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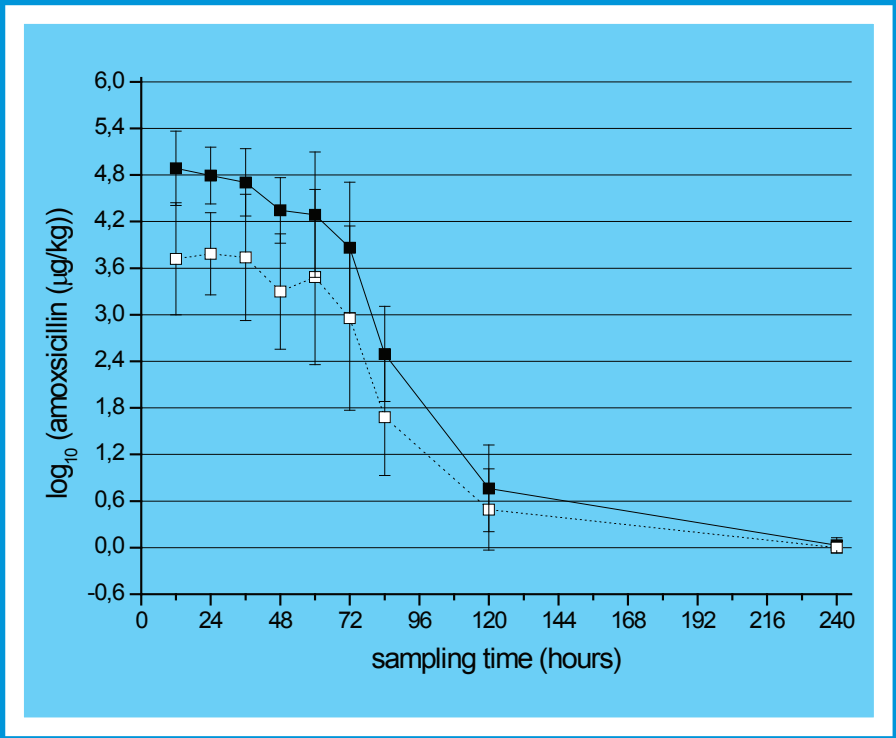

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Review Article

MONENSIN – A REVIEW OF FACTORS INFLUENCING ITS PRESENCE IN THE ENVIRONMENT AND RECOMMENDATIONS FOR SAFE STORAGE AND USE OF MONENSIN-CONTAMINATED MANURE

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Summary: Monensin is a polyether carboxylic ionophore used in the veterinary medicine for treatment and prevention of coccidiosis in poultry. It is the most often used coccidiostat in Slovenia. It is only partially metabolised in the treated animals and gets excreted mostly in its active form. By using monensin-contaminated manure on agricultural soil, monensin enters the environment and can be potentially damaging to soil-dwelling organisms and thereby to the production potential and sustainable use of agricultural soil. There is no regulation regarding the use of manure containing monensin and much uncertainty about its dissipation and effects in the environment. We have reviewed the available literature data on the effects of monensin to soil organisms and on the expected concentrations of monensin in manure and in soil in order to ascertain what (if any) actions would be necessary to mitigate the potential harmful impacts of monensin in soil. By the most realistic scenario, if no manure storage were used, the predicted monensin concentrations in soil would exceed the no-effect levels and could therefore pose a risk to soil organisms. Manure needs to be stored not only because of monensin contamination, but also because use of manure without adequate storage poses a risk of spreading potential diseases and environmental contamination, as well as considerable loss of nutrients. Using an aging period of at least one month would sufficiently reduce the monensin levels in poultry manure to render its use safe for beneficial soil organisms. Composting is preferable to aging manure in a pile, because monensin degradation is faster.

