperm Sorting

Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (22). Semen samples, prepared as described above, were filtered through a 51 µm Cell Strainer grid (Falcon Becton Dickinson and Company, Franklin Lakes, NY, USA) and then supplemented with 1 µL food dye solution FD&C#40 (Warner Jekinson Company Inc. St. Louis, MO USA). Sorting was performed with a high speed flowcytometer MoFlo SX, (Dakocytomation Fort Collins, CO, USA, equipped with an argon UV-Laser (Coherent Laser®, Inova I 909-6, Dieburg, Germany), set to 200 mW output. Samples were sorted in the presence of Tris-sheath fluid at an average event rate of 25000 cells/sec giving a sorting rate of 3300 cells/sec. Spermatozoa were collected into 10 mL conical plastic tubes (Greiner, Nürtingen, Germany) pre-filled with 500 µL TEST-yolk extender (23). After collection of 8 millions sorted spermatozoa in each collection tube, samples were centrifuged at 840xg for 20 min. The supernatant was discarded and the sperm pellet was resuspended with TRIS egg-yolk freezing extender I and II, then filled into straws and frozen in liquid nitrogen. Time of centrifugation and the temperature of glycerol addition were adjusted according to the experimental design.

Experimental design

Tubes containing sorted spermatozoa were submitted to three different protocols. Sorted sperm samples of the first group (group A) were kept at room temperature until the end of daily sorting (4-6 h after onset of sorting). Centrifugation was performed at the end of this holding period. The sperm pellet was gradually diluted with TRIS freezing extender I and II to a concentration of $20.5x10^6$ spermatozoa/mL. Plastic straws (Fine pailette, 0.25 mL, Minitüb, Tiefenbach, Germany) were filled with 3.3 millions spermatozoa and cooled to 5 °C over a 2 h period. Freezing in the vapour of LN_2 was performed approximately 4 h after glycerol addition (Table 1).

Sorted semen samples in the second group (group B) were processed similarly to samples of group A, except that centrifugation was performed immediately after collection tubes were filled with 8x106 spermatozoa. The difference between group B and C was the addition of TRIS freezing extender II at 5 °C, 45 min before freezing in LN_2 (Table 1). Control samples of unsorted semen were frozen as described for group A.

Table 1: Processing of the sorted spermatozoa

	Centrifugation	Glycerol addition
Group A	4-6 h after onset of sorting	Room temperature
Group B	Immediately after sorting	Room temperature
Group C	Immediately after sorting	5 °C
Control	Unsorted	Room temperature

All sorted semen samples and unsorted controls from one sorting day were frozen at the same time according to the previously described experimental design. Frozen samples were kept in LN_2 and analysed or used for insemination within 2-5 months after freezing. The straws were thawed at 37 °C for 17 sec.

Analysis of frozen thawed samples

Reanalysis of sorting purity

Reanalysis to identify sort purity was performed after thawing. Aliquots of 1 million spermatozoa were taken and diluted to 1 mL with TRIS-sample fluid, supplemented with 20 µl of a 0.8 mM solution of Hoechst 33342 and incubated for 20 min at 37 °C. Thereafter, samples were sonicated and filtered as described before (24). Reanalysis was performed at 60 events/sec. The analysis of purity was performed by a curve fitting model.

Motility analysis

Motility of raw semen samples as well as frozen/thawed semen samples was analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) equipped with heating plate at 37 °C. Two drops and at least three fields per drop of each sample were evaluated at 100x magnification.

Analysis of sperm morphology

Morphological abnormalities (MAS) and acrosome integrity were analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) at 1000x magnification after fixation in Hancock's solution. At least 200 spermatozoa were examined per sample. Abnormalities of spermatozoa were divided into damaged acrosomes and morphological abnormal spermatozoa.

Viability and membrane stability of spermatozoa (CFDA/PI)

Samples were prepared by mixing 3.3 µL CFDA (51.04 mM 6-carboxyfluorescein diacetate diluted in dimethyl sulfoxide) and 6.6 µL propidium iodide solution (PI) (mixture of 200 µL of 7.48 mM 3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 400 µL fixative solution: 68 µL of a 37 % formaldehyde solution per 10 mL distilled water) with 10 µL semen sample. Samples were incubated at room temperature in darkness for 10 min. From each sample two drops and at least 200 spermatozoa were analysed under a fluorescence microscope (Olympus BX 60; U-MNIB filter, Hamburg, Germany) at 400 x magnification. Spermatozoa were divided into two groups: viable spermatozoa with intact plasma membrane (CFDA positive and PI negative) and morbid spermatozoa (CFDA positive and PI positive).

Capacitation status of the spermatozoa (FITC-PNA/PI; LPC)

Capacitation status of spermatozoa was assessed with a modified protocol as described by Mcnutt and Killian (25). One Eppendorf cup was filled with 1 mL TRIS-sample fluid and another with 800 µL TRIS-sample fluid and 200 µL LPC solution (500µg L-α-Lysophosphatidylcholine and 1 ml Tris-sample fluid). Tubes were equilibrated for 30 min at 39 °C and then supplemented with 50 µL of semen and incubated for another 10 min at 39 °C. Centrifugation of both tubes was then performed at room temperature at 500xg for 5 min. Supernatant was removed to 100 µL and mixed with the sperm pellet, supplemented with 2 µL FITC-PNA (2 mg FITC-PNA in 2 ml PBS) and 4 µL PI solution (1.50 mM 3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 154 mM NaCl in double distilled water) and incubated another 5 min at 39 °C. The samples were then supplemented with 10 µL paraformaldehyde (1 % in PBS) and analysed immediately. At least 200 spermatozoa in two drops were examined under a fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400 x magnification. Spermatozoa were divided into three groups: viable (PNA-negative, PI-negative), acrosome reacted (PNA-positive) and membrane damaged with intact acrosomes (PNA-negative, PI-positive. Percentage of capacitated spermatozoa was calculated from the difference between the percentage of acrosome reacted spermatozoa before and after addition of LPC.

