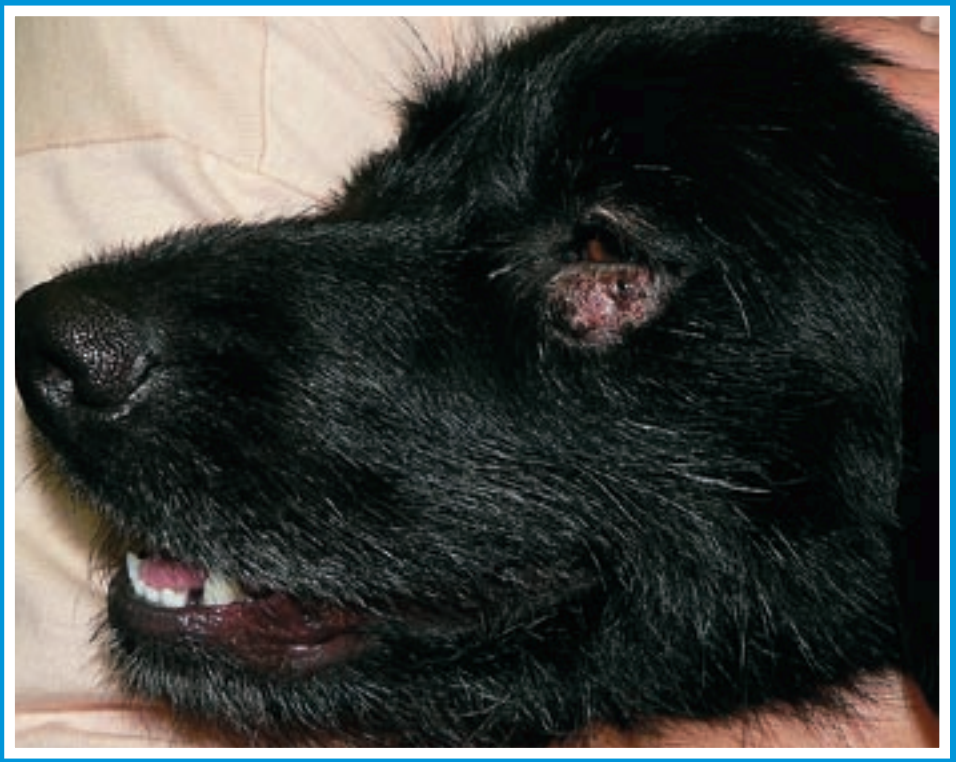
ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK



Slov Vet Res 2007; 44 (3): 55-96

Volume 44 3

Slov Vet Res • Ljubljana • 2007 • Volume 44 • Number 3 • 55-96

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Slov Vet Res • Ljubljana • 2007 • Volume 44 • Number 3 • 55-96

The Scientific Journal of the Veterinary Faculty University of Ljubljana

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno

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Sponsored by the Slovenian Research Agency
Sofinancira: Agencija za raziskovalno dejavnost Republike Slovenije

ISSN 1580-4003
Printed by / tisk: Birografika Bori d.o.o., Ljubljana
Indexed in / indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory
http://www.vf.uni-lj.si/veterina/zbornik.htm
Cover photograph / Fotografija na naslovnici: Tina Kotnik, canine dermatophytosis / dermatofitoza pri psu

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2007; 44 (3)

Slov Vet Res 2007; 44 (3): 55-62
UDC 636.02:57.089

Review Paper

THE USE OF ANIMALS IN BIOMEDICAL RESEARCH

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Summary: Throughout the history invertebrate and vertebrate models have been used in fundamental and goal-oriented scientific research to gain new information on cell and organ anatomy, mechanisms of the diseases and methods to prevent them, behavioral research, for production, development, testing of quality and safety of drugs, food, cosmetic and other products, and to answer scientific questions that would have been impossible to be gathered directly from humans. Although researchers are continually developing non-animal models, research on complex multigenic diseases and therapeutics testing sometimes require the use of in vivo models. It is generally recognized that in the absence of human data, animal research in many cases can offer most accurate approximations and predictions of human responses.

Key words: ethics, medical; animals, laboratory–experiments; research; disease models, animal

Introduction

The use of animals in scientific research and education inevitably raises moral and ethical issues. Many studies have been done to assess the validity of alternative methods (cell lines, computer simulations, etc.), but complex biological processes and testing of therapeutics often require in vivo analysis (1). Inarguably, humanity owns many benefits of modern medicine and countless advances in basic scientific knowledge to animal experimentation (2). The conflicts between the claims of science and medicine and those of humanity in our treatment of lower animals have undoubtedly no easy solution (3, 4). When, in the late nineteenth century, these divergent ideas appeared, the British members of Parliament introduced the famous Cruelty to Animals Act (1876) that balanced the rival claims (2, 5). The rapid development of several biomedical disciplines in the twentieth century caused an increase of animal usage (6). Nowadays, establishment of animal care, legislation and ethical committees, which are responsible for approval of experiments, have a great impact on animal use and welfare.

In complex diseases, finding all the mutated genes is vital for understanding and consequently treating multigenic disorders (1, 7). Therefore, laboratory animals have been used as experimental models to discern biological mechanisms leading to the development of the diseases, detection of potential carcinogens, testing different drugs, cancer therapeutics and consumer products, such as cosmetics, household cleaning products etc., determining the right doses for treatment and many more. In fact, there are no real substitutes for laboratory animals, although extensive research is being done in the direction of replacing them with appropriate in vitro systems (5, 8). Cell cultures, bacteria, yeast or even computer simulations can provide useful information, but the complexity of multicellular organisms still requires research and testing on animals. Cancer for example, is, in essence, a genetic disease characterized by a pathological breakdown in the processes which control proliferation, differentiation and death of particular cells (8). The use of modern and classical molecular biology tools revealed many important genes, which are directly or indirectly responsible for the genesis of various cancers due to the accumulation of multiple genetic alterations, inheritance of susceptible alleles and environmental stimuli (9, 10). However, the clear genetic basis revealing these molecular events in tumor development and progression is still unclear. Much of our understanding of carcinogenesis was (and still is) obtained from the studies on estab-

Received: 2 July 2007
Accepted for publication: 27 August 2007

lished cell lines prepared from human tumors (11). However, these cells are unable to form multicellular forms identical to those found in humans and are therefore inappropriate for studying biological and molecular processes underlying complex diseases.

This review briefly covers the use of animal models in biomedical research of the diseases, mainly cancer, the benefits and limitations of laboratory animals and discusses ethical issues and legislation, concerning animal use.

A short history of using animal models

Humans "use" animals in several different ways. In addition to their use in research, testing and education, they are also used for food and fiber production, for sports and entertainment. Animals can also be kept as pets for the purpose of companionship.

They are also used in virtually every field of biomedical research, which covers a long list of disciplines (molecular biology, anatomy, anesthesiology, biochemistry, biomedical engineering, cell biology, dentistry, developmental biology, endocrinology, entomology, genetics, gerontology, histology, immunology, metabolism, microbiology, neurology, nutrition, oncology, parasitology, pathology, pharmacology, physiology, psychology, radiology, reproductive biology, surgery, teratology, toxicology, veterinary science, virology, zoology,...), behavioral research (depression, drug addiction, aggression,...), testing of products for toxicity and for education of students (medical, veterinarian, advanced life sciences students) (6). Almost all medical knowledge, understanding of the structure and function of organs, treatments and vaccines, has involved the use of experimental animals. The ancient Egyptians acquired basic anatomical knowledge through embalming practices (5). The first attempts to classify and systematize knowledge of the natural world, although with many errors, were undertaken by the Greeks. Galen, the Greek physician and philosopher, is believed to be among the first scientists to perform vivisections and post-mortems on animals, mostly apes and pigs (http://www.zephyrus.co.uk). He extrapolated his discoveries directly to humans, thus initiating many mistakes, which due to the prohibition of Church of post-mortem dissections of human body, were perpetuated well into the 16th century (12). In the medieval Europe, the influence of the Church obstructed scientific research and almost all science was based upon ancient Greek and Egyptian authorities, Aristotle, Ptolemaeus, Galen, Hippocrates, Herophilos and Erasistratos. The quest for medical discoveries continued more than one thousand years later, when in 1543 Vesalius published the first complete textbook of human anatomy, De Humanis Corporis Fabrica (12). He studied medicine and through dissecting the human corpses he discovered the Galen's errors. He is considered as a beginner of modern medicine and was succeeded by William Harvey whose book On the motion of the heart and blood (1628) revealed the basic mechanisms of these two organs (5). His explanation of blood system led to a more extensive use of animals in Europe (5). In 1865, French physiologist Claude Bernard published a book Introduction to study of experimental medicine, which advocated the chemical and physical induction of disease in experimental models (13). Next, the discovery of several types of anaesthesia in the 19th century (ether, nitrous oxide, chloroform, cocaine and its derivatives) also promoted the use of laboratory animals (14). The increasing use of experimental animals in the 19th and 20th century was not universally applauded, but the works of Louis Pasteur, Robert Koch and many others on developing vaccines and discerning the mechanisms of diseases, such as cholera and tuberculosis, advocated and justified the use of »animal models« (5).

The concept of animal models in biomedical research

Despite the widespread use of human cancer-derived cell lines, their limitations sometimes compel the scientists to use animal models (15). The term animal model is loosely defined as: "An animal with a disease either the same as or like a disease in humans. Animal models are used to study the development and progression of diseases and to test new treatments before they are given to humans. Animals with transplanted human cancers or other tissues are called xenograft models" (NCI Dictionary of Cancer Terms).

The researchers use different animal models to study the molecular mechanisms, the cause and cure of human disorders (4). According to Rand they may be conveniently classified into five groups (4):

1. Induced (experimental) disease models
2. Spontaneous (genetic) disease models
3. Transgenic disease models
4. Negative disease models
5. Orphan disease models

Induced models are healthy animals in which the pathologic condition is experimentally induced (for instance, infections or induction of diabetes mellitus with encephalomyocarditis virus). On the other hand, spontaneous models have naturally occurring genetic variants which resemble or can be xenografted to resemble diseases in humans (for instance, nude mice, which enable the study of heterotransplanted tumors). Majority of these models are mice and rats. Transgenic animals (rodents, rabbits, farm animals, fish, etc.) have been developed with genetic engineering and embryo manipulation methods, however, because many diseases are polygenic in nature, the use of these models will require more research to establish the contribution of all genes involved in the development of pathological conditions. Negative models are used in studies on the mechanisms of resistance, since these animals do not develop the investigated disease, and finally, orphan models are animals with the disease, which has not yet been described in humans, such as feline leukemia, papillomatosis, bovine spongiform encephalopathy, but the research done might be of use, if similar conditions should be described in humans (4).

One of the most important considerations when the scientists determine that the use of laboratory animals is necessary is the selection of the species, breed and strain to be used in experiment (4). In many fields of biomedical research and also in cancer research, mice and rats have been traditionally used, because they are relatively cheap, have short life span, high reproduction rate and are easy to handle. However, other animal species are also used, but either they are not as cost-efficient or many ethical issues were raised, especially in the case of non-human primates. Another important reason for the widespread use of rodents is that advances in genetic engineering have enabled scientists to develop "humanized" mice, which are either immunodeficient (engrafted with human haematopoietic cells, tissues or stem cells), or transgenic, which express human genes that were inserted in the mouse genome (1). The first type can be xenografted with human tumors or used to study the effect of immunity to tumor or viral growth, AIDS, lupus, psoriasis and other diseases (1, 16). Also, the researchers have developed "humanized" mice strains to study infections with viruses, bacteria and parasitic protozoa (Dengue virus, EBV, HCV, Mycobacterium tuberculosis, Plasmodium falciparum), the development and function of the immune system, autoimmunity and human haematopoiesis (1). Nevertheless, working with animals requires that scientists take into consideration: a careful design of the experiment, the responsible use of laboratory animals and when this is scientifically appropriate and valid – a reduction in the number of animals used for research and testing, and finally, when possible, to develop and use alternative methods (5, 8, 17, 18).

Laboratory models in cancer research

Animal models have been critical in the study of the molecular mechanisms of cancer and in the development of new antitumor agents (19). Although the mice, especially "humanized" ones, stay as the most important animal model, several other organisms are also used for cancer research. To name just a few, Drosophila flies were used to study and identify genes involved in growth regulation, yeast research opened new views on mechanisms of chromosome fragility, signaling pathways and several other aspects of the disease pathology and RNA interference studies in Caenorhabditis elegans revealed approximately 350 genetic interactions between genes functioning in signaling pathways, which are also frequently mutated in human diseases (7, 20, 21). These genetic maps could be used in identifying new components of specific disease-deregulated pathways (7).

Nevertheless, the majority of knowledge about carcinogenesis, cancer therapy, angiogenesis and metastasis comes from studies with "humanized" murine models (1, 16). The first such models were immunodeficient nude mice, which supported the engraftment of human tumor cells (16). CB-17-scid strain was discovered in 1983, when Bosma and co-workers identified a mutation in a protein kinase Prkdc^{scid}, causing a severe combined immunodeficiency (22). These mice could be engrafted intravenously or subcutaneously with some human neoplasms, whereas solid tumors were grown under the renal subcapsule (1, 16). However, innate immunity - the activity of natural killer cells (NK-cells) - limited tumor growth and prevented metastasis (16). Next developed model, non-obese diabetic-severe combined immunodeficiency (NOD-scid) mice allowed growth of human lymphomas and leukaemias, due to a more humanized microenvironment, achieved by injection of human peripheral blood or bone marrow cells (1, 16). The first such model was described in 1995 and was generated by crossing the *scid* mutation from CB-17 mice onto the NOD

background (NOD mouse is an animal model of spontaneous autoimmune T-cell-mediated insulin-dependent diabetes mellitus) (1, 23). Several other strains have been developed since then, allowing the research of myeloma, breast, colon, prostate and brain tumors (1, 24). Moreover, since observations showed that some subcutaneously injected tumor cells did not mimic the entire human pathology, tumor xenografts have been grown orthotopically (i.e. colon carcinomas injected into colon, melanomas into skin, mammary into mammary fat pad etc.) (16). Orthotopic implantations seemed to be more representative and allowed more accurate analysis of tumor growth, metastasis and evaluation of chemotherapy (4, 16).

Extrapolation from animals to humans

Stretching the observations provided by animal models to understand human pathology has been in many cases proven to be wrong (4, 25). For example, monkeys are resistant to emetogenic (vomiting) and thrombocytopenic properties of conventional anticancer drugs, while ill reputed drug Thalidomide does not cause birth defects in mice and rodents, but does so in primates and humans (1, 16). In short, animals are not human copies and care should be taken when interpreting obtained results (25). For example, mice were initially chosen as a representative model because they are relatively cheap, have high reproductive cycle and supposedly have similar developmental, physiological, biochemical, and behavioral patterns to humans. It is also worth noting that at the genotypic level - 99% of mouse genes have homologs in humans (16). But, results showed that mice have very different biochemical reactions, metabolic pathways and other physiological differences, such as dichotomic receptors, specific adhesion molecules and different levels of liver enzymes (16, 25). Furthermore, even “humanized” mice and rodents can not recapitulate all aspects of the human disease and they provide only approximations, but on the other hand, they enable insights into in vivo genetic and molecular mechanisms of various processes that would otherwise not be possible due to technical restrictions of in vitro systems or ethical constraints (1). Researchers also showed that rodents could reliably predict a safe starting dose for phase I studies, and with the help of mathematical models could also provide data on toxicology and pharmacology, although some vital requirements should be taken into consideration: because drugs and toxins affect organisms by the way they are metabolized and the way they are distributed in the body tissues and finally excreted, therefore the differences in the metabolic rate (rodents have higher metabolic rates then humans), metabolic patterns and other physiological differences (increased capillary density, higher heart frequency,...) between humans and rodents should be taken into account when one calculates the dosages of tested compounds (16). Scientists should always keep in mind, when working with animals, that they are only systems for predicting responses in humans and that extrapolation of obtained results should be carefully validated, either in vivo, using another animal species or in vitro, if possible.