Key words: monensin; manure; environmental risk assessment

Introduction

Monensin is a carboxyl ionophore antibiotic produced by the bacterium *Streptomyces cinnamonensis*. It is used in veterinary medicine for the prevention and treatment of coccidiosis in turkeys and chickens. Animals are treated orally via feed mixtures at a maximum dose level of 125 mg/kg dry feed. The administration to broiler chickens is recommended during the entire lifetime (up to 45 days) and to turkeys for 16 weeks, with the withdrawal period of at least one day (1). According to information gathered with a survey among the feed mixing facilities in Slovenia (unpublished data), approximately 2.4 tons of monensin were used in Slovenia in 2011.

Monensin is only partially metabolised in the treated animals and is excreted almost entirely via faeces (2-5). When the excreta are used as manure on agricultural land, monensin enters the terrestrial ecosystem. The reports on its predicted environmental concentrations (PEC) in soil vary greatly and are between 0.05 mg/kg (6) and 0.59–1.12 mg/kg (7).

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Responsible use of veterinary pharmaceuticals includes the proper management of contaminated wastes. In Slovenia, the amount of manure used on arable soil is regulated by the Decree concerning the protection of waters against pollution caused by nitrates from agricultural sources (8), which is based on the EU Nitrates Directive (9). There is, however, no legislation specifically dealing with the use of manure containing coccidiostats in general or monensin in particular. We therefore reviewed the available literature regarding the kinetics of monensin in treated broiler chickens, its fate and behaviour in the excreta, and its predicted levels and effects in the environment in order to provide a comprehensive basis for establishing the best method and the optimum time of storing monensin-contaminated chicken manure.

Pharmacokinetics of monensin in chickens

Despite the economic importance of chickens and the widespread use of monensin, there are only scarce reports on its kinetics. In 1969, Herberg and Van Duyn (2) studied the excretion and tissue distribution of ^{3}H-labelled monensin in chickens. Most of the administered radioactivity was excreted in the faeces, with less than 2% present in urine and tissues. However, most of the tissue radioactivity was associated with the tissue water, indicating that the results were not effective in defining monensin residues in tissues due to tritium exchange from the ^{3}H-monensin. Further studies therefore employed different methods. Donoho et al. (3) used ^{14}C-labelled monensin to study its pharmacokinetics in broilers. They fed the animals 120 ppm [^{14}C]monensin and measured radioactivity in edible tissues and excreta. The balance-excretion studies revealed that monensin is excreted rapidly and nearly quantitatively (94%) in the urine and faeces with the majority (60.8–83.1% of the total recovered radioactivity) excreted within the first day after treatment. Most of the residue radioactivity in the tissues was measured in the liver, but only about 7% of this radioactivity (0.06 mg/kg) was ascribed to parent monensin.

Davison (5) also used radiolabelled monensin to trace its pharmacokinetics in broilers. Chickens were fed from 2.6 to 100 mg of monensin in a single dose. Between 59.3 and 71.8% of the radioactivity was recovered in the droppings, whereas of the monensin in the tissues, most (up to 5.93 mg/kg) was recovered in the liver, followed by kidneys (up to 2.76 mg/kg), muscle (up to 1.87 mg/kg) and abdominal fat (up to 1.06 mg/kg). The elimination period of monensin from the tissues was not determined.

The development of more precise analytical methods enabled lower detection limits and measurements of trace amounts of substances. Atef et al. (10) used thin layer chromatography to detect monensin in the serum and tissues of broiler chickens after a single dose and after feeding the chickens for 2 weeks with a supplemented monensin premix. The mean elimination half-life of monensin from serum following a single intracrop administration of 40 mg/kg body weight was 2.11 ± 0.08 h. Feeding the monensin premix (120 mg/kg feed) resulted in lower tissue residues and faster elimination from the body. Most of the drug was detected in serum and liver. No monensin was detected within 48 hours after ingestion.

Similar results were obtained by Okada et al. (11) who used quantitative thin layer chromatography to determine monensin residues in broiler tissues under practical conditions of use (feed containing 120 mg/kg monensin during entire lifetime). The highest residues were determined in the fat (0.110 mg/kg body weight) and in the liver (0.039 mg/kg body weight). No monensin was detectable in tissues except fat after a 1-day withdrawal period and in any tissue after 2 days withdrawal.