Artificial insemination

Sorted frozen/thawed spermatozoa of groups A and B were used only for laboratory assessment, whereas spermatozoa of group C and unsorted controls were also used for artificial insemination. Heifers and cows were submitted to routine in-

semination on one farm and were divided into two groups. Animals in the first group were inseminated into the uterine body 12-24 h after onset of natural oestrus. Cows and heifers of the second group were synchronised with GnRH, and PGF-2α (26). In detail, cows received 100 µg GnRH (Depherelin Gonavet Veyx®, Schwarzenborn, Germany) at day -10, further 0.5 mg Cloprostenol (PGF-2α analogue, Essex, München, Germany) at day -3 and again 100 µg GnRH at day -1. Timed artificial inseminations into uterine body were performed 24 h after the second dose of GnRH on day 0. Heifers were synchronised with single injection of 0.5 mg Cloprostenol and animals coming into heat were inseminated 72 h later into uterine body.

Pregnancies were diagnosed 30-60 days after insemination by transrectal ultrasonography (Aloka®; 5 MHz). All pregnant animals were allowed to go to term.

Statistical analyses

Data were analysed with the statistics programme SigmaStat 2.03. Descriptive statistic was used for analyses of means and standard deviations. Laboratory results were tested for normal distribution and then analysed by One-way ANOVA or ANOVA on ranks and Tukey's test. Fertility results were tested with the chi-square test. Results are presented as mean ± standard deviation.

Results

Frozen/thawed semen samples were analysed after incubation at 37 °C for 15 min (Table 2). Percentage of motile spermatozoa was significantly lower in group A compared to groups B, C and control samples. A higher percentage of motile spermatozoa was observed for bull 2 in comparison to bull 1 in unsorted control samples (P= 0.012).

Table 2: Total motility of spermatozoa (%) after thawing

n=12	Group A	Group B	Group C	Control
Bull 1	16.7 ± 14.5^a	55.0 ± 8.4	65.0 ± 3.0^b	60.8 ± 6.6^b
Bull 2	32.9 ± 22.9^a	57.5 ± 7.6	64.6 ± 5.4^b	70.0 ± 3.2^b
Together	24.8 ± 20.5^a	56.3 ± 7.7^b	64.8 ± 4.3^b	65.4 ± 6.9^b

Values within rows with different superscripts differ (P<0.05).

The semen samples from group C, which were superior to other two groups of sorted spermatozoa, and unsorted control samples, were thawed separately and subjected to a thermo-resistance test at 37 °C. Analysis of motility in frozen/thawed samples, after incubation on 37 °C for 15 min also did not reveal any significant difference between sorted samples and unsorted controls. After further incubation at 37 °C, the motility decreased faster in sorted samples and was significantly higher in control samples after 3 and 6 h of incubation (Figure 1).

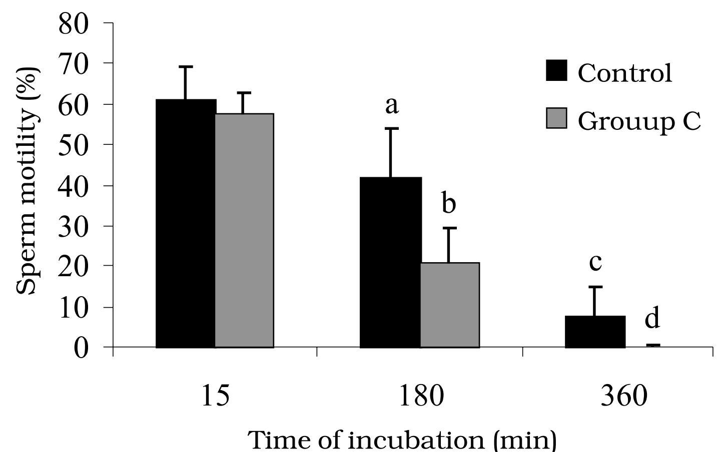

Figure 1: Motility of spermatozoa in frozen/thawed semen samples during incubation over a 6 h (360 min) period at 37 °C (n= 12); Superscripts a and b differ (P<0.001); c and d differ at P<0.05.

Significantly higher percentages of spermatozoa with damaged acrosomes (Table 3) were observed in all groups of sorted spermatozoa compared to unsorted control (P<0.001). The difference between the groups of sorted spermatozoa was not statistical significant. A significant difference between sorted semen and unsorted controls was also observed for the MAS (P<0.001).

Table 3: Percentage of spermatozoa with damaged acrosomes and morphologically abnormal acrosomes (MAS)

(n= 12)	Group A (%)	Group B (%)	Group C (%)	Control (%)
Acrosomes	32.6 ± 7.0^{a}	32.0 ± 7.6^{a}	29.2 ± 5.3^{a}	17.9 ± 7.3^{b}
MAS	43.3 ± 7.8^{a}	44.0 ± 8.1^{a}	37.4 ± 6.8^{a}	28.6 ± 7.0^{b}

Values with different superscripts differ (P<0.001).

Compared to bull 2, bull 1 had higher percentages of damaged acrosomes in group A (P<0.001) and B (P=0.041) (Table 4) and a higher percentage of MAS in group A (P= 0.009). Bull 1 had significantly higher percentage of damaged acrosomes and MAS in all groups of sorted spermatozoa compared to unsorted semen. For bull 2 this differences were not statistically significant.

Table 4: Differences in the percentage of damaged acrosomes and morphologically abnormal spermatozoa (MAS) between bull 1 (B1) and bull 2 (B2)

(n=6)	Group A (%)	Group B (%)	Group C (%)	Control (%)
Acrosomes (B1)	37.2 ± 5.2^{a}	36.3 ± 7.9^{a}	29.9 ± 3.7^{a}	14.2 ± 5.5^{b}
Acrosomes (B2)	28.1 ± 5.7	27.7 ± 4.4	28.4 ± 6.6	21.7 ± 7.4
MAS (B1)	47.3 ± 6.0^{a,e}	47.3 ± 8.4^{a,e}	38.7 ± 5.7^{c,f}	26.0 ± 4.8^{b,d}
MAS (B2)	39.3 ± 7.6	40.7 ± 6.9	36.2 ± 7.6	31.2 ± 8.3

Values within rows with superscripts differed a:b (P<0.001); c:d and e:f (P<0.05).