Ethical considerations regarding research in animals

Interest in moral status of animals and their protection is by no means modern. For example, several ancient religions treated selected animals as sacred and almost all of them suggested that humans are not permitted to treat them in any way they please. In medieval Europe they have been acknowledged as subjects and have been even sent for trials and usually accused of committing a crime and brutally murdered. For example, in 1474 in Bassel a rooster was accused of laying an egg and was of course killed (http://www.ius-software.si/Novice/prikaz_Clanek.asp?id=23728&Skatla=17). On the other hand, the philosophical doctrine of Orient was totally different and regarded animals as equal beings (6).

In the Western countries, although the use of animals for experiments has always been a matter of great concern in the society, different tradition took root, one that states that animals exist only to serve human beings (6, 26, 27). French philosopher René Descartes (1596-1650) maintained that animals are nothing more than automatons, or robots, created by God, therefore it would be absurd to talk about humans having any moral or legal obligations to animals (6). Immanuel Kant (1724-1804) thought that animals are things, but people shouldn’t be cruel to them, because this cruelty could extrapolate to us (6). The Darwin’s theory of evolution (1859) provided a scientific rationale for using animals to learn about humans, and Darwin endorsed such use, although he was troubled by the suffering that experimentation could cause (28). The rising use of animals in scientific research inspired animal-protection movements, but the phenomenal success of medicine

silenced most of them (6, 28). The British Cruelty to Animals Act, introduced in 1976, balanced the rival claims and animal lovers receded into background until 1970, when a utilitarian philosopher Peter Singer started to advocate the rights of animals and generally opposed the use of animals in biomedical research (2, 28). Other important contemporary proponents of animal rights, but with slightly different views consistent more with deontological theory are Tom Reagan and Christopher D. Stone, who believe that animals have inherent rights (6, 28). There are several philosophical viewpoints that attempt to explain the moral status of animals, but all these major theories and their derivatives are subject to several objections. Classical utilitarianism, for example, has often been used to justify the use of animals in biomedical research, by making the argument that the benefits gained (e.g. development of vaccines for deadly diseases) from using animals outweighs the pain and suffering that animals must endure (6). On the other hand, as Singer says, this doctrine promotes equality, therefore all living beings are equal, so to count human suffering and ignoring animal suffering violates this rule (29, 30). Clearly, the present debate over animal use in research, testing and education is marked by different explanations of philosophical doctrines, different religious views and ethics based arguments (6, 27, 31). Only a few philosophers have lent their voices to researchers. One of them, Michael A. Fox, author of The Case for Animal Experomentation (University of California Press, 1986), was later convinced by the critics and became an advocate for animal rights (28). Other supporters of research noted that nature is cruel (cats play with mice, etc.), that humans eat animal meet, raise animals for food and that evolution has placed us on top, so it is natural for us to use other creatures (28, 31).

Nonetheless, a substantial majority of scientists believe that the use of laboratory animals is justifiable for the benefit of humankind (health, knowledge and safety), but they should be treated as humane as possible and they should not be suffering. The range of public and scientific opinions on the rights and wrongs of using animals in research is broad and is based on philosophical and religious views (6). On one side there is the liberty of humans to use animals for important research (knowledge, health and safety) and on the other side, a moral dilemma that animals are free beings and that we have no rights over them (6). To date, these questions remained unsolved. Scientists have been justifying the use of animals by stating that it is necessary for maintaining human and animal health, protection of the environment, and that in the absence of human data, animal research is the most reliable means for estimating the risks of new compounds (6, 8). On the other side, the growing number of animal protection groups throughout the world voiced considerable opposition to the use of whole animals for scientific purposes and even some scientists were skeptical: they stressed that our understanding of human cancer and other diseases cannot be gleaned from animal studies because genetic changes and control seem different (4, 5, 8).

Despite this, laboratory animals have been used extensively as experimental models in virtually all fields of biomedicine (8). After the famous bill Cruelty to Animals Act in 1876, several attempts have been made to write laws that regulate animal rights and welfare in science research. The book, published in 1959, The Principles of Humane Experimental Technique marked the beginning of determining ethical issues, humane endpoints and setting the general guiding principles for the use of laboratory animals (2, 5). In USA, the Animal Welfare Act of 1966 with amendments, sets the standards for the proper care and treatment of research animals (6). In Europe, the EU Animal Welfare Directive (Council Directive 86/609/EEC with amendments) and the Council of Europe Convention ETS123 guide welfare of animals used for experimental and scientific purposes (6, 32). The European Commission has been developing animal welfare legislation for over 30 years. The first Community legislation on farm animal welfare was adopted in 1974 and concerned the stunning of animals before slaughter. Since then, EU has already taken various steps to improve and supplement initial policies. Some of the main objectives of the Commission in the future are: to communicate on animal welfare in Europe and abroad, to upgrade existing minimum standards for animal protection and welfare, to promote policy-orientated future research on animal protection and welfare, to introduce standardized animal welfare indicators, to ensure that animal keepers/ handlers as well as the general public are more involved and informed on current standards of animal protection and welfare, and to continue to support and initiate further international initiatives to raise awareness and create a greater consensus on animal welfare (http://ec.europa.eu/index_en.htm). In Slovenia, Veterinary Administration of Republic of Slovenia regulates the area of animal welfare with the Ani-

mal Protection Act - official consolidated text (slo., Zakon o zaščiti živali, ZZZiv) (24). The legal foundations for animal rights were introduced in 1993 with the Environmental Act (slo., Zakon o varstvu okolja) and since then it has been improved and reconciliated with EU legislation (25, Ur.l. RS, št. 3972006, 04.04.2006). ZZZiv is part of this important Environmental Act, which regulates basic principles of protecting the nature, responsibility of humans for animal protection and welfare, defines the rules of proper animal care and lays down the directions for future amendments and extension acts. Moreover, several other international conventions bound Slovenia in the case of parliament ratifications, among them are Ramsar, Bonn, Bern and Washington convention, Conventions about biological diversity and protection of migratory animals, European convention for the protection of animals kept for farming purposes, etc. (http://ec.europa.eu/food/animal/welfare/references_en.htm).

The field of animal welfare is constantly evolving and through research, promotion of dialogue and general awareness of people that animals are not things or our property, but equal inhabitants of planet Earth and irreplaceable link in nature's equilibrium European commission is trying to establish the principle of humane treatment for all animals and how it should be applied in different fields of animal use (26, 32). More and more countries are adopting this point of view, which regards animals as independent beings and surpasses the established comprehension of animals as objects, originating from Roman times (6). It is commendatory that Slovenian Animal Protection Act regards animals in this way and thus we granted them status sui generis (Latin expression indicating an idea, an entity or a reality that cannot be included in a wider concept; independent entity) (Ur.l. RS, št. 3972006, 04.04.2006).

Conclusion: pro et contra

Currently, research involving laboratory animals is absolutely essential for maintaining human health and for the development of new treatments (2, 26, 33-35). Nevertheless, the emergence of sophisticated technologies in molecular and cell biology has enabled the development of reliable in vitro tests which could replace animal experiments (5, 6). Some scientists argue that these models lack a few critical points of multicellular systems, such as microenvironment, cooperation of all organs in the body and responses to different environmental stimuli. Next, the inability to study integrated growth processes, biochemical and metabolic pathways and loss of original phenotype in immortalized cell cultures, even more restrict their usage (6). On the other hand, opposing parties state arguments against animal use, such as: moral and ethical issues concerning the animal rights, physiological, genetic and epigenetic differences between animals and humans, which confer false positive or false negative results (6, 16).

But for now, because the use of animals in research is by law still justifiable, minimizing unnecessary suffering and use of animals in laboratories via the implementation of the three Rs (replacement, reduction, and refinement) are the most important goals researchers tend to achieve (2). Replacement refers to using sophisticated in vitro technologies when possible, reduction refers to minimizing the number of animals used for research and testing, when this is scientifically appropriate and valid, and lastly, refinement stands for optimizing the existing experimental protocols in a way that animals are subjected to less pain and distress (5, 6). Debate on these moral and ethical questions regarding animal use in research is bound to continue, but most, if not all, parties agree that promoting and implementation of the three Rs is desirable, when scientists must use animals for research (6). For now, human population must accept that in vitro methods act together with in vivo (whole-animal and clinical (human)) studies to advance science, develop products, drugs, treat, cure and prevent disease. However, the use of animals must be regulated by the strictest moral and ethical standards, and when scientifically possible, their use should give way to in vitro methodologies.

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UPORABA LABORATORIJSKIH ŽIVALI V BIOMEDICINSKIH RAZISKAVAH

P. Hudler

Povzetek: Poskusne živali se že dolgo uporabljajo v temeljnih in aplikativnih znanstvenih raziskavah, predvsem pri preučevanju anatomije, delovanja organov in celic, za izogibanju boleznim, njihovemu preprečevanju in zdravljenju, pri preučevanju obnašanja, pri razvoju, izdelavi, preizkušanju kakovosti, učinkovitosti in varnosti zdravil, živil in drugih snovi ali izdelkov ter tudi pri izobraževanju in usposabljanju študentov in delovnega osebja. Le z laboratorijskimi živalmi lahko dobimo odgovore na vprašanja, na katera drugače ne bi mogli odgovoriti, saj so poskusi na ljudeh nehumani in neetični. Kljub temu da raziskovalci poskušajo razviti ne-živalske modele, nekatere zapletene večgenske bolezni in testiranje zdravil zahtevajo uporabo živih modelov in analize zapletenih odzivov na preučevane dražljaje pri in vivo sistemih. Na splošno velja, da s poskusi na laboratorijskih živalih torej dobimo le približne odgovore na zastavljene raziskovalne probleme, vendar jih vseeno lahko uporabimo za predvidevanje in določanje odzivov pri človeku.

Ključne besede: etika, medicinska; živali, laboratorijski –poskusi; raziskave; bolezen, živalski modeli

Slov Vet Res 2007; 44 (3): 63-73
UDC 636.09:616.5-002.828:616.993-084

Review Paper

DERMATOPHYTOSES IN DOMESTIC ANIMALS AND THEIR ZOONOTIC POTENTIAL

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Summary: Dermatophytoses in domestic animals in this article are discussed from the zoonotic and practical point of view. Data on animal infections are compared to data from human research. Latest human and veterinary research on dermatophytes is presented with special emphasis on Slovenian research. The article explains the very reason why cats, among all different domestic animals, are the main reservoir of M.canis infection for people. It also explains the reason why among all dermatophytoses M.canis infection is the most difficult to control. .

Key words: zoonoses–microbiology; dermatomycoses-epidemiology-transmissions–pathology; diseases reservoirs; infection control-methods; dogs; cats; horses

Introduction

In the scope of the zoonotic aspect it is necessary to discuss dermatophytoses being the group of the most common fungal diseases in dogs and cats. Most of them are zoonoses (1). But from the practical point of view we need to realize that dealing with the patient at the very beginning of the disease we don't know about the final diagnosis. Therefore one need to consider more than one differential diagnosis while observing the alopecia anywhere on the body. At least the most important among them will be mentioned in the article (2).

Among all very different species of domestic animals people live probably in the closest contact with dogs and cats, as they don't take a cow or a horse into their beds. Dislocated habitat of economic animals enables veterinarians to manage dermatophytoses in these groups of animals differently. Trichophytosis in cows, for example, can be managed using live vaccines but they cannot be used in pet animals because of the close contact with the people (3). Close contact is also the reason that the main source of infection to the people represents cats and less frequently dogs (2).

Received: 4 July 2007
Accepted for publication: 30 August 2007

Classification of dermatophytes

Fungi are omnipresent in our environment. Amidst thousands of different species of fungi only a few have the ability to cause disease in animals. The great majority of fungi are either soil organisms or plant pathogens; however, more than 300 species have been reported to be animal pathogens (1).

The term *fungus* includes yeasts (unicellular) and moulds (multicellular-filamentous). Dimorphic fungi are capable of existing in both morphologic forms that may depend on the temperature or type of the media. Dogs and cats harbour many saprophytic moulds and yeasts on their hair and skin. The most common of these fungi isolated from dogs are species of *Alternaria, Aspergillus , Aureobasidium, Chrysosporium, Mucor, Penicillium and Rhizopus*. In cats, the most commonly isolated fungi are the species of *Alternaria, Aspergillus, Chrysosporium, Cladosporium, Mucor, Penicillium, Rhodotorula and Scopulariopsis*. Most of these saprophytic isolates presumably represent repeated transient contamination by airborne fungi or by fungi in soil (2) and are important as contaminants on fungal cultures, making interpretation difficult.

Fungal skin diseases (mycoses) can be divided into superficial, subcutaneous and systemic. The superficial mycoses are fungal infections that in-

volve superficial layers of the skin, hair and claws. The organisms may be dermatophytes such as *Microsporum* and *Trichophyton* which are able to use keratin. However other fungi such as *Candida (Monilia), Malassezia (Pityrosporum)* and *Trichosporon (piedra)* may also produce superficial mycoses.

The filamentous fungi which invade skin (derma) and keratinized tissues (hair, nails, etc.) are dermatophytes. They are a group of closely related fungi, classified into three genera:

Microsporum (Nannizzia)

Trichophyton (Arthroderma)

Epidermophyton (no sexual form described yet)

The dermatophytes are traditionally placed into the *Fungi Imperfecti* but for some of them the perfect (sexual) stage has been described and they are classified as *Ascomycetes. Nannizzia* sp. is the teleomorph for *Microsporum* sp. and *Arthoderma* sp. is the teleomorph for any *Trichophyton* species.

Today near 40 species of dermatophytes are known. In the pathological processes the conidial (asexual = imperfect) form is taking part. Therefore the human-, and veterinary literature is still using the so called „imperfect" names (i.e.: *Microsporum* instead of *Nannizzia*).

Three species cause the great majority of clinical cases of dermatophytosis in dogs and cats: *Microsporum canis, Microsporum gypseum*, and *Trichophyton mentagrophytes. M.canis* and *T. mentagrophytes* are zoophilic dermatophytes that have become adapted to animals and are rarely found in soil. *M. gypseum* is a geophilic dermatophyte that normally inhabits soil. *M.canis* is in general the most common cause of dermatophytosis in cats and dogs (2).

Trichophyton equinum is the most common causative agent of dermatophytosis in a horse. Other agents that may cause dermatophytosis in a horse are: *T. mentagrophytes* (natural reservoir are rodents), *T. verrucosum* (natural reservoir are ruminants), *M. canis* (natural reservoir are cats) and *M. gypseum*, the latter being a geophilic dermatophyte. A horse can get infected with *M. gypseum* when coming into contact with the soil but it may exceptionally be transmitted from another infected horse as well (4).

According to the extensive research, done in people from 1995-2002 on 42494 samples from University Medical centre Ljubljana, *M.canis* was the most frequent dermatophyte isolated (46,8%), followed by *Trichophyton rubrum* (36,7%), *T. mentagrophytes var. interdigitale* (7,9%), and *T. mentagrophytes var. mentagrophytes* (4,9%), while other species, including *M. gypseum*, were isolated less frequently (5).

Pathogenesis of dermatophytoses

Dermatophytes can grow only on the hairs that are in anagen phase (6,7). In the tellogen phase follicles behave as saprophites (8). Hyphae of the fungus are embedded into stratum corneum, infundibulum of hair follicle and hair. To be successfully attached, dermatophyte should stay in the contact to the skin for about 2-3 hours (9). That's the period when people holding the infected cat, for example, can prevent infection if they act according to the common hygiene praxis. Four hours after attachment reproduction of the fungus already begins. (9). This is probably the reason as well for the difficulties researchers had encountered with while establishing experimental infection in cats that were allowed to groom. It is possible that grooming may be an under recognized host defence mechanism (10). Dermatophytes grow on the surface of the hair and migrate toward root. Proteolytic enzymes also enable them to grow in the medulla. When hair enters tellogen phase, the production of keratine slows down and finally stops. With this dermatophytes stop growing as well. Infectious arthrospores may persist on the hair for a long time (at least 18 months) but they may not re-infect the same follicle until the new hair begins to grow (6, 11).

Incubation period in animals infected with *M. canis* is not well defined. It ranges between 4 days and 4 weeks (5) in cats and 1 – 4 weeks in horses (5). The cause for such a loosely defined incubation period might be that we hardly control free roaming cats therefore the time of infection is hard to establish. Additional problem in cats are frequent asymptomatic carriers. Establishing incubation period in experimental conditions can be of great help and it was found to be between 7-14 days in cats (2, 12).

Because of their weaker specific and nonspecific immunity young animals and children become infected more often (4, 6, 13, 14, 15). One survey, done on 1011 humans being treated at University clinical centre of Ljubljana, revealed that 95,5% of dermatophyte infected were younger of 15 years old (15). We know that concurrent FIV, FeLV, Ehrlichia or Leishmania infections, cancer diseases or immunosuppressive therapy predisposes for dermatophyte infection or worsens the clinical course of the disease (6, 16, 17, 18). Dermatophytosis is three times more prevalent in cats with feline immunodeficiency virus than in uninfected cats (16). Among dog breeds surveyed in southern Italy Yorkshire terriers showed the highest positiveness (14) while

Persian cats are well known to exhibit chronic dermatophyte infections.

Clinical presentation in a dog

As the infection is almost always follicular the most consistent clinical sign in a dog is one or many circular patches of alopecia with variable scaling. Lesions occur most commonly on the face, pinnae, paws, and tail. Pruritus is usually minimal or absent. Dogs most often exhibit the classic ring lesion with central healing and fine follicular papules and crusts at the periphery. However, less common syndromes with occasionally marked pruritus are frequent enough that dermatophytosis should be considered in the differential diagnosis of any annular, papular, or pustular eruption (2).

Based on the history and clinical signs one make the list of differential diagnoses that in classical cases include bacterial folliculitis and demodicosis. In less commonly described clinical presentations the differential diagnoses should be as follows:

- symmetric nasal or facial folliculitis and furunculosis (diff. ▼ pemphigus complex)
- generalized infection with seborrhea-like eruptions (diff. ▼ seborrheic diseases)
- dermatophyte kerion (diff. ▼ histiocytoma)
- onychomycosis (diff. ▼ bacterial nail infection, autoimmune disease)
- dermatophytic pseudomycetoma (diff. ▼ mycetomas caused by other fungi)

Clinical presentation in a cat

Feline dermatophytosis most often appears as one or more irregular or annular areas of alopecia with or without scales. Hairs in these areas often appear broken and frayed. The alopecia may be severe and widespread, accompanied by little evidence of inflammation.

Cats occasionally have more inflammatory areas of folliculitis characterized by alopecia, erythema, scale, crust, and follicular papules.

Other clinical presentations are:

- miliary dermatitis (pruritic, papulocrustous dermatitis, diff. ▼ flea allergy dermatitis)
- chin folliculitis (diff. ▼ feline acne)
- dermatitis of the dorsal tail (diff. ▼»stud tail«)
- onychomycosis (diff. ▼ bacterial nail infection, autoimmune disease)
- generalized infection with seborrhea-like eruptions (diff. ▼ seborrheic diseases)
- exfoliative erythroderma (diff. ▼ endocrine disorders)
- eroded lesions due to self-grooming (diff. ▼ eosinophilic plaque)
- dermatophyte kerion (diff. ▼ neoplasm)
- otitis externa (diff. ▼ other causes of otitis)
- dermatophytic pseudomycetoma (diff. ▼ mycetomas caused by other fungi, neoplasm) Last feature was described only in Persian cats (19, 20).

The nature of the dermatophyte cannot be determined from the clinical presentation. Moreover, cats may often be asymptomatic carriers of the disease (8, 21): among show cats, cats from shelters and those that are often taken to the veterinarians, 6.5 to 100% has been found to be asymptomatic carriers (6). Half of the infected cats can be without clinical signs of the disease (11). In opposite, asymptomatic carriers among dogs are rarely found (22) and may represent 5% of infected dogs (23). Lately, 2.16 per cent of asymptomatic *T. mentagrophytes* carriers were found among 169 clinically healthy cats in the southeast of England. Asymptomatic animal carriers should be considered when treating humans with trichophytosis (24).

Clinical presentation in a horse

Not all horses in contact got infected (more often young and immunosupressed animals). Majority of cases occur from autumn to spring. Skin changes may vary in their appearance but are prevalent on the head, neck and extremities. Alopecic spots of different size, with or without erythema and squames are typical. Rarely pruritus or pain may be present.

When dealing with alopecic changes in a horse we need to consider differential diagnoses as follows:

- demodicosis (*Demodex Cabali, Demodex equi*)
- dermatophylosis (*Dermatophylus Congolensis*)
- bacterial folliculitis (*Staphylococcus aureus, hyicus, intermedius*)
- hypersensitivities (5).

Diagnosis of dermatophytosis

As already pointed out, one cannot establish final diagnosis merely on the base of the clinical features of the disease. In cats symptoms may often be absent and one gets the cat presented for examination because infection in humans has been confirmed. According to some data, infection of at least 1

member was confirmed in 30 to 70% of households keeping infected cat (2). In opposite, one should not exclude dermatophytosis on the fact that none of the humans in contact yet got infected. Successful infection may depend on many factors, like immune status of the recipient and the time of exposure to the contagious material. History-taking may be of limited value unless exposure is known to have occurred; this is so because clinical dermatophytosis is so variable and the incubation period is incompletely defined. The number, types, and sources of contact animals should be determined.

Wood lamp examination

For fluorescence causes only certain strains of *M. canis, M. audouinii, M. distortum, and Trichophyton schoenleinii* to produce a positive yellow-green colour on infected hairs. The Wood's lamp is an ultraviolet light with a light wave of 253.7 nm that is filtered through a cobalt or nickel filter (2). The Wood's lamp should be turned on and allowed to warm up for 5 to 10 minutes because the stability of the light's wavelength and intensity is temperature dependent (1, 25). The animal should be placed in a dark room and examined under the light of the Wood's lamp. When exposed to the ultraviolet light, hairs invaded by *M. canis* may fluoresce in about 50% of the isolates (6, 11,13, 25). Hairs should be exposed for 3 to 5 minutes because some strains are slow to show the obvious yellow-green colour. The fluorescence is due to tryptophan metabolites produced by the fungus (26). Positive fluorescence should be distinguished from false positive fluorescence due to presence of certain bacteria (*Pseudomonas aeruginosa, Corynebacterium minutissimum*), keratin, soap, petroleum, and other medication. These fluorescing hairs should be plucked with forceps and used for inoculation of fungal medium or for microscopic examination (2).

Microscopic examination

One perform microscopic examination by adding 20 % KOH to the hair, scales, and claw material on microscope slide, adding the cover slip and heating (but not boiling) the sample for 15-20 seconds. Instead of heating the preparation may be allowed to stand for 20 minutes at room temperature (6). Alternatively to KOH, lactophenol can be used without heating (11).

Direct examination may reveal hyphae and arthrospores in 40-50% of the cases (6, 11) but cannot distinguish between different dermatophyte species (26). When the result is positive it is a definitive evidence of dermatophytosis (6).

Microscopic examination with fluorescent microscope

Material (hairs) is placed on the microscopic slide, 2 drops of 10 % KOH solution are added and then mixed. Then 2 drops of calcofluor sre added, mixed and slide covered. Calcofluor is colorless fluorescent stain that fixes to B1-3 and B1-4 polisaccharides that build in the cellulose and chitine. Stained preparation is then exposed to ultraviolet light and green fluorescent fungal elements can be seen. (11).

Microscopic examination with fluorescent microscope is rarely used because of the need of special equipment but it can be useful. According to some data it can be efficient in more than 50% of cases (11).

Fungal culture

Fungal culture is needed for species of dermatophyte to be identificated (11). Collecting the hairs may be done by plucking the damaged hairs from the margin of the alopecic lesion or by brushing the haircoat all over the body, whenever asymptomatic infection. One should avoid taking hairs from all over the body if skin modifications are detected. Collecting the hairs in this manner encourages contamination of the specimen with saprophitic fungi.

Sabouraud's dextrose agar and dermatophyte test medium (DTM) are traditionally used in clinical veterinary mycology for isolation of fungi (2). SDA is a classical Sabouraud's dextrose agar containing penicillin and streptomycin that most of fungi grow on it. The antibiotics are added to prevent growing of bacterial contaminants. Sabouraud's dextrose agar containing chloramphenicol and actidion (SCA) is a selective culture plate because chloramphenicol prevents growing of most of the bacteria and actidion prevents growing of most of the saprophytic fungi (11). Dermatophyte test medium (DTM) is essentially a Sabouraud's dextrose agar containing cycloheximide, gentamicin, and chlortetracycline as antifungal and antibacterial agents. The pH indicator phenol was added. Dermatophytes first use protein in the medium with alkaline metabolites turning the medium from yellow to red. When the protein is exhausted the dermatophytes use carbohydrates giving off acid metabolites. The medium changes from red to yellow. The majority of other fungi use carbohydrates first and proteins only later; they too may produce a change to red in DTM – but only af-

ter a prolonged incubation (10 to 14 days or longer). Consequently, DTM cultures should be examined daily for the first 10 days. Fungi such as *Blastomyces dermatitidis, Sporothrix schenkii, H. capsulatum, Coccidioides immitis, Pseudoallescheria boydii,* and some *Aspergillus* species may cause a change to red in DTM, therefore microscopic examination is essential to avoid an erroneous presumptive diagnosis (2).

Skin scrapings, claws, and hair should be inoculated onto Sabouraud's dextrose agar and DTM. Desiccation and exposure to ultraviolet light hinder growth. Therefore, cultures should be incubated in the dark at 30° C with 30% humidity. A pan of water in the incubator usually provides enough humidity. Cultures should be incubated for 10-14 days and should be checked daily for fungal growth. Proper interpretation of the DTM culture necessitates recognition of the red colour change simultaneously with visible mycelial growth (2). One study showed that increased incubation temperature (24-27°C) had resulted in a more rapid colour change on a DTM developed for animals and suggested that incubation at room temperature might account for false negative culture results (27). The interpretation of the positive fungal culture results should be taken with care since dermatophytes are also isolated from the hair coats and skin of normal dogs. It is likely that dermatophytes isolated from normal dogs and cats – such as *M.gypseum, T.mentagrophytes* – simply represent recent contamination from the environment. This is particularly true in outdoor animals or hunting dogs (6). In one study anthropophilic dermatophytes were isolated from about 10% of the stray cats in various animal shelters indicating that cats can automatically carry human pathogens. *M. canis*, however, is undeniably present as a persistent infection in many asymptomatic infected cats (2).

ELISA diagnostic method

ELISA diagnostic method has been developed for the diagnostics purposes of *M. canis* infection in cats. Antibodies against *M.canis* were measured in the group of naturally infected cats and compared to the group of healthy cats that were brought to the clinic for vaccination or sterilisation purpose. Significantly higher antibody titres had been measured in the group of infected cats compared to the group of healthy cats. The presence of certain amounts of the antibodies in the blood of healthy cats had been explained with possible cross-reactions with saprophytic fungi or the possibility that reactive animals had recovered from infection in the past (28, 29 30). The method that has been developed at Veterinary faculty of Ljubljana exhibited 75,0% of sensitivity and 91,7% of specificity. Prediction value for the negative result was 68,8% and represented the possibility that the animal was healthy if the test was negative. Prediction value for the positive result was 93,8 % and meant the possibility, that the animal was infected if the test positive. Using this method one would be able to treat presumably infected cat before getting the fungal culture results with the minimal risk of misdiagnosis if ELISA test would be positive (30). Unfortunately the test is not routinely available.

Clinical management of dermatophytosis

Dermatophytosis in healthy dogs and short-haired cats often undergoes spontaneous remission within 2 months (dogs) to 4 months (cats) (8, 31). Dermatophytosis in a horse with a few solitary lesions is often a self-limiting disease. When treated it usually takes 6 – 8 weeks to resolve. Kos and Kramarič reported on 4 cases that were clinically solved after 3 - 10 weeks of treatment (32).

Cats infected with *M. canis*, however, can undergo chronic infection and usually require aggressive therapy. Cats represent the main host for this fungus and *M. canis* is well adapted to them therefore when infected only minor inflammatory reaction evolves in the majority of cases. This is probably attributed to the fact that cats are often asymptomatically infected (1, 8, 25). Even longhaired cats can undergo spontaneous resolution but it may take 1.5 - 4 years (2). The goals of therapy are (1) to maximize the patient's ability to respond to the dermatophyte infection (by the correction of any nutritional imbalances and concurrent disease states and by the termination of systemic anti-inflammatory and immunosuppressive drugs), (2) to reduce contagion (to the environment, other animals, and humans), and (3) to hasten resolution of the infection. A critical feature of clinical management is the treatment of all dogs and cats in contact with the infected animal and the treatment of the environment (2).

Every confirmed case of dermatophytosis should receive topical therapy (2). In cats and dogs it is instructed that hair should be clipped from a wide margin (6 cm) surrounding all lesions. Although clipping may worsen and/or spread the lesions it is more important to get rid of infected hairs (8). Owners should use clippers at home since clipping in

the veterinary practice may contaminate the room (2). Clipping is not routinely instructed when treating dogs and cats at Veterinary faculty of Ljubljana. Although it is not necessary in all cases of dermatophytosis, clipping of the hair coat is optimum (10).

A wide variety of topical antifungals is available and there is no particular advantage of one product over another. Creams and lotions are available for use on focal lesions. For dogs with multifocal or generalized skin involvement and always in cats and horses, antifungal rinses (dips) are indicated. Rinses are preferred because the entire body surface can be treated, rubbing of the hair coat is minimized, and the antifungal agent can be allowed to dry on the skin. Lime sulphur (1:16), enilconazole and miconazole have been consistently effective and captan, chlorhexidine (as a single agent), and povidone iodine have been consistently ineffective antifungal agents. Sodium hypoklorite has shown mixed results (10). Chlorhexidine in combination to miconazole, however, expressed synergistic effect to *M. canis* (33). Topical medicaments should always be continued until two or preferably three fungal cultures at weekly intervals are negative (2).

Zoniton^{R} (enilconazole) solution for topical treatment is used at Veterinary faculty of Ljubljana. Though it is not registered for use in cats it has yet been in use for years. Clinically any serious side effects were noted. In two studies enilconazole was evaluated as a sole topical therapy (post whole body clipping) for the treatment of naturally occuring *M.canis* infection in Persian cats. The treatment twice a week has resolved infection in 4-5 weeks, whereas l' of placebo treated cats were still culture positive at end of 10 weeks of monitoring. Enilconazole was well tolerated but may have been associated with hypersalivation, anorexia, weight loss, emesis, idiopathic muscle weakness, and slightly elevated serum alanine aminotransferase (ALT) concentrations (34, 35).