Percentage of viable (CFDA-positive) spermatozoa was higher (P<0.001) in group C (45.7 ± 6.3 %) compared to group A (28.3 ± 4.9 %), B (30.6 ± 8.1%) and control samples (33.5 ± 6.7 %). Percentage of morbid spermatozoa (CFDA and PI positive) was lower (P<0.001) in group C (36.5 ± 5.8 %) and control (35.8 ± 6.3 %) compared to group A (51.4 ± 7.1 %) and B (46.0 ± 8.3 %). Compared to bull 2, bull 1 had lower percentages of viable spermatozoa in all tested groups (P<0.05) and a significantly (P<0.05) higher percentage of morbid spermatozoa in group C. As presented in table 5, both bulls had significantly higher percentages of viable spermatozoa in group C compared to group A, B and control samples. Both bulls had significantly higher percentages of morbid spermatozoa in group A compared to group C and control samples. Bull 2 had also a significantly higher percentage of morbid spermatozoa in group A compared to group B and in group B compared to group C.

Table 5: Difference in the percentage of viable (CFDA+) and morbid (CFDA+/PI+) spermatozoa between bull 1 (B1) and bull 2 (B2)

(n=6)	Group A (%)	Group B (%)	Group C (%)	Control (%)
CFDA+ (B1)	26.0 ± 4.7^{a}	25.3 ± 4.7^{a}	43.0 ± 4.7^{b}	28.7 ± 4.2^{a}
CFDA+ (B2)	30.6 ± 4.0^{a}	35.9 ± 7.4^{a}	48.3 ± 6.7^{b,c}	38.2 ± 5.1^{d}
CFDA+/PI+ (B1)	50.8 ± 8.7^{c}	48.8 ± 9.3	40.0 ± 3.7b^{d}	37.0 ± 7.4^{d}
CFDA+/PI+ (B2)	52.0 ± 5.6^{a,c}	43.2 ± 7.0^{d,e}	33.3 ± 5.6^{b,f}	34.6 ± 5.5^{b}

Values within rows with superscripts differed a:b (P<0.001); c:d and e:f (P<0.05).

A significant higher percentage of capacitated spermatozoa was observed in group A in comparison to both other sorted groups and control. Bull 1 in comparison to bull 2 had lower percentages of capacitated spermatozoa in group B (9.0 ± 3.6 % and 13.8 ± 3.4 %, respectively) and C (9.1 ± 2.6 % and 13.6 ± 5.0 %, respectively).

Table 6: Percentage of capacitated spermatozoa (PNA/LPC-Test)

Group A (%)	Group B (%)	Group C (%)	Control (%)
16.3 ± 4.0[a]	11.4 ± 4.2[b]	11.3 ± 4.5[b]	7.9 ± 4.3[b]

(n= 12) Values with different superscripts differ (P<0.05).

Significantly more pregnant animals were observed after AI with unsorted semen compared to groups with flowcytometrically sorted semen. More pregnancies were achieved in bull 2 compared to bull 1 for the sorted semen (P<0.005) and in heifers than in cows for the unsorted controls (P<0.007).

Table 7: Pregnancy rates and number of animals inseminated in the natural oestrus with flowcytometrically sorted spermatozoa and unsorted controls

	Sorted spermatozoa		Controls	
	(%)	n	(%)	n
Bull 1	12.1[a]	66	57.0[b]	128
Bull 2	26.2[c]	42	55.7[d]	79
Cows	18.8[a]	69	47.3[b]	110
Heifers	15.4[a]	39	67.0[b]	97
total	17.6[a]	108	56.5[b]	207

Values within rows with different superscripts differed a:b (P<0.001); c:d (P<0.05).

Insemination with unsorted semen in animals with synchronized oestrus led to significantly lower pregnancy rates compared to animals inseminated in natural oestrus (P= 0.012). No difference was observed between animals inseminated in synchronised and normal oestrus with sorted spermatozoa (P= 0.629). Overall pregnancy rates in the synchronised animals did not differ between sorted spermatozoa and unsorted controls (P= 0.076). Significantly more pregnancies were observed for cows and bull 1 after insemination with unsorted compared to sex sorted spermatozoa. Sex ratios, as predicted by resort analysis, differed from the 50:50 ratio (P<0.001); 84.5 % of the offspring born were females.

Table 8: Pregnancy rates and number of animals inseminated in the synchronised oestrus with flowcytometrically sorted semen and unsorted controls

	Sorted spermatozoa		Controls	
	(%)	n	(%)	n
Bull 1	12.2[a]	49	38.5[b]	26
Bull 2	32.5	40	34.5	29
Cows	16.9[c]	65	35.7[d]	42
Heifers	33.3	24	38.5	13
total	21.3	89	36.4	55

Values within rows with different superscripts differed a:b (P<0.001); c:d (P<0.05).

Discussion

The goal of this study was to analyse the effects of different modifications in sperm processing after sorting on the quality of frozen/thawed spermatozoa and to investigate the differences in the quality and fertility between sorted and unsorted frozen/thawed semen. Different processing of sorted spermatozoa

had a significant impact on the quality of the frozen/thawed spermatozoa. Post thaw motility of spermatozoa was significantly higher in group B, C and unsorted controls compared to the samples in group A. Further incubation at 37 °C showed significant reduction of motility in sorted samples compared to controls. This may reflect the situation in the female genital tract, and sorted spermatozoa that have been processed especially according to group C, may have little lifetime after AI to reach the oocyte in the oviduct. Therefore, it has been proposed to inseminate closer to the expected time of ovulation and to inseminate deep into the uterine horn in order to avoid sperm losses during the transport through the uterus, and to shorten the time interval between insemination and the passage through the utero-tubal junction.