Dogs and cats that have multifocal lesions, all longhaired animals, and those in multiple animal settings should receive systemic antifungal therapy. Animals that are not responding to topical therapy after a 2 to 4 week course of treatment should also receive systemic therapy (2). It is rarely necessary that systemic treatment should be used in a horse (4).

Griseofulvin is still the drug of choice in US but it has lately not been available in Slovenia. In Slovenia the registration for ketokonazole that is active against many fungi and yeasts, including dermatophytes, *Candida*, *Malassezia*, and numerous dimorphic fungi responsible for systemic mycoses has expired as well.

Itraconazole is a triazole and compared with ketokonazole, has increased potency, decreased toxicity, and wider spectrum of action. At low doses it is fungistatic and at higher doses fungicidal. Susceptible organisms include dermatophytes, *Candida* spp., *Malassezia*, those causing many intermediate and deep mycoses, *Aspergillus, Sporotrichum*, and the protozoans *Leishmania* and *Trypanosoma*. Doses for cats are 10mg/kg q12h-20mg/kg q48h. Doses for dogs are 5-10mg/kg/day. In two studies, using itraconazole as a sole therapy, 13 of 14 cats, either naturally or experimentally *M. canis* infected, were cured after 56 days (8 weeks) of therapy. One (naturally infected) cat has been cured after 70 days of therapy (36, 37). Anorexia, nausea, and hepatotoxicity are the primary side effects, while teratogenic effects haven't appeared at therapeutic doses. In Slovenia itraconazole in 100 mg capsules or 10 mg/ml oral solution (Sporanox^{R}) is available. In Austria veterinary product Itrafungol (10mg/ml) is available as an oral solution for dogs and cats.

Terbinafine is an allylamine that is well absorbed orally in the presence or absence of food. Terbinafine is active against dermatophytes, *Candida* spp., *Sporotrichum*, and *Aspergillus* spp. Latest research in dogs shows the activity against Malassezia yeasts as well (38, 39). The major side effects are gastrointestinal. No embryonic or fetal toxicity or teratogenicity has been demonstrated. Effect of terbinafine in humans is fungicydal while terbinafine in veterinary infections exhibits primary cidal activity against only 66% of *Microsporum canis* isolates but almost complete cidal effect in *Trichophyton* (40).

In our study done at Veterinary faculty of Ljubljana three groups of cats were experimentally infected with *M. canis* and monitored for 120 treatment days. Two doses of terbinafine were compared with each other and untreated control group. There was no difference when low dose terbinafine (10-20 mg/kg) was compared with the untreated control group. The cats receiving high dose terbinafine (30-40 mg/kg) were considered cured after > 120 days (16 weeks) of therapy (41). Eleven of 12 naturally infected cats, treated with terbinafine 30 mg/kg once daily for 14 days, were cured in 60-90 days (8-12 weeks) (42). In one study 41 naturally *M. canis* infected dogs and 24 naturally *M. canis* infected cats were treated with terbinafine at a dose of 10-30 mg/kg once daily. The mean length of therapy for mycological cure for

dogs was 53 days (21-126) and 63 days (28-84 days) for cats (43). Even in prolonged treatments with terbinafine no resistance of fungi is expected (42). One should use higher doses (30-40 mg/kg every 24 h) and expect longer treatment courses in *M.canis* infection compared to other dermatophytoses, as it should be expected also in children with tinea capitis (12 weeks of treatment with 60% healed) (10, 44).

Clinical management of dermatophytoses in ruminants is successfully implemented through chemoprophylaxis. Vaccines that are most often used in Slovenia nowadays for this purpose are Trihoben^{R} (Bioveta, Chech Republic) and InsolTrichophyton^{R} (Intervet, Netherlands).

Optimum treatment protocol for dogs and cats

According to recommendations the optimum treatment protocol for dogs or cats with dermatophytosis involves a combination of clipping of the hair coat, twice a week topical antifungal therapy, concurrent systemic antifungal therapy, and environmental decontamination. Fungal culture monitoring should be performed every 2-4 weeks until mycological cure (two or three negative consecutive fungal cultures) (10).

Prevention of infection

After resolution of infection animals and humans remain immune to re-infection for the certain period of time. The longest immunity persists at the skin of the previous infected site (45) while general immunity in cats can last at least 8 months (46). Based on the results of one study delayed intradermal testing (IDT) with *M. canis* extract can be used to asses the cellular immune response of cats with dermatophytosis (47). For prevention as resolution polymorphonuclear neutrophils and macrophages are undoubtedly responsible (3). Unfortunately killed or recombinant vaccines don't protect animals against experimental infection nor they allow the vaccinated animals to recover more quickly than the control ones (3).

Several commercial vaccines against feline dermatophytosis are available but their prophylactic efficacy has not been reported (10). In contrast vaccination against ringworm in other species (cattle, horses, fur-bearing animals) has been spectacularly successful in many countries. Their use has reduced the incidence of the disease in these animals considerably and indirectly contributed to the reduction of human infections. However these attenuated vaccines frequently induce small lesions at the injection site and subsequent dissemination of arthrospores into the environment. In a farm or a ranch situation, this may be of negligible consequence as long as all animals are included in the vaccination programme. However for cats which are generally housed indoors and have frequent and close contact with their owners, a live-fungus vaccine, able of producing active lesions, is highly undesirable because of the zoonotic hazard (3).

Environmental contamination can be important source of recurrent or persistent infection in one household. In one survey 100% environmental contamination of air and surfaces was found where infected cats were kept but less than 50% environmental contamination was found where infected dogs were kept, showing that infected cats appear to cause substantial environmental contamination, and provoke a substantial presence of viable airborne fungal elements (48).

In the household harbouring infected dog or cat one can prevent spreading of infection to other inhabitants by strict environmental disinfection and personal hygiene measures (see treatment chapter). A part of routine management of a cat that have been found and adopted, should always be fungal culturing. Adopted animal should be quarantined till the results of culturing are obtained.

Environmental treatment

Environmental treatment is as important as animal treatment since fungal arthrospores can remain viable and contagious on infected hairs at least 18 months (6, 49). For environmental treatment of cats' and dogs' habitat chlorhexidine or enilconazole was prooved effective. In Slovenia chlorhexidine is available as a powder (Virkon-S^{R}, Krka) mixed in a 5% solution with water. We can also use enilconazole (Zoniton^{R}, Krka) mixed 20 ml with 1 L of water. The first treatment is more cost-effective. The textiles should be washed at minimum 50°C if possible (6).

Environmental treatment of stables and farm facilities is best executed with fungicydal desinfectants, like 3% kaptan, 3% kresol or 50% kalii peroxisulphate (Virkon-S^{R}, Krka) (32).

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DERMATOFITOZE PRI DOMAČIH ŽIVALIH S STALIŠČA ZOONOZ

T. Kotnik

Povzetek: V članku avtorica obravnava dermatofitoze pri domačih živalih s stališča zoonoz in s praktičnega stališča. Podatke o okužbah pri živalih primerja z izsledki raziskav pri ljudeh. Obravnava novejše humane in veterinarske raziskave o dermatofitozah s poudarkom na slovenskih. Članek razlaga, zakaj so od vseh vrst domačih živali ravno mačke glavni rezervoar okužbe za ljudi. Članek tudi pojasni, zakaj je mikrosporoza najteže obvladljiva med vsemi vrstami dermatofitoz.

Ključne besede: zoonoze–mikrobiologija; dermatomikoze–epidemiologija–prenos–patologija; rezervoarji okužbe; infekcija, nadzor–metode; psi; mačke; konji

Slov Vet Res 2007; 44 (3): 73-81
UDC 636.7.09:612.432.062:615.252:615.244:615.073

Original Research Paper

RETINOIC ACID AS A THERAPY FOR CUSHING'S DISEASE IN DOGS: EVALUATION OF LIVER ENZYMES DURING TREATMENT

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Summary: Recent studies have demonstrated that retinoic acid is capable to control the Pituitary-Dependent Hyperadrenocorticism by its action on the ACTH-secreting pituitary tumour. Because the hepatotoxic effects of retinoic acid have been reported, the hepatograms of dogs with Cushing's Syndrome treated with this medicine were analysed during 180 days of therapy and compared with dogs which received Ketoconazole as an alternative treatment. No animal showed hepatoxicity signs with both treatments. Dogs treated with retinoic acid showed a decrease (not significant) of the alkaline phosphatise (AP), without any changes in the other group. Alanine amine transferase (ALT) was reduced after 180 days vs. 0 days (P = 0.04) in the retinoic group. On the other hand, this enzyme increased in the Ketoconazole group (P<0.02). Alanine aspartic transferase (AST) did not show any variations in the retinoic group, remaining within the baseline values. In the Ketoconazole group a significant increase (P<0.03) was seen after 180 days vs. 0 day. The reduction of ALT and AP in the retinoic group is related to the decrease of ACTH (r=0.41, P=0.026 and r=0.37; P=0.035, respectively), there are also correlation between ALT and urine cortisol: urine creatinine ratio (r=0.40, P=0.028) in this group. These variables do not correlate in the Ketoconazole group. In conclusion, retinoic acid shows no risk of hepatoxicity after180 days of treatment in dogs with PDH.

Key words: cushing syndrome–physiopathology–therapy; pituitary ACTH hypersecretion; tretionin–therapeutic use; liver–drug effects; dogs

Introduction

Pituitary-Dependent Hyperadrenocorticism (PDH), is caused by the corticotroph adenoma of the pituitary (1). Medical treatments are the most frequently used and are based on the drugs that exercise their effects over the adrenal gland. These drugs have either cytostatic action, such as the o,p'-DDD, or are enzyme inhibitors that act in different steps of the corticosteroid synthesis , such as aminoglutemide, ketoconazole (Ktz), and trilostane (2, 3, 4, 5). Ketoconazole is routinely used in Argentina for the treatment of PDH because the other mentioned drugs are not available. It has been reported that Ktz has hepatotoxic effects which are demonstrated by the increase of transaminases and clinical signs of hepatic insufficiency, such as anorexia, vomiting and jaundice (6).

It is known that retinoic acid (RA) acts through its receptors at genomic level, regulating the expression of genes for transcription factors involved in the synthesis of the proopiomelanocortin-peptide (POMC), precursor of adrenocorticotrophin hormone (ACTH) (7, 8). It also interacts with factors that intervene in cell development and mitosis, particularly the bone morphogenetic protein 4 (BMP-4), as recently described (9). Paéz-Pereda and colleagues (10) reported that RA inhibited the function (synthesis of POMC and ACTH) and growth (inhibition of

Received: 16 May 2007
Accepted for publication: 22 June 2007

the cell mitosis) of the tumoral cell, inducing its apoptosis in experimental rats. Based on this report, the therapeutic action of RA was studied in dogs with PDH and proven presence of corticotrophinoma, resulting in the control of the disease (11).

It is known that dogs with PDH present hepatomegaly with an increase of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP) due to an increase in their enzymatic activity (12). This is caused by an effect of the glucocorticoids on different functions of the hepatocytes, especially on gluconeogenesis and the glycerogenesis (13, 14).

As RA has been described as having a hepatotoxic effect (15), the aim of the present study was to determine if the aforementioned drugs in the effective dose for the treatment of PDH can provoke change on the liver enzyme activity (as indicators of possible liver damage) compared with Ktz. Furthermore, we aimed to determine if the control of hypercortisolism has a relationship to the changes in the hepatic enzymogram according to the treatment received.

Material and methods

Study population.

40 dogs with PDH were studied (11 male and 29 intact female). Average age of the animals at the time of the diagnosis was 9 years (range 3–14 years old), there were no significant differences between males and females. Dogs included in the study showed the following clinical signs, characteristics and consistent with PDH (12): polyuria–polydipsia, urinary density less than 1010, polyphagia, dermatologic problems, pendulous abdomen and anoestrus. Routine biochemical analysis was indicated as a control of the general status of the dogs. PDH was confirmed with specific endocrine test (ACTH stimulation test, urine cortisol:urine creatinine ratio and high-doses of dexamethasone suppression test). Besides that, the presence of the pituitary tumour has been visualised by Magnetic Nuclear Resonance Imaging (MNRI).

At the time of the diagnosis, none of the animals showed signs of concurrent diseases except for the hepatomegaly and the corresponding increase of ALT, AST and AP

A simple blind study was performed and dogs were randomly distributed into two groups according to the drugs they received over 180 days: A) Retinoic Group (RG): 20 dogs (5 male and 15 female). They were administered RA as isotretinoin 9-cis (2 mg/kg/once a day) because this is the form that binds to both isoforms of the recipient (8, 16). B) Ketoconazole Group (Ktz): 20 dogs (6 male and 14 female): They were administered ketoconazole 20 mg/kg/once a day.

Evaluation of the hepatic enzymogram

Hepatic enzymes AP (normal up to 250 UI/L), AST and ALT (both normal up to 80 UI/L) were evaluated in time "0" (at the time of the diagnosis) and 180 days after start of treatment. Basal and day 180 values were compared. Enzymatic determinations were performed by means of the automated kinetic method (ByoSystems®, Metrolab Autoanalizer Merck, Germany). The inter and intra-assay coefficients of variation were 1.1% and 4.5% for AP, 1.8% and 5.3% for ALT and 1.4% and 5.9% for AST.

Criteria for suspension of treatment

It was decided to separate any animal from the study protocol in case of: a) observing at least three of the following signs indicating hepatic insufficiency: jaundice, vomiting, anorexia, ascites, cachexia, hypocholia or acholia, increase of urobilinogen and/or bile pigments in urine. Presence of petechiae or echimosis and alterations of the coagulation tests (Quick time, Activated partial thromboplastin time, Altered bleeding time), b) 3-time increase of the AP and ALT, and 2-fold increase of AST during the treatment period, over the value found at the time of diagnosis (with presence or absence of the aforementioned signs).

Measurement of plasmatic ACTH, urine cortisol / urine creatinine ratio (C/C) and ACTH stimulation test

Basal plasma ACTH (22-250 pmol/L) and C/C (10-65, data from Laboratory of Nuclear Medicine, School Animal Hospital, Faculty of Vet. Sci.-U. Buenos Aires) determinations were carried out as was previously described (11, 17). Both were evaluated at day 0 (time of diagnosis) and day 180. ACTH (Nichols Advantage ACTH Assay, Nichols Institute Diagnostics, Bad Vilbel, Germany), and urinary cortisol (DPC Corporation, San Diego, California, USA) were measured by immune-radiometric assay and radioimmuno-assay respectively. The intra-assay variation coefficient of ACTH was 3%, with an inter-assay variation coefficient of 6.8%. The inter- and intra-assay coefficient of the cortisol was 8% and 5%, respectively. Creatinine (meas-

ured by automated kinetic method, ByoSystems®, Metrolab Autoanalizer Merck, Germany) inter and intra-assay coefficient of variation was 1.5% and 5.3% respectively.

High-doses of dexamethasone suppression test were performed in accordance with the method described by Rijnberk (17) and Galac (18). The urine samples (an aliquot of 2 ml for each) from each dogs were home collected in a non stressed environment into 2 flasks as follows: Flask 1 (as diagnostic of hypercortisolism and previous dexametasone intake), the second voided urine in the morning, the last one at night and the first urine of the following day (all mixed in the same flask). After collection of the last sample of urine on the second day, 0.1 mg/kg/w PO each 8 hours of dexametasone were given during one day. Flask 2: the first morning urine on the third day after dexametasone intake was obtained. It was consider suppression when the C/C of the flask 2 is less than 50% than C/C of flask 1.

ACTH stimulation test was performed as described previously (3) in order to confirmed the C/C result obtained from flask 1. Both ACTH stimulation test and high-doses of dexamethasone suppression test (data not shown) were carried out only at diagnosis time.

Statistical Analysis

Results are expressed as median and range. The comparisons between intra- and inter-group were made by Wilcoxon signed rank test (time "0" vs. 180 days), being significant $P<0.05$.

AP, ALT and AST values prior and post-treatment were correlated with the ACTH and C/C in each group, and also correlation between ACTH and C/C were performed (Spearman's correlation test).

The statistical program used was GraphPad Prism, version 3 (GraphPad Software, Inc.).

Ethical procedures in experimental animals

This study was approved by the Ethics Committee of the School of Veterinary Sciences and the Secretariat of Science and Techniques (UBACyT) of the University of Buenos Aires (project V045), adapting itself to the laws in force as to experimental animals and the recommendations of the WHO. Written consent was obtained from the owners of the dogs to participate in this project.

Hepatic biopsy was not performed because the owner and Ethical Committee did not authorise this procedure, considering it unnecessary risk.

Results

Clinical aspects

None of the animals treated with one of the drugs presented signs of hepatic insufficiency during the treatment. An increase of AP or transaminases 3 times greater than the PDH value at the time of the diagnosis was not observed in any of the dogs in both groups. No side effects with isotretinoin-9 cis or Ketoconazole during the time of therapy were observed. Eight dogs from Ktz group died during the experiment because of the poor control of hypercortisolism. Thus, 12 animals of Ktz group ended the study.

With respect to the evaluated clinical signs, dogs under treatment with retinoic acid showed an improvement in all clinical signs (normalization of the water intake resulting in normal diurises, normal food intake, diminution of the abdomen size, hair growth and return of the oestrus in 10 of 15 females). In Ktz group polydipsia-polyruria and polyfagia and hair loss were presented in 7 dogs, the oestrus did not return in any females and no change in the abdomen size of the 12 dogs was observed.

Hepatic enzymogram, plasmatic ACTH, C/C.

Dogs with PDH (both groups) had an increase of AP and ALT at the time of the diagnosis, while AST was found, except for 2 dogs in RG and 1 in Ktz, within the reference range for the method. There is an individual decrease of AP (time "0": 805 UI/L [156-2998]) after 180 days (620 UI/L [136-1970]) of treatment in the RG group (but not significant if comparing the median values of the group), reaching its lower serum concentration compared to the previous values (Fig. 1a). This was not observed in the Ktz group, where values practically do not change ("0" day: 683.5 UI/L [164-2990] vs 180 days: 960 UI/L [200-3000])

ALT (Fig 1b), shows a decrease in the RG group, which is significant when comparing the time 0 days vs. 180 day (106.5 UI/L [16-393] and 95.5 UI/L [27-283]; $P= 0.04$). In the Ktz group, comparing the same time a significant increase is observed ("0" day:144 UI/L [25-392] vs 180 days: 152 UI/L [25-284]; $P<0.02$). Comparing 180 days vs 180 days, ALT concentration is greater in Ktz group in comparison to the RG group ($P<0.02$)

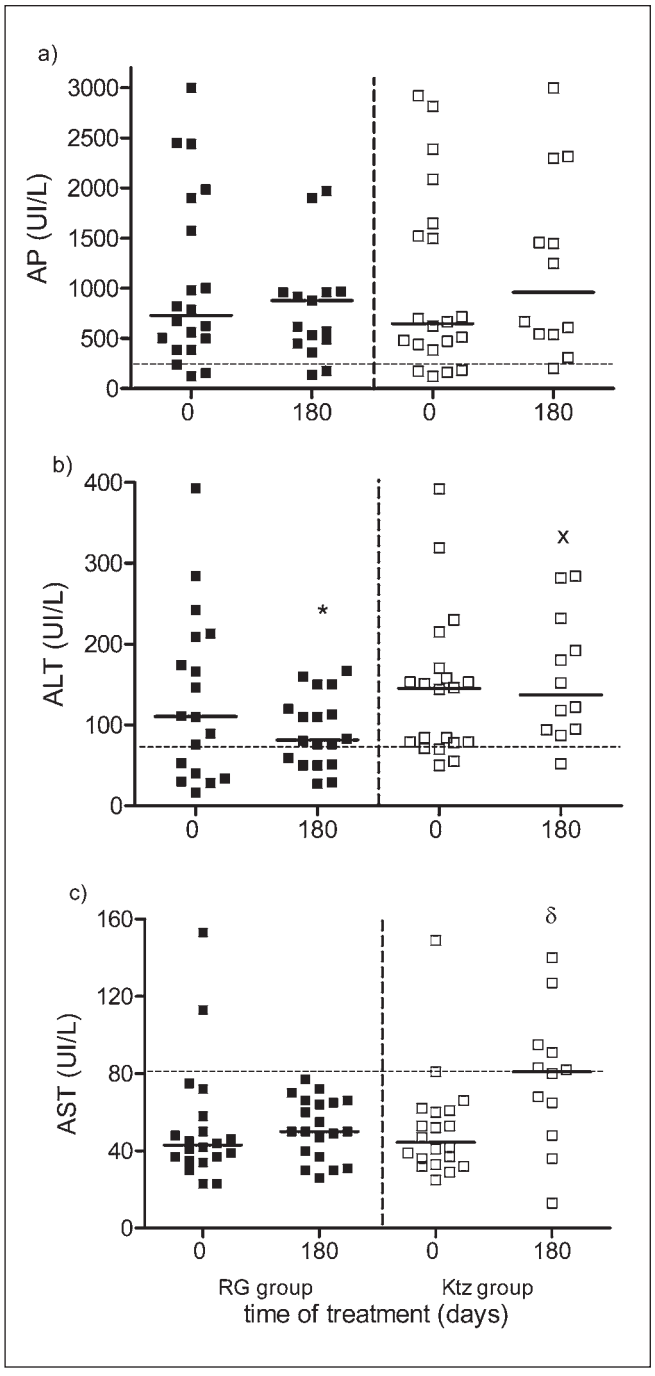

Figure 1: Variation in the plasma concentration of AP (a), ALT (b), and AST (c) in dogs treated with retinoic acid (full square) and ketoconazole (open square). In spite of individual decrease of AP in RG group (a), the difference is not significant. In (b), decrease of A LT (*P = 0.04) is evident after isotretinoin 9-cis therapy. On the other hand, in the Ktz group, there is a slight increase of ALT (xP< 0.02) in comparison to the RG group at the same time period. In (c), AST serum concentration is not changed in the RG group, but increased in the Ktz group(δ P<0.03). Doted lines indicate the upper limit of each enzyme

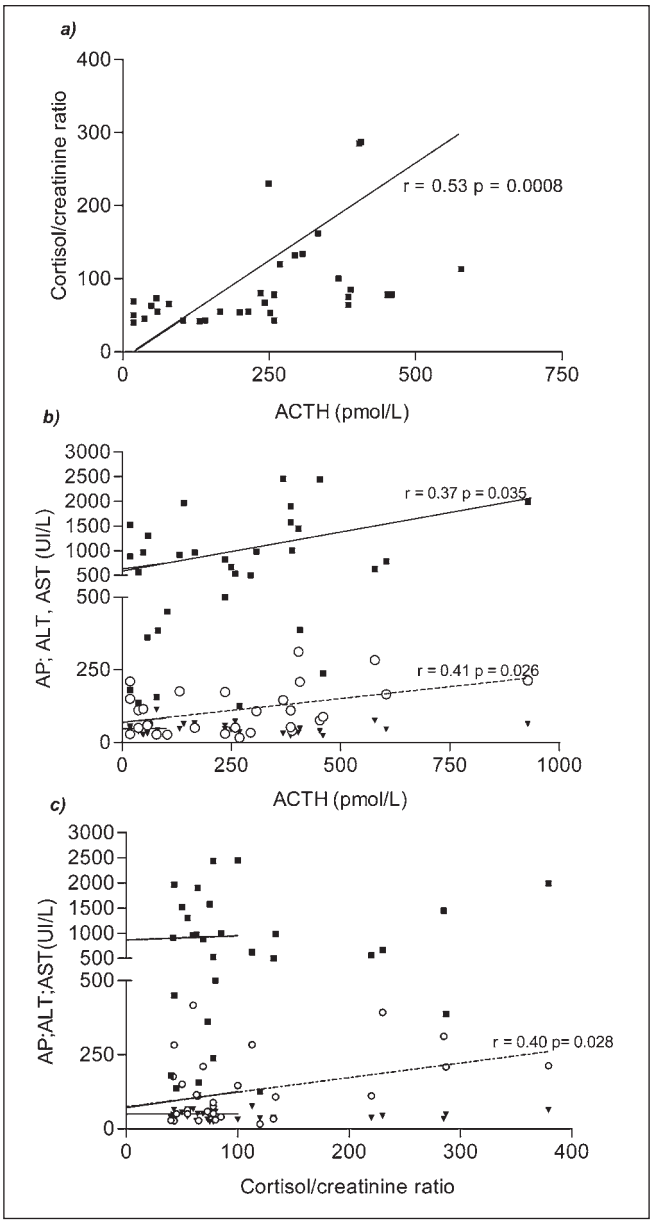

Figure 2: Correlation between ACTH vs. C/C (a), ACTH vs AP, ALT and AST (b) and C/C vs AP, ALT and AST in dogs treated with retinoic acid. Correlation between ACTH and C/C (a) demonstrated the effectiveness of RA to control PDH through its action on the corticotroph adenoma. As a result of this control, a positive correlation (b) between AP (full squares), ALT (open circles) and ACTH was observed, while there was no correlation between AST (full triangles) and ACTH

AST (Fig 1c) showed a significant increase (P< 0.03) after 180 days vs. 0 day (75 UI/L [13-127] vs 42 UI/L [18-149]) in the Ktz group and in comparison to values for 180 days (50 UI/L [26-77]) RG group (P< 0.03). There were no significant differences in the RG group ("0" day: 42 UI/L [23-153]. In this group 2 dogs normalized its previous elevated values.

Both plasma ACTH and C/C (Table 1) decreased after 180 days vs 0 day (P=0.0002 and P<0.0001 respectively) in RG group. In contrast, plasmatic ACTH augment at the end of treatment with KTZ vs 0 day (P=0.001) and C/C diminished at 180 day vs 0 day (P=0.0001). Nevertheless, the C/C in this group was significant higher (P=0.012) compared with RA group at 180 days.

In the RG group correlation was found between ACTH vs. C/C (r=0.53; P=0.0008) (Fig. 2a), with the decrease of the C/C accompanying the decrease of ACTH. In this group, a correlation (Fig. 2b and c) was also seen among ACTH vs. AP and ALT (r=0.37; P=0.035 and r=0.41; P=0.026 respectively) and between C/C vs. ALT (r=0.40; P=0.028). These correlations were not found in the Ktz group.

Table 1: Variation of ACTH and C/C in dogs treated with isotretinoin 9-cis vs ketoconazole

	RG		KTZ	
	0 day	180 days	0 day	180 days
ACTH (pmol/L)	375.7 (50-940.7)	100.5 [a] (18-258.4)	296.2 (52.1-904.2)	480 b (170-700)
C/C	126 (64-1200)	53 [c] (40-78)	114 (65-420)	77.5 d,e (52-120)

a) P=0.0002 (vs 0 day); b) P=0.001 (vs 0 day); c) P<0.0001 (vs 0 day); d) P=0.001 (vs 0 day), e) P=0.012 (vs 180 days of RG group)

Values are expressed as median and range. Wilcoxon signed rank test.

RG: retinoid group (N=20), Ktz: ketoconazole group (N=20 at 0 day and N=12 at 180 days). C/C: cortisol: creatinine ratio

Discussion

In the present study, we found that treatment with retinoic acid at the doses and in the studied time did not produce any significant alterations on the liver enzymes status.

Adverse effects of hypercortisolism in the organisms are well known. The same happens with the drugs prescribed for its control, especially due to their effect on the hepatic function (19, 20). These pharmacological products are inductors of the transaminases activity, and may cause damage in the hepatocytes (5). Taking into account that in PDH there is an increase of the hepatic enzymes activity, as well as in the size of the liver (1, 12), it is plausible to be concerned about the potential risk of the use of these drugs in dogs. It is known for AP, ALT and AST that their concentration in serum can be increased by corticosteroid induction (increase of enzymatic activity). On the one hand, the hepatic AP-isoenzyme is associated to the membrane of the hepatocytes and the bile epithelium, increasing its concentration in serum by induction and further release in the cholestatic liver disease (21, 22, 23).

In case of hepatopathy, the serum increase of ALT is related to the number of hepatocytes affected (cytoplasmic rupture), resulting also in an increase of AST (19, 22). The later enzyme is indicative of the hepatocellular damage and necroinflammation (22, 24). Corticoids also induce a greater activity of both enzymes (ALT and AST), due to their action on the neoglucogenesis and the glyceroneogenesis, metabolic pathways where both enzymes intervene (13, 14). The fact that ALT and AP were already high at the time of the diagnosis, while AST was within its normal range (except for three dogs, see Figure 1) could indicate that cortisol has a larger inducing effect on the ALT and AP than on AST.

The values obtained and the correlation analysis between ACTH and C/C in RG group shows that the isotretinoin 9-cis control PDH by inhibiting the secretion of ACTH and, consequently, cortisol. On the contrary, this does not occur in the Ktz group. Although there is a decrease of C/C, the ACTH remains high because the corticotroph adenoma is not targeted. As a result, the C/C is higher in the Ktz group than in RG. Similar effect has also been

observed before with o,p'-DDD and trilostane (26, 27). Thus, when the secretion of both hormones is controlled, this event will be reflected in the correlations observed with regards to the analyzed hepatic enzymes. The cortisol-induced effect on ALT is diminished with treatment, which is demonstrated in its significant decrease in the RG. With regard to AP, individual decrease is observed, with values remaining above 1,500 UI/L only in two dogs (see Figure 1). Considering the correlation between ACTH and AP, the cortisol action on the isoenzyme (23) is probably less important. This would explain the absence of correlation between AP and C/C. However, it is not known at the present if AP would decrease after longer time period than the one reported in the present study.

The lack of correlation between AST, ACTH and C/C would indicate that this enzyme is only marginally influenced by cortisol and that the isotretinoin 9-cis do not produce, at the indicated dose and time, hepatic damage. Therefore, when PDH is controlled as described in the RG group, the metabolic pathways where both enzymes interact are normalized and the activity of ALT and AST decrease in RG.

It is evident in the second group that the lack or loss of correlation between studied variables is due to the effects of ketoconazole. On one hand, as it does not completely normalize the synthesis of glucocorticoids, its inducing effect over these enzymes persists, adding to the choleostasis caused by hepatomegaly. On the other hand, this effects could be due to initial stages of liver damage after 180 days of treatment. The significant increase of ALT and AST in this group could be due to lesser control of the hypercortisolism, however, it could be also by necroinflammatory event with the consequent passage of the enzymes to the portal circulation (6, 24).

The behaviour of the studied enzymes and their relationship with the ACTH and C/C in the RG indicates that the isotretinoin 9-cis in the prescribed doses does not produce hepatocyte damage during the treatment period (180 days), being safe in PDH therapy. The elevated serum concentration of ALT and AP at the diagnosis time is not a contraindication for use of this drug. By contrast, Ketoconazole could imply a certain hepatoxicity risk according to the behaviour of ALT and AST, and therefore its use should be interrupted in case of a sustained increase in the activity of these two enzymes. Therefore, we conclude that AST enzyme must be closely monitored during PDH therapy as this enzyme could be a marker for hepatic damage in these patients.