A reason for the lower quality and the shorter lifespan could be the sorting itself (7). However, as the sorting was identical for groups A, B and C, the post-sorting process also has a major impact on semen quality. The observed reduction of motility in group C may have been caused by reactive oxygen species (ROS) production. Exposure of spermatozoa to higher temperatures and centrifugation are known to increase the production of ROS and consequently lipid peroxidation (13, 27). Removal of seminal plasma by high dilution during sorting promotes the lower resistance of sorted spermatozoa against such damage (11, 28, 29). Increased morphological damage, especially of acrosomes, may also indicate a higher ROS production (30). The percentage of capacitated spermatozoa was calculated from the difference between acrosome reacted spermatozoa before and after LPC treatment. A statistically higher percentage of capacitated spermatozoa was observed in group A in comparison with unsorted control samples. One of the mechanisms for capacitation and the acrosome reaction is the lipid peroxidation of the sperm plasma membrane (30-32). The results indicate that immediate centrifugation and replacement of sorting extender with an extender containing egg yolk, which has an antioxidant ability (16, 33), offered protection against ROS.

Differences between groups B and C are mainly caused by the presence of glycerol at room temperature. It is well known that glycerol is toxic to spermatozoa and therefore should be added to sorted semen shortly before the freezing process starts (19, 34).

Labelling of spermatozoa with CFDA and PI resulted in three distinct populations: green (enzyme active with intact plasma membrane), green/red (enzyme active with damaged plasma membrane), red (dead) (35-37). The most important information in this test is the percentage of viable and membrane intact spermatozoa, represented in the green population. The highest percentages of viable and membrane intact spermatozoa were detected in group C. In groups A and B significantly more cells were damaged; it is also interesting that control samples were more damaged than the sorted sperm of group C. The reason for this result is discarding damaged sperm during sorting. Food dye was added to the spermatozoa before sorting, and only enters into spermatozoa with damaged plasma membranes. This reduces the emission of Hoechst 33342 stain and enables exclusion of damaged sperm during sorting (18).

A statistical significant effect of the bull was found for motility, morphological changes, viability and the percentage of capacitated spermatozoa. These results are in agreement with other studies showing significant bull effect on the quality and the fertilizing capacity of spermatozoa, especially if the spermatozoa are highly diluted (38, 39). Further, they also indicate the importance of bull selection for success of flow-cytometrical sorting (40).

Sperm fertilizing abilities were further tested in the field insemination trial. Pregnancy rates after AI during natural oestrus were lower for sorted spermatozoa (17.6 %) compared to unsorted controls (56.5 %) and were significantly affected by the bull ($P<0.005$). In synchronised animals the difference in pregnancy rates between sorted and unsorted semen dropped to 15 % and pregnancy rates for bull 2 did not differ between sorted samples and unsorted controls.

This results showed a significant effect of the bull on the pregnancy rates after artificial insemination with sex sorted spermatozoa, which is in agreement with other reports indicating high importance of bull selection for insemination with reduced number of spermatozoa (38, 40, 41, 42). Since the differences between the bulls were observed only for the sex sorted and not for the unsorted semen samples, the results also indicate the variability in resistance of spermatozoa to flow-cytometrical sorting between the semen donors. Further, the pregnancy rates in unsynchronised animals were significantly lower in the groups inseminated with sex sorted compared to unsorted semen. The reports from other studies also showed a reduction of pregnancy rates after artificial insemination with sex sorted semen, but mostly in

all studies this reduction of fertility as compared to unsorted semen was within 90% for heifers (4, 43). A high reduction of pregnancy rates after artificial insemination with sex sorted semen in this study could be due to reduced quality of the sorted semen or to management failure. Since the preparation and the quality of the sorted semen used for artificial insemination in this study only differed marginally from these two parameters as reported by other authors (4, 18), it is not likely that this would affect the pregnancy rates to a large extent as obtained in our study. The pregnancy rates in synchronized animals were significantly lower compared to animals inseminated in normal oestrus, but the difference between sorted and unsorted semen was smaller and not significantly different. Lower pregnancy rates in animals with synchronised oestrus could be due to a lack of synchronization in some animals (44). We did not find any significant reduction of overall fertility after artificial insemination of synchronized animals with sorted semen. The reduction of fertility was observed only in bull 1 and in bull 2 fertility was comparable to unsorted semen. These results also indicate the difference between bulls in their resistance to flow-cytometrical sorting. A lower effect of flow-cytometrically sorting on pregnancy rates in synchronised as compared to unsynchronized animals, could be explained by management failure. In our study the animals were inseminated under normal farm conditions and the inseminations with sorted semen were performed at the same time in oestrus as for unsorted semen. Shorter viability of sorted compared to unsorted semen (45, 46) is probably responsible for the reduction of the fertilizing capacity of flow-cytometrically sorted spermatozoa in this study.

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VPLIV LOČEVANJA BIKOVIH SEMENČIC PO SPOLU NA KVALITETO IN OPLODITVENO SPOSOBNOST PO ODMRZOVANJU

P. Klinc, D. Rath

Izvleček: Namen raziskave je bil, ugotoviti vpliv različnih postopkov priprave sortiranega semena pred zamrzovanjem v tekočem dušiku na kakovost in oploditveno sposobnost odmrznjenega semena ter ugotavljanje razlik med sortiranim in nesortiranim semenom. Seme je bilo pridobljeno od dveh bikov črno-bele pasme. Kakovost odmrznjenih vzorcev je bila preiskovana s pomočjo gibljivosti, morfologije, s testom za ugotavljanje stabilnosti membran (6-CFDA/PI) ter kapacitacije (barvanje s pomočjo FITC-PNA/PI z dodatkom L-α-Lysophosphatidylcholine). Podaljšana inkubacija visoko razredčenega sortiranega semena je imela statistično značilen negativni vpliv na gibljivost (P<0,001) in kapacitacijo (P<0,05). Stabilnost celičnih membran je bila statistično značilno (P<0,001) višja pri sortiranih vzorcih, pri katerih je bil glicerol dodan pri 5 °C, v primerjavi z dodatkom glicerola pri sobni temperaturi. Neodvisno od postopka priprave je bil v sortiranem semenu v primerjavi z nesortiranim ugotovljen višji odstotek poškodovanih akrosomov in skupnih morfoloških nepravilnosti (P<0,001). Poleg osnovnih raziskav je bil opravljen tudi termorezistentni test pri 37 °C. V test je bilo vključeno sortirano (centrifugiranje neposredno po sortiranju in dodatek glicerola pri 5 °C) ter nesortirano odmrznjeno seme. Neposredno po tajanju razlika v gibljivosti med sortiranim in nesortiranim semenom ni bila statistično značilna. Po podaljšani inkubaciji pri 37 °C je bil po 3 (P<0,001) in 6 urah ugotovljen statistično značilen nižji (P<0.05) odstotek gibljivih semenčic v sortiranem v primerjavi z nesortiranim semenom. Preiskava in primerjava oploditvene sposobnosti sortiranega in nesortiranega semena je bila opravljena s pomočjo poskusnih osemenitev krav in telic v času normalnih in sinhroniziranih pojatev. Pri osemenitvah med normalnimi pojatvami je bila ugotovljena statistično značilno višja (P<0,001) brejost z nesortiranim semenom (56,5 %) v primerjavi s sortiranim (17,6 %). Pri živalih s sinhroniziranimi pojatvami je bil odstotek brejih živali, osemenjenih z nesortiranim semenom, statistično značilno nižji (p= 0,012) v primerjavi z živalmi, osemenjenimi med naravnimi pojatvami, vendar pa pri tej skupini ni bilo statistično značilne razlike med nesortiranim (36,4 %) in sortiranim semenom (21,3 %). Poleg tega smo ugotovili statistično značilen vpliv bika na oploditveno sposobnost sortiranega semena (P<0.05).

Ključne bsede: sperma - analize; spermatozoa - fiziologija - ultrastruktura; kontrola kvalitete; sperma, konzerviranje; ose - enjevanje, umetno - veterinarsko - metode; brejost; nosečnost, število; biki

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Case Report

CHLAMYDOPHILA FELIS INFECTION IN CATS – CLINICAL CASES

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Summary: Cats, infected with *Chlamydophila* felis, formerly known as *Chlamydia psittaci*, can present many different clinical signs.

The study included eleven domestic shorthaired cats presented at our clinic between 2003 and 2005. The physical examination and hematology was performed in all patients, thoracic radiographs in two cats and FeLV/ FIV test was performed in 5 of 11 cats. Clearview Chlamydia MF test (11/11) and Chlamydia Direct IF test (5/11) were performed in oropharyngeal swabs. Specific antibodies against *Cp. felis* were determined in serum or plasma of 5 cats using indirect immunofluorescence test.

Most of them (9/11) were presented with mild clinical signs (conjunctivitis (4/11), acute nasal discharge (3/11), intermittent chronic recurrent nasal discharge (3/11), coughing (2/11)) and without any changes in hematology. Two cats were presented with acute and severe clinical signs of lower respiratory tract involvement and systemic signs of infection, marked changes on thoracic radiographs and elevated WBC count.

Based on our study we conclude, that *Chlamydia sp.* can be considered primary or secondary pathogen, which can potentially cause severe signs of respiratory tract infection in cats, especially in younger animals. The infection in cats could be successfully treated using doxycycline.

The results using different laboratory tests confirmed the possible infection with *Cp. felis.* It is not excluded that possible cross-reaction between different chlamydial antigen can occur.

Key words: Chlamydia infections - diagnosis; pathology, clinical; serodiagnosis – methods; cats

Introduction

Chlamydophila felis (*Cp. felis*), formerly known as Chlamydia psittaci, is one of the most important etiologic factors of feline conjunctivitis. *Cp. felis* is an obligate intracytoplasmic parasite, a coccoid bacterium with a rigid lipid-containing cell wall, which is similar in structure and content to the wall of gram-negative bacteria. *Cp. felis* contains nuclear DNA and RNA but cannot replicate and survive autonomously in the environment (1).

The cycle of *Cp. felis* is specific, with intracellular and extracellular phases producing unique forms of the organism: elementary bodies, initial bodies and reticulate bodies. Elementary bodies, which are supposed to be the infectious form, are released from ruptured infected cells and can survive in the environment for up to one week at room temperature. Elementary body enters a new host cell by endocytosis and forms a membrane-bound phagosome, where it forms initial body, a specialized reproductive noninfective form of the organism. Initial bodies undergo intracellular division by means of budding and double fission, followed by a period of rapid growth and finally form a reticulate body. A reticulate body is a large, metabolically active membrane bound population of initial bodies, which are differentiating and maturing towards infective elementary bodies, which are released from the host cell (1, 2).

Cp. felis may persist on conjunctival and upper respiratory epithelium as resident flora. Transmis-

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sion from one cat to another is usually by direct contact and with infective occular secretion (1).

Cats, infected with *Cp. felis*, can present many different clinical signs, such as conjunctivitis, nasal discharge, and other signs of upper respiratory tract disease. Clinical signs are influenced by the age of the cat, immunocompetence, the tissue inoculated and the volume of the inoculum (1, 2, 3).

When conjunctivitis occurs, the signs are profuse serous ocular discharge with chemosis, hyperemia of the palpebral conjunctiva, and blepharospasm. One or both eyes can be affected. In case of unilateral conjunctivitis, spread of clinical signs to the unaffected eye can be expected within 5-21 days. If occular discharge starts to change its character than we can suspect that other resident and transient opportunistic bacterial organisms may be present in conjunctiva (1, 4).

Signs of respiratory tract disease are much less common. They include intermittent recurrent nasal discharge, sneezing, coughing and in severe cases possible lower respiratory tract involvement can be expected (1, 2). These signs are usually present in young cats in the age between 5 weeks to 9 months (5).

Diagnosis of *Cp. felis* infection can be performed using different diagnostic procedures, based on either isolation of the infectious organism, amplification of chlamydial DNA by polymerase chain reaction (PCR), or detection of anti-chlamydial antibodies by immunofluorescence Assay and enzime-linked immunosorbent assay (ELISA). The PCR showed to be the most sensitive assessment, even more than isolation itself. Serology was of limited use in predicting which cats were infected (3, 6, 7).

Material and methods

The study included eleven domestic shorthaired cats from nine different owners presented on our clinic between 2003 and 2005. They are in-and outdoor living cats of different age and of both sexes.

Physical examination and hematology

In all eleven patients physical examination was performed. Blood for CBC and white blood cell differential count was taken from vena jugularis. Blood tests were performed using the automated laser hematology analyser (Technicon H*1, Bayer, Germany) with species-specific software (H*1 Multi-Species V30 Software).