Acknowledgements

This study was supported by grant from Science and Technique of University of Buenos Aires (UBACyT, ref. V045). We thank Dr. Stalla of Max Planck Institute, Germany, to measure plasma ACTH.

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RETINOJSKA KISLINA KOT ZDRAVILO ZA CUSHINGOVO BOLEZEN PRI PSIH: OVREDNOTENJE JETRNIH ENCIMOV V ČASU ZDRAVLJENJA

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Povzetek: Nedavne študije so pokazale, da je možno z retinojsko kislino nadzorovati od hipofize odvisni hiperadenokorticizem (PDH), saj deluje na hipofizni tumor, ki izloča adenokortikotropni hormon (ACTH). Ker pa obstajajo poročila o hepatotoksičnosti retinojske kisline, smo se odločili analizirati hepatograme psov s Cushingovim sindromom. Primerjali smo pse, ki so 180 dni prejemali retinojsko kislino, s psi, ki so bili zdravljeni s ketokonazolom. Pri nobeni živali ni bila ugotovljena hepatotoksičnost. Pri psih, zdravljenih z retinojsko kislino, smo ugotovili (statistično neznačilen) padec alkalne fosfataze (AP), pri skupini, zdravljeni z ketokonazolom, pa ni bilo razlik. Pri retinojski skupini je bila po 180 dnevih (v primerjavi z dnem 0, P=0,04) znižana alanin amin transferaza (ALT). V ketokonazolni skupini je bil ta encim povišan (P<0,02). Alanin aspartanska transferaza (AST) se v retinojski skupini ni spreminjala, vrednost je ostala na osnovni ravni. V ketokonazolski skupini pa je AST po 180 dneh v primerjavi z dnevom 0 opazno narasla (P<0,03). Zmanjšanje vrednosti ALT in AP v retinojski skupini je povezano s padcem vrednosti ACTH (r=0,41, P=0,026 in r=0,37; P=0,035), obstaja pa tudi povezava med ALT in razmerjem urinskega kortizola in urinskega kreatinina (r=0,40, P=0,028). Te spremenljivke se v ketokonazolni skupini niso ujemale. Zaključimo lahko, da zdravljenje psov, obolelih s PHD, po 180 dnevih tretiranja z retinojsko kislino ne povzroča hepatotoksičnosti.

Ključne besede: cushingov sindrom–patofiziologija–zdravljenje; hipofizni ACTH, hipersekrecija; tretionin–terapevtska uporaba; jetra–učinki zdravil; psi

Slov Vet Res 2007; 44 (3):
UDC 614.31:615.282:667.28:543.645:639.2.09

Original Research Paper

DETERMINATION OF MALACHITE GREEN AND LEUCOMALACHITE GREEN IN TROUT AND CARP MUSCLE BY LIQUID CHROMATOGRAPHY WITH VISIBLE AND FLUORESCENCE DETECTION

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Summary: A fast and specific method for determination of malachite green (MG) and its major metabolite leucomalachite green (LMG) in trout and carp muscle is described. MG and LMG residues were extracted from fish muscle with an acetonitrile-buffer mixture and isolated by partitioning into dichloromethane. Extracts were then cleaned up on solid-phase extraction (SPE) columns. Chromatographic separation was achieved by using reverse-phase column with an isocratic mobile phase consisting of acetonitrile and acetate buffer (0.01M, pH 4.1). MG was detected with an absorbance detector (λ= 618 nm), while a fluorescence detector (λ_{ex} = 265 nm and λ_{em} = 370 nm) was used for detection of LMG. Both detectors were connected on-line which allowed simultaneous analysis of a sample extract for MG and LMG. The method was validated according to Commission Decision 2002/657/EC. The mean recoveries of MG and LMG from muscle fortified at three levels (2, 3, 4 µg/kg) were 55% and 74%, respectively. Relative standard deviations of the mean at all fortification levels were less than 15% and 13% for MG and LMG, respectively. With the described method 33 samples of fish bought in local shops and fish farms between August 2004 and April 2005 were analysed. Seven samples showed detectable amounts of residues.

Key words: antifungal agents–therapeutic use; rosaniline dyes–chemistry; aniline compounds–chemistry; drug residues–analysis–methods; food analysis; chromatography, liquid; trout; carps; fish

Introduction

Malachite green (MG) is a triphenylmethane dye, originally used as a dyeing agent in the textile industry, but it have been also widely used in aquaculture industry as an anti-fungal, anti microbial and anti-parasitic agent for many decades (1). It is used in the form of bath treatment, either on its own or synergistically with formalin (1, 2). MG is easily absorbed into tissues during waterborne exposure and rapidly transformed to its reduced form, leucomalachite green (LMG). LMG in tissues may be eliminated at a rate that is dependent on the fat content (3, 4). Because of its suspected carcinogenic, mutagenic and teratogenic properties, MG has never been registered as a veterinary drug for fish treatment in the European Union (5). The ban on its use necessitated a robust and reliable analytical method for determination of residues of MG in fish muscle. According to the European Commission, methods for determining MG in fish tissues should meet the minimum required performance limit (MRPL) of 2 µg/kg for the sum of MG and LMG (6). Several analytical approaches for determination of MG residues have been published. For the determination of MG residues, high-performance liquid chromatography (HPLC) with post-column unit for oxidation of LMG and an absorbance detector for the detection of MG has been commonly used. The post-column reactors were filed with lead (IV) oxide (7-11) or 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (12). As an alternative to lead (IV) oxide, an electrochemical cell was used (13). As mass spectrometers

Received: 29 May 2007
Accepted for publication: 22 August 2007

become more common, methods based on mass spectrometry (MS) have also been reported for the confirmation of suspected MG residues (4, 14-17). However, the post-column reactor has been used with mass spectrometry as well, because detection of MG is more sensitive compared to LMG (4, 16, 17). The use of a fluorescence detector for the detection of LMG has also been reported (13, 18).

Although MS methods provide greater sensitivity and residue confirmation for the detection of MG and LMG in fish, reliable and robust methods are needed to routinely screen numerous laboratory samples without straining the resources of sophisticated LC-MS instruments. In this report, we present a selective, sensitive and relatively fast LC method with visible and fluorescence detection for simultaneous determination of LMG and MG in trout and carp. Because LMG is detected with a fluorescence detector, the post-column oxidation procedure is not needed. The method was validated according to Commission Decision 2002/657/EC (19), it is suitable for routine analysis and provides a detection limit below 1.0 μg/kg. To check if MG is still illegally used in the fish farming industry due to the low cost, easy availability, and high efficacy against fungus, bacteria and parasite, 33 samples of fish collected randomly at different fish farms, fish shops and fish markets were analysed with the presented method.

Materials and methods

Chemicals

Organic solvents used were LC grade and other chemicals were of analytical grade unless stated otherwise. Acetonitrile and methanol were obtained from J.T. Baker (Deventer, the Netherlands), hydroxylamine (HA) hydrochloride, p-toluenesulfonic acid (p-TSA), ammonium acetate (extra pure), triethylamine (TEA), glacial acetic acid and dichloromethane were from Merck (Darmstadt, Germany).

An acetate buffer (0.1 M, pH 4.5) was prepared by dissolving 7.708 g ammonium acetate in about 800 ml of water, adjusting the pH to 4.5 with acetic acid and diluting the solution to 1000 ml. An acetate buffer (0.01 M, pH 4.1) used for the mobile phase was prepared by dissolving 0.771 g ammonium acetate in about 800 ml of water. 2 ml TEA was added before a pH adjustment to 4.1 with acetic acid and dilution to 1000 ml.

Standards and standard solutions

MG oxalate and crystal violet chloride (CV) were purchased from Riedel-de Haën (Seelze, Germany) and LMG and leuco crystal violet (LCV) from Aldrich (Steinheim, Germany). Individual stock solutions of MG, CV, LMG and LCV at 100 μg/ml were prepared in methanol, taking into account the active substances. These solutions were combined and diluted in methanol to prepare an intermediate standard solution of 1 μg/ml. Working standard solutions were prepared by several dilutions of intermediate standards with methanol for recovery experiments and with a mixture of acetate buffer (0.1 M, pH 4.5), acetonitrile and hydroxylamine hydrochloride solution (2.5 mg/ml) (40:40:20, v/v/v) for calibration.

Sample preparation equipment

The instruments used were an Ultra-Turrax T25 (IKA-Labortechnik, Janke & Kunkel, Germany), a temperature controlled Minifuge T centrifuge (Heraeus, Osterode, Germany) and a vacuum rotary evaporator Büchi Model R-205 (Osterode, Germany). For the extraction, a linear shaker Vibromix 314 EVT (Tehtnica, Železniki, Slovenia) was used. Solid-phase extraction was carried out on a vacuum manifold for Visiprep™ (Supelco, Bellefonte, USA).

Sample collection and preparation

33 samples of fish were collected randomly between August 2004 and April 2005 at different fish farms, fish shops and fish markets in Slovenia. Among 33 collected samples, 8 were imported from other EU countries. 13 samples of rainbow trout, 12 samples of brown trout, 6 samples of brook trout and 2 samples of carp were examined in the study. Fish samples (2–3 fish/sample) were filleted and the bones removed. The muscle tissue with skin was homogenized, frozen and stored at –18°C before analysis.

Extraction and clean-up

A homogenized sample (10 g) was weighed into a 50 ml centrifuge tube. The spiked sample was prepared by adding a known amount of working standard solution to the fish muscle. Three milliliters of aqueous 0.25 g/ml HA, 5 ml of aqueous 0.05 M p-TSA and 5 ml of 0.1 M ammonium acetate buffer (pH 4.5) were added to each sample and homogenized for 1 min with an Ultra-Turrax at 13000 rpm. Then 20 ml of acetonitrile were added, the tube was capped and shaken vigorously on a platform shaker for 5 min. The tube was centrifuged at 2000 *g* for 10 min at 20°C. The supernatant was decanted into a 100 ml centrifuge tube. Another 20 ml acetonitrile

were added to the sample pellet and the sample was shaken and centrifuged under the same conditions as before. The supernatants were combined in a 100 ml centrifuge tube.

To the supernatant, 20 ml of deionised water and 20 ml of dichloromethane were added, and the tube was shaken vigorously on a platform shaker for 5 min and centrifuged at 1400 *g* for 10 min at 10°C. The lower dichloromethane layer was transferred into a 500 ml round bottom flask. The extraction with 20 ml of dichloromethane was repeated and the lower layer was transferred into the same flask. The combined dichloromethane extract was then concentrated on a rotary evaporator at 65°C to approximately 5 ml. At this point, the sample was kept overnight in the dark.

J.T Baker neutral alumina (6 ml, 1 g) and Varian Bond Elut PRS-SPE columns (3 ml, 500 mg) were pre-washed with 5 ml acetonitrile. With an adapter, the alumina SPE column was placed on top of the PRS-SPE column. This assembly was then attached to the solid-phase extraction vacuum manifold. To the sample extract in the 500 ml round bottom flask, 2 ml of dichloromethane were added. The flask was swirled to dissolve the residue. 5 ml of acetonitrile were added to the flask prior to pouring the sample extract onto the columns. The flask was rinsed 2 times with 5 ml of acetonitrile, which was also applied to the columns. At this moment the alumina SPE column was discarded and the PRS-SPE column was rinsed with 2 ml of water followed by 1 ml of a mixture of ammonium acetate buffer (0.1 M, pH 4.5) and acetonitrile (50:50, v/v). The MG and LMG were eluted from the PRS-SPE column with 2 ml of the above mixture of buffer and acetonitrile, and collected in a graduated tube containing 0.5 ml of 2.5 mg/ml HA in water. The volume of the eluate was adjusted to 2.5 ml with the mixture of buffer and acetonitrile. The content was mixed well and filtered through a 0.45 μm filter before HPLC analysis.

Liquid chromatography

An Agilent 1100 HPLC system (USA) consisting of a quaternary pump, a vacuum degasser, an automatic injector, a column thermostat, a fluorescence detector (GA1321A) set at λ_{ex} = 265 nm and λ_{em} = 370 nm and Hewlett Packard (Atlanta, USA) LC-95 UV/Vis detector set at 618 nm were used. Both detectors were connected on-line. The ChemStation software controlled the LC system and processed the data.

Three different analytical columns were tested: SynChropak SCD-100, 5 μm, 150x4.6 mm was obtained from Eprogen (Darien, USA); Luna Phenyl-Hexyl, 5 μm, 250x3.0 mm from Phenomenex (Torrance, USA); and PerkinElmer-HS 5 C-18, 5 μm, 150x4.6 mm from PerkinElmer (Boston, USA). The optimal mobile phase was selected by varying the proportion of acetonitrile and pH of 0.01 M acetate buffer.

SynChropak SCD-100 analytical column was selected for the determination of MG and LMG in fish. A C18 guard cartridge (4x3 mm, Phenomenex, Torrance, USA) was used prior to the analytical one. The mobile phase was acetonitrile and ammonium acetate buffer (0.01 M, pH 4.1) (62:38, v/v). The injected volume was 100 μl and the separation of the analytes was accomplished with a flow of 1 ml/min at 27°C. Quantification was performed using the external standard method and was based on peak area.

Method validation

Validation of the procedure was carried out in accordance with Commission Decision 2002/657/EC (19). The linearity of the LC-Vis/FLD response was checked across a wide concentration range from 5 to 40 ng/ml. Concentrations of standards were applied to the abscissa and the corresponding peak areas to the ordinate. The least squares method was used to create the calibration curves, which were evaluated by regression and correlation. Linearity in matrix was also checked. The chromatographic response was recorded from the samples of trout meat with standard additions in the whole range from 1 μg/kg to 5 μg/kg, with five calibration points. The absence of interfering endogenous compounds around the retention times of the analytes was verified with an analysis of blank samples of different carp and trout muscle samples and also with an analysis of blank samples fortified with CV, LCV, MG and LMG at 4 μg/kg. Precision (repeatability and within-laboratory reproducibility) was checked with an analysis of blank samples of trout and carp. Samples were fortified with MG and LMG at 2, 3 and 4 μg/kg. At each level the analyses were performed with six replicates. The analyses of fortified blank samples of trout were repeated on two other days close to each other, with the same instruments, batches of reagents and the same operators and on two other days with the same instruments but with different batches of reagents and different operators. Repeatability and within-laboratory reproducibility were expressed with standard deviation and relative standard deviation. The decision limit (CCα) was de-

termined as the corresponding concentration at the y-intercept of the calibration curve plus 2.33-times the standard deviation of the within-laboratory reproducibility at 2 μg/kg. The detection capability (CCβ) was the corresponding concentration at the CCα plus 1.64 times the standard deviation of the within-laboratory reproducibility at 2 μg/kg.

Results and Discussion

Method development

Most methods published so far are based on the post-column oxidation of LMG to MG and detection of MG using visible light absorption. As mentioned before, oxidation has been commonly performed using PbO_2 (7-11). While the methods that used PbO_2 reactor provide adequate sensitivity and recovery, the manually prepared lead-oxide reactor can be plagued by problems, including rapid depletion and peak broadening, which lead to a decrease in method sensitivity (12). For this reason we avoided the oxidation of LMG to MG and thus both analytes were detected as such. MG and LMG were detected simultaneously, MG with an absorbance detector and LMG with a fluorescence detector. To optimize the analytical procedure the absorption spectrum of the MG solution and fluorescence spectra of the LMG solution was measured. According to the absorbance spectrum, the absorbance detector was set at 618 nm. That wavelength is usually used for detection of MG (7, 8, 11). With regard to previously published methods (13, 18) and to the apex in the recorded fluorescence spectrum, the wavelength of emission was raised from 360 to 370 nm, while the wavelength of excitation was left the same (256 nm). This change improved the sensitivity of the method.