Other diagnostic procedures

In two cats with severe systemic disease and signs of lower respiratory tract disease thoracic radiographs were performed.

Detection of feline leukemia virus antigen and antibodies against feline immunodeficiency virus was performed in 5 of 11 cats using rapid ELISA test (Feline Leukemia Virus Antigen/Feline Immunodeficiency Virus Antibody Test Kit®, IDEXX, Westbrook, Maine, USA). The test is used for the simultaneous detection of feline leukemia virus (FeLV) antigen and antibodies to feline immunodeficiency virus (FIV) in serum, plasma or whole blood.

Diagnosis of chlamydial infection

1. Detection of chlamydiae antigen.

Clearview Chlamydia MF test (Clearview Chlamydia MF, Unipath Limited® Bedford, United Kingdom) was performed in oropharyngeal swabs of all eleven cats. This test is a rapid immunoassay for the direct genus specific qualitative detection of *Chlamydia trachomatis* (*C. trachomatis*) antigen.

Chlamydia Direct IF test (Chlamydia Direct IF, Biomerieux® Lyon, France) was performed on oropharyngeal swabs of five cats. It enables detection of *Chlamydia* antigen using two different monoclonal antibodies, one directed against the antigen of the genus *Chlamydia*, and the other against the species *C. trachomatis*.

2. Detection of antibodies against *Chlamydophila felis.*

Specific antibodies against Cp. felis were determined in serum or plasma of 5 cats using indirect immunofluorescence test (Feline Chlamydia IgG IFA KIT, Fuller Laboratories® Fullerton, CA USA). This test provides detection and quantitative determination of IgG class antibodies against *Cp. felis.* Titer 1: 40 was considered as a margin titer.

Treatment

All patients were treated with doxycycline per oral or with inhalation of the drugs.

Results

Four of eleven patients were younger cats (6 to 12 months old) and seven of them were older (from 2 to 13 years old), five female and six male. Six cats

Table 1: Clinical signs, age and test results for cats infected with chlamydial infection

Patient			Diagnostic test - Chlamydia			Other tests	Outcome (in months)	
Nr.	Clinical signs	Age/Sex	ORLs[1]	ORLs[2]	Serum[3]	FeLV/FIV	Follow up	Without clinical signs
1	Dyspnea	6 m /F	Pos	Pos	1: 320	-20	12	12
*2	Sneezing and coughing	1 y / M	Pos	Pos	ND	ND	12	10
*3	Coughing 1 month, febrile	6 m /F	Pos	Pos	1:320	-20	12	10
**4	Occasional sneezing and coughing, inapetence	2 y / M	Pos	ND	Neg	-20	12	12
**5	Intermittent recurrent nasal discharge for2-3 years	6 y/ M	Pos	Pos	1:160	Neg/Neg	12	12
6	Inapetence, vomiting, occasional coughing	2 y / M	Pos	ND	ND	ND	4	4
7	Coughing 1 week, sneezing	6 y / M	Pos	ND	ND	ND	18	12
8	Coughing, ocular discharge	7 m /F	Pos	ND	ND	ND	3	3
9	Intermittent recurrent nasal discharge for 2-3 years	13 y /F	Pos	Pos	1:40	-20	18	10
10	Sneezing, occular discharge	12 y/M	Pos	ND	ND	ND	12	12
11	Sneezing, inapetence	13 y/F	Pos	ND	ND	ND	6	2

Legend:

ORLs oropharyngeal swab

1 Clearview Chlamydia MF test (Clearview Chlamydia MF, Unipath Limited® Bedford, United Kingdom)

2 Chlamydia Direct IF test (Chlamydia Direct IF, Biomerieux® Lyon, France)

3 Feline Chlamydia IgG IFA KIT (Fuller Laboratories® Fullerton, CA USA)

m month
y year
F female
M male
Neg negative
Pos positive
ND not done

* the first owner
** the second owner

were fully vaccinated against feline rhinotracheitis, calici virus infection, panleukopenia (Felocell CVR®, Pfizer, New York, USA) and feline leukemia virus infection (Leukocell 2®, Pfizer, New York, USA), but none of them received vaccination against Cp. felis.

Most of them (9/11) were presented without signs of systemic illness. Clinical signs of these cats included conjunctivitis (4/11), nasal discharge of acute onset (3/11), intermittent chronic recurrent nasal discharge (3/11), and coughing (2/11). Only one cat has previously been treated for these problems and it responded well to three consecutive daily inhalations with Terramycin/LA® (oxytetracycline 200mg/ml, Pfizer, Amboise, France). Every inhalation lasted for 30 minutes, with 2.0 ml of Terramycin/LA® dissolved in 3.0 ml of injectable water.

All of these cats had unremarkable complete blood count and differential white blood cell count. Three of nine patients without systemic clinical signs of infection were tested for FeLV and FIV and were negative.

Another two cats were presented with acute and severe clinical signs of lower respiratory tract involvement and systemic signs of infection. The first patient was 6 months old female and fully vaccinated mainly indoor cat with dyspnea. CBC was within reference limits. Thoracic radiographs revealed extensive bilateral alveolar infiltrations (Figure 1). The second patient was 7 months old female outdoor non-vaccinated cat presented in febrile state with cough of one-month duration and conjunctivitis. This patient had elevated WBC count of $27 x 10^9/L$

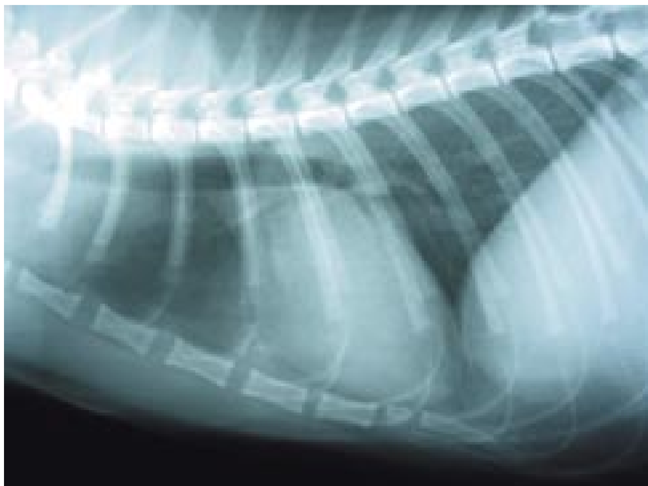

Figure 1: Thoracic radiographs of cat with dyspnea at time of diagnosis

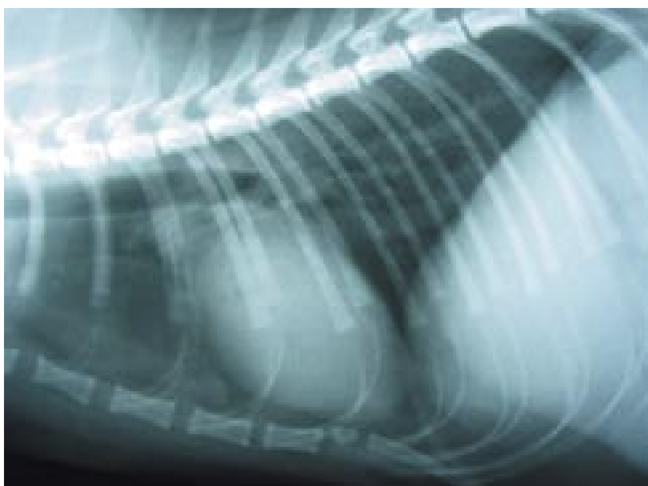

Figure 2: Thoracic radiographs of cat with dyspnea during treatment

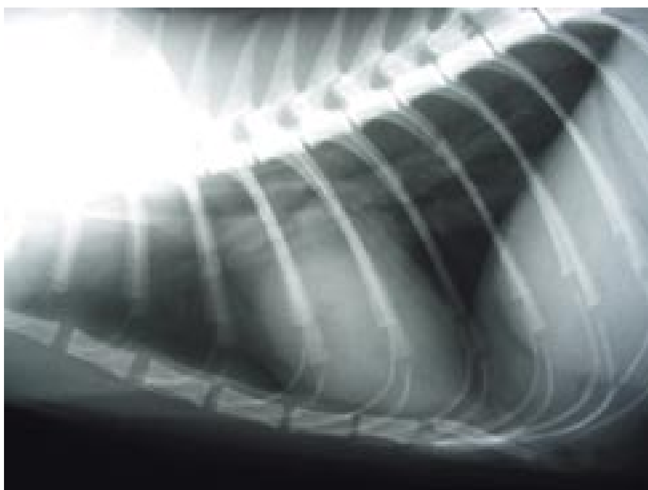

Figure 3: Thoracic radiographs of cat with dyspnea after completed therapy

(reference 5.5-19.5x109/L) and radiographic changes showing generalized interstitial infiltrations of lungs. Both cats were tested for FeLV and FIV and were negative.

All eleven patients were successfully treated with doxycycline (5 mg/kg b.w. /day for 3 weeks). They showed rapid clinical improvement shortly after therapy was instituted. The majority remained without any clinical signs of respiratory infection for 3 to 18 months, depending of follow up. Results of clinical examinations are presented in table 1.

Discussion

According to literature, the most common clinical sign, associated with *Cp. felis* infection, is unilateral or bilateral conjunctivitis (8). Clinical signs of upper respiratory tract disease and systemic signs, especially in adult cats, are considered to be less common (9). The first report about chlamydiosis in cats in Slovenia was in 1994. There were 30% positive reactors in direct immunofluorescence test (Chlamydia Direct IF, Biomerieux® Lyon, France) and 40% positive reactors in indirect immunofluorescence test (Chlamydia Psittaci spot, Biomerieux® Lyon, France) of 10 clinical suspected cats. Most of them had conjunctivitis (8/10) and rhinitis (5/10), three of them had pneumonia (10). In the next study between 1994 and 1997 the 38 cats were tested using direct immunofluorescence test (Chlamydia Direct IF, Biomerieux® Lyon, France), indirect immunofluorescence test (Chlamydia Psittaci spot, Biomerieux® Lyon, France) and enzyme immune test (Clearview Chlamydia MF, Unipath Limited® Bedford, United Kingdom). In this period in cats with acute symptoms upper respiratory tract of 57.7% immunoreactive cats were found. In two breeds acute chlamydiosis was confirmed . Pathoanatomical and pathohistological lesions were also performed. In conjunctival swabs chlamydiae were presented in 22.2%. In 1997 thirteen breeders of cats were tested with microimmunofluorescence and all owners were seronegative to *Cp. psittaci* (11).

In group of our patients with *Cp. felis* infection, conjunctivitis appeared not to be the most frequent presenting problem. Clinical signs of upper and lower respiratory tract disease, such as coughing, nasal discharge and sneezing were much more common. The nature and severity of clinical signs in *Cp. felis* infection are influenced by the age of infected cat (1).

All treated cats responded well, even two, which were most severely affected (signs of lower respirato-

ry tract involvement with systemic signs) were young cats 6 months of age. However, despite the young age and severe clinical condition, they responded extremely well to per oral antibiotic therapy with doxycycline, with improvement of clinical signs in about 2-3 days after beginning of treatment. The infection in cats could be successfully treated using doxycycline in water solution inhalation of the drugs, too.

Chlamydial infection in group of our patients was diagnosed using three different diagnostic procedures. The presence of *Chlamydia sp.* antigen was detected in all eleven oropharyngeal swabs using Clearview Chlamydia MF test and in five oropharyngeal swabs (5/5) using Chlamydia Direct IF test.

The presence of chlamydial antigen confirmed the diagnosis of chlamydial infection. The tests that we were using in our study are not specific for *Cp. felis*. Detection of *Cp. felis* specific antibodies was performed in 5 of 11 cats using Feline Chlamydia IgG IFA KIT test. Four of five cats had specific IgG antibodies (titer 1:40 to 1:320). The highest titer (1: 320) was observed in two cats with signs of systemic illness. The results confirmed the possible infection with *Cp. felis*. Possible cross-reaction between different chlamydial antigens is not excluded. PCR or other more specific tests should be done to confirm the *Cp. felis* antigen.