Three different analytical columns were tested for effective resolution of MG, CV, LMG and LCV. CV is a triphenylmethane dye with very similar structure to MG and anti-parasitic and anti-microbial properties (13). On all tested columns MG and CV were separated with baseline resolution, but the separation of LMG and LCV was not easy to achieve. The best resolution of LMG and LCV was achieved on a Phenyl-Hexyl column at 27°C with a mobile phase of 55% acetonitrile and 45% 0.01 M acetate buffer (pH = 3.6) and a flow-rate at 1 ml/min. At this condition, the resolution (R) between LMG and LCV was higher than 2.5, but the retention time was relatively long (t_r for LMG was 22 min). On a SynChropak SCD-100 column at 27°C with 62% acetonitrile and 38% 0.01 M acetate buffer (pH = 4.1) as mobile phase and a flow-rate at 1 ml/min, near-baseline separation between LMG and LCV was achieved (R = 1.4) in less than 11 min (Figure 1). But on a PerkinElmer-HS 5 C-18 column using a different mixture of ammonium acetate buffer (0.01 M) and acetonitrile as the mobile phase, the resolution between LMG and LCV was always less than 1. Because the aim of our study was to develop a fast and economical method, we chose the SynChropak SCD-100 column, which was also used by Rushing and Hansen (13).

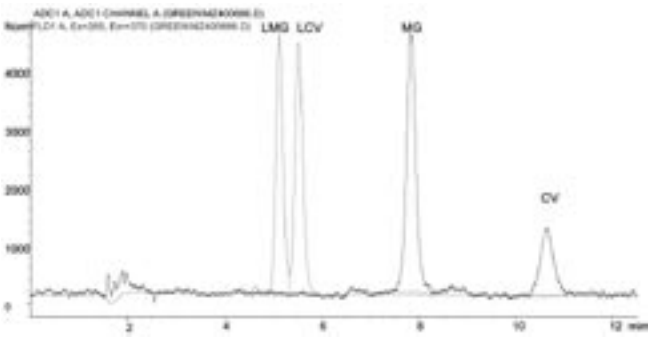

Figure 1: Typical chromatogram of mixed solution (50 ng/mL)

We tested two procedures of sample extraction and sample clean-up. First we followed the procedure of Rushing and Hansen (13). With this procedure, extraction of MG and LMG was performed using a mixture of ammonium acetate buffer and acetonitrile; liquid-liquid extraction into dichloromethane and solid-phase extraction followed. With regard to the original method we decreased the weight of the sample and the volumes of chemicals used for the extraction by a factor of two. Although the method was performed more economically, the consumption of organic solvents was still very high and the time used for sample preparation was long, especially because separatory funnels were used for liquid-liquid extraction. For these reasons we performed liquid-liquid extraction according to the procedure described by Halme and co-workers (4). The sample extraction in this method is performed with smaller volumes of organic solvents. Instead of separatory funnels for liquid-liquid extraction, solvents were separated by centrifugation and the lower layer was transferred using the pipette. All these changes reduced the time used for sample preparation and decreased the recovery of LMG from 90% to 78%, which was still acceptable for the determination of veterinary drug residues in food. The recovery of MG remained the same (around 50–60%).

Method validation

Chromatograms demonstrating the selectivity of the procedure are shown in Figure 2, 3 and 4. In Figure 2 chromatograms of blank sample and spiked sample of trout with LMG and LCV at 4 μg/kg are shown. LMG and LCV were separated with almost baseline resolution. From chromatograms of blank and spiked sample of trout (Figure 3) and blank and spiked sample of carp (Figure 4) with MG and LMG at 4 μg/kg, it is evident that no interfering peaks from endogenous compounds were found at the retention times of the target analytes. Hence the selectivity of the procedure is considered satisfactory.

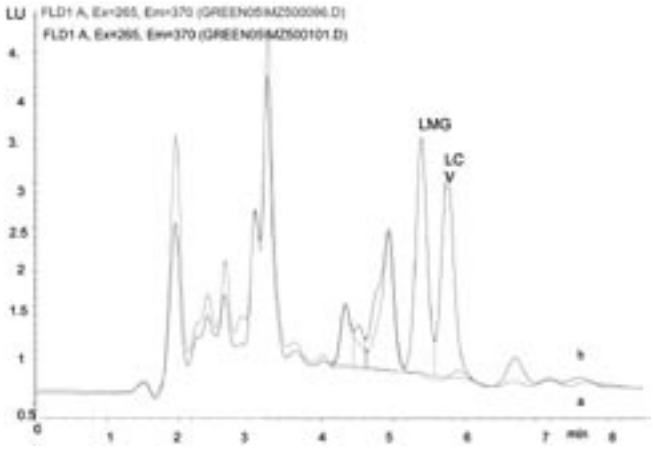

Figure 2: Chromatograms of (a) trout muscle and (b) trout muscle with a standard addition of LMG and LCV (4 μg/kg)

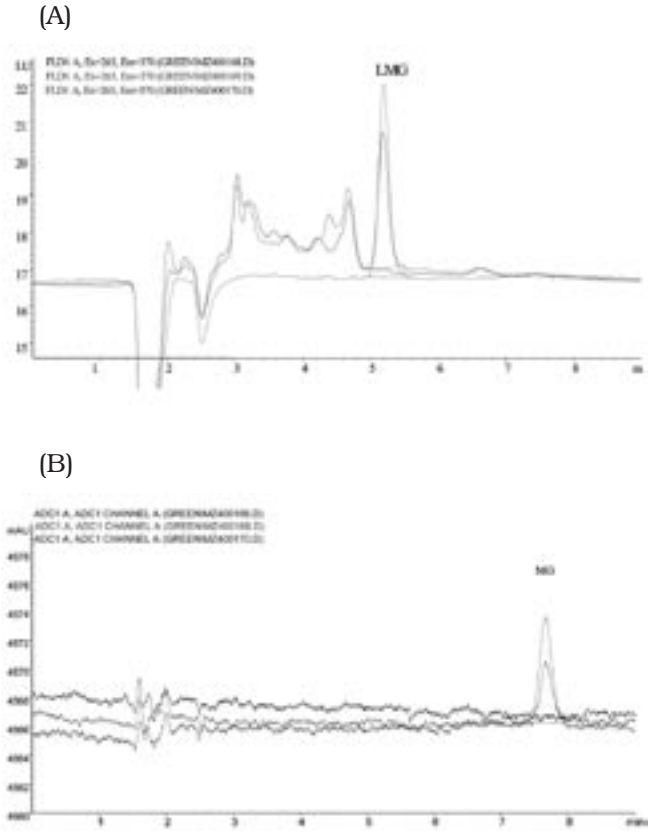

Figure 3: Selectivity for the determination of MG and LMG in trout muscle tissue. (A) LMG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg (B) MG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg.

The results of the linearity of the LC-Vis/FLD response and matrix calibration curve are reported in Table 1. The standard calibration curves are linear over the range 5-40 ng/ml and the matrix calibration curves were linear over the range 1-5 μg/kg for MG and LMG. The correlation coefficients of the standard and matrix calibration curves were greater than 0.9993 for both MG and LMG.

Table 1: Linearity of MG and LMG determination on standard and matrix level

	Analyte	Slope	Intercept	Correlation coefficient
Standards	MG	6.59	-3.67	0.9995
	LMG	3.49	-1.16	0.9999
Trout muscles	MG	8.56	-3.87	0.9993
	LMG	5.62	1.32	0.9998

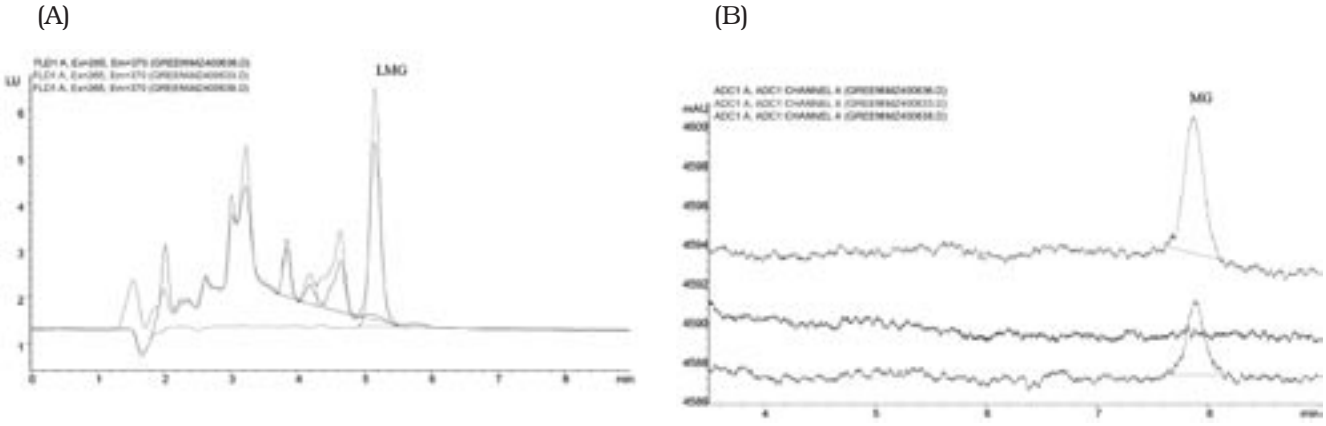

Figure 4: Selectivity for the determination of MG and LMG in carp muscle tissue. (A) LMG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg. (B) MG: standard mixture 16 ng/mL, blank and blank with standard addition of 4 μg/kg.

The average recoveries and precision of repeatability and within-laboratory reproducibility at 2, 3 and 4 μg/kg are summarised in Table 2 for trout samples and in Table 3 for carp samples. For MG the average recovery was in the range between 47% and 62%. The recovery for LMG was higher than for MG and ranged between 67% and 78%. In Table 2 and 3 we can see that there were almost no differences between trout and carp samples in recovery and standard deviation of measurements. Accuracy of the method was checked by participating in the proficiency testing programme organized by FAPAS® (Series 2 Round 59, 2004). The z-score lied between -2 and 2, hence the result was considered as satisfactory.

Table 2: Accuracy and precision of MG and LMG determined in fortified trout muscle at three concentration levels (n = 18 at each level)

	Parameter	Fortification level (μg/kg)		
		2	3	4
MG	Repeatability			
	Mean concentration (μg/kg)	1.23	1.83	2.32
	s (μg/kg)[a]	0.15	0.17	0.30
	RSD (%)[b]	11.9	9.2	3.0
	Recovery	62	61	58
	Within-lab reproducibility			
	Mean concentration (μg/kg)	1.10	1.69	2.21
	s (μg/kg)	0.16	0.21	0.26
	RSD (%)	14.5	12.8	11.6
	Recovery	55	57	55
LMG	Repeatability			
	Mean concentration (μg/kg)	1.44	2.02	2.94
	s (μg/kg)	0.17	0.10	0.16
	RSD (%)	12.0	4.6	5.5
	Recovery	72	67	74
	Within-lab reproducibility			
	Mean concentration (μg/kg)	1.54	2.10	2.94
	s (μg/kg)	0.19	0.23	0.18
	RSD (%)	12.1	10.7	6.2
	Recovery	77	72	74

[a] s = Standard deviation
[b] RSD = Relative standard deviation

The CCα's were 0.6 μg/kg for MG and 0.5 μg/kg for LMG. The CCβ's were 1.0 μg/kg and 0.9 μg/kg, respectively. The method thus meets the European Commission Performance requirements of 2 μg/kg. This sensitivity is also in accordance with those reported for similar HPLC analysis approaches (7, 9, 10, 12), besides the method that was published by Mitrowska and co-workers (18). CCα's of that LC-UV/Vis method were lower (0.15 μg/kg for MG and 0.13 μg/kg for LMG), and similar to CCα's of the LC-MS/MS methods (4, 14, 15).

Table 3: Accuracy and precision of MG and LMG determined in fortified carp muscle at three concentration levels (n = 6 at each level)

	Parameter	Fortification level (μg/kg)		
		2	3	4
MG	Repeatability			
	Mean concentration (μg/kg)	0.93	1.45	2.05
	s (μg/kg)[a]	0.10	0.21	0.19
	RSD (%)[b]	10.8	13.6	9.3
	Recovery	47	52	51
LMG	Repeatability			
	Mean concentration (μg/kg)	1.54	2.33	3.01
	s (μg/kg)	0.10	0.05	0.10
	RSD (%)	4.7	2.3	3.3
	Recovery	77	78	75

[a] s = Standard deviation
[b] RSD = Relative standard deviation

Fish Samples Analysis

The method presented was tested on a number of samples collected randomly at different fish farms, fish shops and fish markets. Together with each series of samples a fortified blank sample with MG and LMG at 2 μg/kg to control recovery was analysed. The results were corrected for recovery of respective series and then used as final results. Surprisingly, 7 out of 33 samples contained residues of MG (Table 4), i.e. in excess of CCα, and 1 out of 7 samples containing residues of MG was imported from another EU country. In four samples the sum of MG and LMG was higher than 2 μg/kg (MRPL-value). In all seven samples we detected LMG, but in two of them we also found MG. These two samples of brown trout contained high concentrations of LMG (28 and 18 μg/kg), and therefore the presence of MG was not surprising.

Table 4: Summary of results from the analysis of 33 samples

Species	MG (μg/kg)	LMG (μg/kg)	Species	MG (μg/kg)	LMG (μg/kg)
Brown trout	nd[a]	nd	Brown trout	nd	nd
Rainbow trout	nd	1.5	Carp	nd	nd
Brown trout	nd	nd	Brown trout	nd	2.8
Rainbow trout	nd	0.8	Rainbow trout	nd	nd
Brown trout	0.8	28	Brown trout	nd	nd
Brook trout	nd	nd	Rainbow trout	nd	nd
Brook trout	nd	nd	Brown trout	nd	2.2
Brown trout	nd	nd	Rainbow trout	nd	nd
Brown trout	nd	nd	Rainbow trout	nd	nd
Brook trout	nd	nd	Rainbow trout	nd	nd

Species	MG (μg/kg)	LMG (μg/kg)	Species	MG (μg/kg)	LMG (μg/kg)
Rainbow trout	nd	nd	Rainbow trout	nd	nd
Brook trout	nd	nd	Carp	nd	nd
Rainbow trout	nd	nd	Brown trout	nd	nd
Rainbow trout	nd	nd	Brown trout	2.0	18
Brook trout	nd	nd	Rainbow trout	nd	nd
Rainbow trout	nd	nd	Brook trout	nd	nd
Brown trout	nd	1.2			

[a] nd = not detected

Conclusion

A sensitive and specific method for the determination of MG and LMG residues in trout and carp muscle has been described. The obtained validation results indicate the accordance of the method performance with Commission Decision 2002/657/EC (19). The CCα and CCβ for MG and LMG are below the MRPL of 2 μg/kg. The collected data demonstrate that the sample processing and HPLC analysis is amenable in control and inspection programs to secure food free of this veterinary drug.

Acknowledgement

The authors would like to thank to Mrs. Denise Jazbar for her technical assistance. The presented work was supported by the Slovenian Research Agency (P4-0092).