Due to possible zoonotic potential of *Cp. felis*, owners of patients with chronic disease or those, showing severe disease, were advised to visit their medical practitioner for serologic diagnostics of *Cp. felis* infection. Two of the owners responded to our advice and both of them were tested negative for the presence of specific antibodies against *Cp. felis*.

Based on our study we conclude, that Chlamydia sp. can be considered primary or secondary pathogen, which can potentially cause severe signs of respiratory tract infection in cats, especially in younger animals.

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OKUŽBA MAČK Z BAKTERIJO CHLAMYDOPHILA FELIS – KLINIČNI PRIMERI

N. Tozon, S. Suhadolc Scholten, D. Pavlin, A. Dovč

Povzetek: Mačke, okužene s Chlamydophila felis, poprej imenovano Chlamydia psittaci lahko kažejo različne klinične znake.
V študijo je bilo vključenih 11 mačk, ki so se na naši kliniki zdravile med letoma 2003 in 2005. Pri vseh je bil opravljen klinični pregled in hematološka preiskava. Dvema mačkama smo rentgensko slikali prsni koš, pri petih pa je bil opravljen tudi test FeLV/FIV. Clearview Chlamydia MF test (11/11) in Chlamydia Direct IF test (5/11) smo izvedli na žrelnih brisih. Specifična protitelesa proti antigenu Cp. felis pa smo dokazovali v serumu ali plazmi petih živali s testom posredne imunoflourescence.
Pri večini (9/11) mačk so bili le blagi klinični znaki okužbe (konjunktivitis (4/11), akuten nosni izcedek (3/11), ponavljajoči se kronični nosni izcedek (3/11), kašelj (2/11)), in sicer brez odstopanj v hematoloških parametrih. Pri dveh mačkah so bili prisotni akutni in resni klinični znaki spodnjega dihalnega trakta z izraženimi sistemskimi znaki okužbe. Rentgenske slike prsnega koša so potrdile izrazite bolezenske spremembe na pljučih in povišano število levkocitov.
Na podlagi študije lahko sklepamo, da je Chlamydia sp. primarni ali sekundarni povzročitelj okužbe pri mačkah. Še posebej pri mladih živalih lahko izzove resne znake okužbe celotnega dihalnega trakta. Zdravljene z doksiciklinom je običajno uspešno.
Rezultati različnih uporabljenih laboratorijskih metod potrjujejo možnost okužbe s Cp. felis, pri čemer ne moremo izključiti morebitne navzkrižne reaktivnosti z drugimi vrstami klamidij.

Ključne besede: klamidija infekcije - diagnostika; patologija, klinična; serološka diagnostika – metode; mačke

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Examples of references

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Slovenski veterinarski zbornik (Slovenian Veterinary Research) objavlja izvirne prispevke, ki še niso bili objavljeni oz. poslani v objavo drugam. Za vse navedbe v prispevkih so odgovorni avtorji. Uredniška politika obsega publiciranje znanstvenih člankov, preglednih znanstvenih člankov, strokovnih člankov, povzetkov disertacij in drugih prispevkov, kot so kritične presoje o vsebini razprav, objavljenih v zborniku, kratke znanstvene prispevke, pisma uredniku in drugo. Avtorji pošljejo prispevke na naslov uredništva. Glavni urednik pregleda vse prispevke. Za vse članke je obvezna strokovna recenzija, za katero poskrbi uredništvo.

Prispevki naj bodo napisani v angleškem jeziku, z naslovom, povzetkom in ključnimi besedami tudi v slovenščini. Obsegajo naj največ 12 strani, kar pomeni 27 vrstic na stran s približno 75 znaki v vrstici. Prispevki naj bodo poslani v elektronski obliki v katerem koli urejevalniku besedil za okensko okolje. Zaželjena je uporaba elektronske pošte (slovetres@vf.uni-lj.si) in avtorji naj predlagajo tri možne recenzente. Besedilo naj ima dvojni razmik med vrsticami, pri čemer naj bodo vrstice na levi strani oštevilčene. Besedilo naj bo na levi strani od roba oddaljeno 4 cm.

Naslovna stran prispevkov se začne z naslovom, sledi ime in priimek avtorja. Kadar je avtorjev več, jih ločimo z vejicami. V naslednjih vrsticah je v rubriki Addresses of authors: za dvopičjem treba navesti polno ime in priimek ter naslov(e) avtorja(ev), tj. ustanovo, ulico s hišno številko, pošto in kraj. Vse navedene podatke ločujejo vejice. Sledi vrstica, kjer je treba navesti ime ter elektronski (E-mail:) in poštni naslov ter telefonsko številko (Phone:) odgovornega avtorja.

Sledi besedilo povzetka Summary v obsegu 16 do 20 vrstic (približno 1000 do 1500 znakov). V naslednji rubriki Key words: se za dvopičjem navedejo ključne besede. Posamezne besede ali sklopi besed morajo biti ločeni s podpičjem.

Znanstveni članki in tisti, ki so prikaz lastnih raziskav in dognanj, morajo vsebovati še naslednje obvezne rubrike, s katerimi avtor sam naslovi ustrezne dele besedila v prispevku: Introduction, Material and methods, Results, Discussion in References. Pregledni članki naj vsebujejo uvod, poglavja, ki so glede na vsebino smiselno naslovljena, in literaturo. Podatke o financerjih ali drugih zadevah, pomembnih za prispevek, npr. o tehnični pomoči, avtorji navedejo v rubriki Acknowledgements, ki se uvrsti pred rubriko References. Za rubriko References sledijo spremna besedila k slikam.

Priloge, kot so tabele, grafikoni in diagrami naj bodo smiselno vključene v besedilo. Slikovni material naj bo poslan posebej v obliki bmp, jpg, ali tif.

Priloge in slike morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. 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. Če je citirani vir napisalo šest ali manj avtorjev, je treba navesti vse; pri sedmih ali več avtorjih se navedejo prvi trije in doda et al.

Da bi se morebitni popravki lahko objavili v naslednji številki, jih morajo avtorji pravočasno sporočiti glavnemu uredniku.

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