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DOLOČANJE MALAHITNEGA IN LEVKOMALAHITNEGA ZELENILA V MESU POSTRVI IN KRAPOV S TEKOČINSKO KROMATOGRAFIJO S SPEKTROMETRIČNO IN FLUORESCENČNO DETEKCIJO

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Povzetek: Uvedli smo hitro in specifično metodo za določanje malahitnega zelenila (MG) in njegovega glavnega metabolita levkomalahitnega zelenila (LMG) v mesu postrvi in krapov. Ostanke MG in LMG smo iz mesa rib ekstrahirali z mešanico acetonitrila in acetatnega pufra, nato pa iz te mešanice v diklorometan. Ekstrakt smo očistili še z metodo tekoče-trdno. Kromatografsko ločitev smo izvedli na analitski koloni z reverzno fazo z uporabo izokratske mobilne faze, sestavljene iz mešanice acetonitrila in acetatnega pufra (0.01 M, pH 4.1). MG smo zaznali s pomočjo spektrometričnega detektorja (λ = 618 nm), LMG pa s pomočjo fluorescenčnega detektorja (λ_{ex} = 265 nm in λ_{em} = 370 nm). Uporaba obeh detektorjev omogoča istočasno analizo ekstrata vzorca na MG in LMG. Metodo smo validirali po postopku, opisanem v Evropski direktivi 2002/657/EC. Povprečni izkoristek za MG in LMG, dobljen z analizo obogatenih vzorcev mesa rib na treh koncentracijskih nivojih (2, 3, 4 µg/kg), se je gibal med 55 % in 74 %. Relativna standardna deviacija povprečja je bila na vseh treh koncentracijskih nivojih manjša od 15 % za MG in 13 % za LMG. S predstavljeno metodo smo analizirali 33 vzorcev rib, kupljenih v lokalnih trgovinah in na farmah med avgustom 2004 in aprilom 2005. V sedmih vzorcih smo ugotovili prisotnost ostankov MG.

Ključne besede: antimikotiki–uporaba za zdravljenje; rosanilinska barvila–kemija; anilinske spojine–kemija; zdravila, ostanki–analize–metode; kromatografija, tekočinska; hrana, analize; postrv; krapi; ribe

Slov Vet Res 2007; 44 (3): 91-6
UDC 636.2.09:579.62:579.844-074

Original Research Paper

PLASMA PYRUVIC ACID CHANGES IN ZEBU CATTLE EXPERIMENTALLY INFECTED WITH CLOSTRIDIUM CHAUVOEI

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Summary: A study was carried out to investigate the pyruvic acid concentration in Zebu cattle experimentally infected with *Clostridium chauvoei*. In the experimental design, 14 Zebu bull-calves were divided into 4 groups namely: groups A, B, C and D, and administered *C. chauvoei*, toxins, neuraminidase and control respectively. There was pyruvate accumulation in the plasma of all but the control group and the mean pyruvic acid concentrations in the *C. chauvoei*, toxin and neuraminidase groups had some peaks, with the highest peak value in the neuraminidase-administered group, followed by *C. chauvoei* and toxin-administered groups in that order. The control group had no peak pyruvic acid concentration and the values were almost similar throughout the experimental period ($P>0.05$). There was a statistically significant difference ($P<0.05$) between the plasma pyruvic acid levels of *C. chauvoei*, neuraminidase, toxin-administered and control groups, respectively. The significance of pyruvate accumulation in the pathogenesis of blackleg is discussed.

Key words: cattle disease; *Clostridium* infections–pathology–diagnosis; pyruvic acid–blood; neuroaminidase–blood; bacterial toxins–blood

Introduction

Blackleg is a fatal disease of cattle and sheep caused by *C. chauwoei* and was first reported in 1870 (1). In Nigeria, the disease was first reported in 1929 and has remained a major problem of cattle in the country (2,3). The prevalence of blackleg is known to be very high during years of high average annual rainfall (4,5). Vaccination against the disease has been carried out since 1930, but sporadic outbreaks are recorded annually. The economic losses of cattle to blackleg in Nigeria have been estimated at about 4.3 million United States dollars annually (3). The nomadic Fulani pastoralists of rural Nigeria, who own about 70-80% of livestock in the country, rear the Zebu breed of cattle that is highly susceptible to blackleg. They migrate from one place to another in search of pasture for their livestock and many of them request blackleg vaccination for their cattle, only if there are outbreaks of the disease in neighboring herds.

C. chauwoei which is the known cause of blackleg has been reported to produce neuraminidase (6,7). Neuraminidases (sialidases, EC 3.2.1.18) are involved in the pathogenesis of some infectious diseases, whose aetiologic agents produce the enzyme (8-12). The enzyme is of great importance in medicine and the pharmaceutical industry for the analysis of oligosaccharides and development of neuraminidase inhibitors (13-16). There is no consensus on the pathogenesis of blackleg, but toxins and neuraminidase produced by the bacteria are believed to play a significant role in the mechanisms of the disease (17-19). No studies have been carried out so far, on the biochemical changes in Zebu cattle infected with *C. chauvoei*. In this report, we present for the first time, the changes in plasma pyruvic acid concentration in Zebu cattle experimentally infected with *C. chauvoei* and the possible role of neuraminidase and toxins produced by the bacteria in the derangements observed.

Received: 31 July 2007
Accepted for publication: 23 August 2007

Materials and methods

Animal acquisition, acclimatization and grouping

Fourteen (14) Zebu bull-calves were purchased, acclimatized, grazed, aged and grouped into 4 groups as described by Useh (3). Groups A (n=4), B (=3) and C (n=4) were infected with *C. chauvoei* (Jakari strain), toxins and neuraminidase from the bacteria respectively, while group D (n=3) served as control. During the period of acclimatization, the animals were grazed on free range, because of the abundant pasture that characterize the rainy season in Zaria, Nigeria, but when the experiment commenced they were confined in the appropriate experimental pens and fed a combination of groundnut hay and hay prepared from *Andropogon gayanus, Hyprrhenia rufens, Pennisetum pedicellatum and Elionurus probeguinii* until the experiment was terminated. They were supplied feed commensurate with 4% of their individual body weights daily and water *ad libitum*. The weights of the animals were estimated using waist band and ranged between 80-140 kg. The animals were aged using dental eruption (20) and their ages ranged between 19-23 months. Analysis of variance (ANOVA, Duncan multiple range test) was used to compare means ± standard deviations (SD) of the ages cum weights of the experimental animals on day zero of the experiment and there was no statistically significant difference (P>0.05) between the mean age and the mean weight of all the animal groups on day zero of the experiment.

Cultivation of C. chauvoei for infection

Lyophilized *C. chauvoei* (Jakari strain) donated by the National Veterinary Research Institute (NVRI), Vom, Plateau state, Nigeria was used for the experiment. The organism was first isolated from Zebu cattle with blackleg and its pathogenicity indices have been fully determined (21). The preparation of the bacteria and infection of Zebu bull calves was carried out using the method described by Singh *et al.* (22) and the experiment lasted for 21 days.

Culture of C. chauvoei (Jakari strain) for neuraminidase production

Lyophilized *C. chauvoei* (Jakari strain) was obtained from the National Veterinary Research Institute (NVRI), Vom, Plateau state, Nigeria. It was cultivated and neuraminidase was isolated as described previously (12). The neuraminidase was partially purified as described earlier (23) and administered to one experimental group using conventional protocols (3).

Cultivation of C. chauvoei (Jakari strain) for toxin production

The method of Jayaraman et al. (24) was used to cultivate the bacteria and produce the toxins which were administered to one experimental group. The protocol including the amount of toxins administered is described elsewhere (3). Although there are no definite ethical guidelines of animal experimentation in Nigeria, the Zebu cattle were treated as humanely as possible during the experimental period in accordance with international provisions (25). At the end of the experiment, the surviving animals were treated with penicillin (20,000 IU/kg) (Tennyson, China) and they all recovered.

Determination of pyruvic acid concentration in plasma

Blood was collected on days 1 (24 h), 2 (48 h), 3 (72 & 81 h), 4 (105 h), 7 (165 h), 8 (189 h), 9 (214 h), 10 (245 h), 11 (265 h), 13 (293 h) and 21 (413 h) of bacteria, neuraminidase, and toxin-administration respectively and plasma was prepared from the whole blood (26). The same applied to the control group. Pyruvic acid levels were determined from the plasma using the dinitrosalicylic acid (DNS) method (27).

Statistical analysis

Data obtained from the study was computed as mean± standard deviation (SD), analyzed using analysis of variance (ANOVA, Duncan multiple range test) and values of P<0.05 were statistically significant (28).

Results

Pyruvic acid concentrations were higher in the bacteria, neuraminidase and toxin administered groups, compared to the control group. There was a statistically significant difference (P<0.05) between the mean pyruvic acid concentrations of the bacteria (*C. chauvoei*), neuraminidase, toxin-administered and control groups respectively (Fig. 1). From day 1-21 of the experiment, mean pyruvic acid concentration in the control group did not vary significantly (P>0.05). There were 4 peaks of mean pyruvic acid concentration in the bacteria (*C. chauvoei*) administered-group, with the highest peak of 1120.33 ± 186.29 g/L occurring on day 10 (245 h),

followed by 678.58 ± 205.84 g/L on day 4 (105 h), 413.52 ± 103.40 g/L on day 3 (72 h) and 330.68 ± 24.54 g/L on day 1 (24 h) respectively. In the neuraminidase administered-group, there were also 4 peaks of mean pyruvic acid concentration, with the highest peak of 1228.31 ± 198.66 g/L recorded on day 8 (189 h), followed by 649.31 ± 120.67 g/L on day 11 (265 h), 393.89 ± 97.24 g/L on day 3 (72 h) and 99.39 ± 68.72 g/L on day 1 (24 h) respectively. On the contrary, however, three peaks of mean pyruvic acid concentration were recorded in the toxin-administered group, the highest peak of 803.19 ± 94.72 g/L occurring on day 10 (245 h), followed by 452.79 ± 86.09 g/L on day 1 ((24 h) and 423.34 ± 100.60 g/L on day 3 (81 h) respectively. The highest peak of mean pyruvic acid concentration in the neuraminidase-administered group (1228.31 ± 198.66 g/L) was higher than the highest peak in the bacteria (1120.33 ± 186.29 g/L) and toxin-administered (803.19 ± 94.72 g/L) groups in that order. The control group had no peak of mean pyruvic acid levels and the concentrations were almost similar throughout the experimental period (P>0.05) (Fig. 1).

Discussion

This study is a continuation of a series of works to identify the role of neuraminidase in the pathogenesis of blackleg. It is believed that the changes in pyruvic acid levels of ruminants in blackleg, if established, will further assist in understanding the pathogenic roles of neuraminidase and toxins produced by *C. chauvoei* in the disease. Glucose which is a product of carbohydrate metabolism in diet is transformed either to pyruvate in plasma aerobically or lactate anaerobically in muscles (29). In the former, pyruvate is converted to acetyl Coenzyme A which enters the Kreps cycle and is oxidized to yield energy. In blackleg, anaerobiosis occurs following a chain of pathological events: neuraminidase produced by *C. chauvoei* cleaves sialic acid from the muscles and erythrocytes of infected ruminants (18), resulting in the exposure of galactose on the erythrocyte surfaces. Since galactose has a high affinity for lectins, this phenomenon is thought to subject the erythrocytes whose sialic acid is removed to erythrophagocytosis leading to reduced erythrocyte concentration and haemoglobin (3), similar to the erythrophagocytosis reported in trypanosomiasis (30,31) . Since haemoglobin is the oxygen carrying pigment in the body, this results in reduced oxygen tension in the muscles and red blood cells (RBC), leading to impaired cellular (mitochondrial) respiration and hence anaerobiosis. Similarly, toxins produced by *C. chauvoei* triggers necrosis through impaired cellular (mitochondrial) respiration, leading to anaerobiosis (32,33). It can therefore be explained that the anaerobic environment created by neuraminidase and toxins produced by *C. chauvoei* prevents pyruvate metabolism, leading to its accumulation in the plasma as observed in the present study.

Although haemoconcentration has been reported in blackleg (34, 35), recent studies suggest that the pathogenesis of the haemoconcentration is due to the masking effects of anaemia which occurs due to desialylation of erythrocytes (3). The haematological and biochemical changes in blackleg (*C. chauvoei* infection) have been exhaustively reported (34,35), but studies on the variation in plasma pyruvate concentration during the infection have been ignored. In the present study, it was observed that pyruvic acid catabolism was impaired by anaerobiosis in the bacteria, neuraminidase and toxin-administered groups, leading to a build up of high amounts of mean pyruvic acid in the plasma of these animals (Fig. 1), compared to the control animals which were healthy and whose pyruvate catabolism continued unabated. This study suggests that anaerobiosis created by neuraminidase and toxins produced by *C. chauvoei* is the major cause of impaired pyruvate metabolism in blackleg. It has further confirmed that neuraminidase and toxins produced by the bacteria work in tandem with each other in blackleg, and may be playing key roles in the mechanisms of the disease. Further studies should be conducted to investigate the beneficial effects of neuraminidase and toxin inhibitors, if used clinically to manage blackleg.

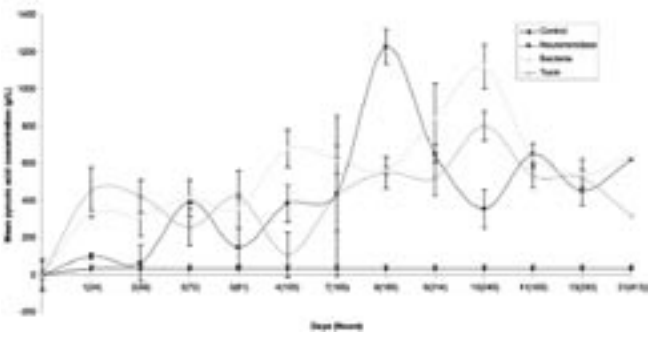

Fig. 1.: Variation in mean pyruvic acid concentration of Zebu cattle experimentally administered *Clostridium chauvoei*, its toxins and neuraminidase

Acknowledgements

Mrs. Chinwe Useh was very supportive when this research was designed and executed. Also, the arrival of Johnmark Kerter Useh provided the intellectual enthusiasm to write this paper.

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SPREMEMBE V NIVOJU PIRUVIČNE KISLINE V KRVNI PLAZMI PRI GOVEDU ZEBU, POSKUSNO OKUŽENIM S *CLOSTRIDIUM CHAUVOEI*

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Povzetek: V raziskavi smo ugotavljali nivo piruvične kisline v krvni plazmi pri govedu zebu, ki je bilo umetno okuženo z bakterijo *Clostridium chauvoei*. Štirinajst moških telet pasme zebu je bilo razdeljenih v 4 skupine. Skupine so bile tretirane z bakterijo *C. chauvoei*, toksinom te bakterije ali encimom nevraminidaza, četrta skupina pa je bila kontrolna in ni bila tretirana. Pri vseh treh poskusnih skupinah smo ugotovili povišanje nivoja piruvične kisline v primerjavi s kontrolno skupino, najvišje koncentracije so bile ugotovljene v skupini, ki je bila tretirana z nevraminidazo. V kontrolni skupini se koncentracija piruvične kisline ni spremenila ves čas poskusa, v ostalih skupinah pa je bila ves čas trajanja poskusa statistično zanesljivo višja ($p < 0,05$) kot v kontrolni skupini. V članek je vključena tudi razprava o morebitnem pomenu dviga piruvične kisline pri patogenezi bolezni črnih nog.

Ključne besede: govedo, bolezni; klostridij infekcije–patologija–diagnostika; piruvična kislina–kri; nevraminidaza–kri; bakterijski toksini–kri

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Examples of references

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

Chapterorar ticle in a book: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

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

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Načini citiranja

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

Poglavje ali prispevek v knjigi: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

Članek iz revije ali časopisa: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

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Slov Vet Res 2007; 44 (3)

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