Henri et al. (12) measured the bioavailability of monensin to chickens and its residues in tissues using mass spectrometry (HPLC-MS/MS). They found that the bioavailability of monensin to broilers is between 28 and 34% and concluded that the rest of the active compound is excreted in the droppings. In the body, monensin was distributed mostly in fat, followed by plasma, liver and muscles. It was still possible to quantify monensin in fat 12 h after the end of treatment (feeding the monensin premix containing 125 mg/kg of feed *ad libitum* for 33 days), whereas no residues were detectable after 6 h in plasma, liver and thigh muscle and after 2 h in breast muscle.

Presence and degradation of monensin in poultry manure

According to EFSA (13), unchanged monensin represents less than 20% of the whole metabolites in chicken excreta. However, no data could be

found on the actual amounts of monensin in broiler manure. An investigation was therefore performed on manure from a farm in Pivka, Slovenia (6), where monensin was measured in composite samples of broiler excreta every week throughout the rearing period (45 days). The results revealed that monensin levels are between 0.72 and 8.91 mg/kg wet manure. This range of antibiotic concentrations can typically be expected in the manure of treated animals (14).

The fate of monensin in manure is also not well known. Only a few studies have been reported and the results are highly variable. An overview of the reported half-lives of monensin in manure and compost is given in Table 1.

The degradation of monensin was studied in manure from chickens treated with monensin at the maximum recommended dose (7). Excreta from five consecutive twenty-four-hour periods (collected between 8-12, 13-17 and 18-22 days of treatment) were analysed for monensin. Half of the wet manure was dried at temperature 70°C, ground and analysed for monensin content. After 30 days, up to 22 and 30% of monensin was no longer detectable in wet and dry manure, respectively.

In another study, also reported by EFSA (7), monensin in manure appears to degrade much faster. After five days, approximately 50% was degraded. After 20 days the concentration in manure was below the detection limit of 0.01 mg kg^{-1}.

Experiments on monensin degradation in chicken faeces were performed on one month old chickens fed with 127 mg monensin kg^{-1} feed (13). Faeces (dry matter 26-28%) were incubated at 27 and 37°C. Levels remaining after 6 days ranged from 7 to 31% of the initial amount, but data showed a large variability.

The only comprehensive and detailed study on monensin degradation in manure was performed on turkey litter by Dolliver et al. (15). Monensin degradation was studied under different conditions of manure storage (manure pile, manure with weekly mixing and moisture adjustment, and vessel composting). The average half-life of monensin was 17 days. The fastest degradation was observed in compost.

Fate of monensin in the environment

The actual concentrations of monensin that can be expected in soil are generally unknown and no measured values can be obtained in literature regarding monensin from poultry manure on agricultural soil. All we can rely on are calculated values obtained from data on the dosage and metabolism of monensin in broilers and its manure content. The predicted environmental concentrations of monensin in agricultural soil were calculated to be between 650 µg/kg in a worst-case environmental exposure scenario obtained on the basis of doses applied in the European broiler production, and 63.4 µg/kg when taking into account data on elimination of the parent compound (16). Based on the measured monensin concentrations in manure in Slovenia (6) and the legislation on the permissible amounts of manure used on agricultural soil in the EU (9), the highest predicted environmental concentrations of monensin we might expect in Slovenia would be around 50 µg/kg soil if the manure were used without prior storage (6).

The measured soil adsorption distribution coefficients (ratio between the amount of substance absorbed to soil and the amount dissolved in water) for monensin are between 1 and 80 and are pH-dependent (7, 17). The corresponding organic carbon partition coefficients are between 125 and 5,700. This indicates low mobility of monensin between environmental compartments – the majority of the substance that enters the soil absorbs to particles and is not dissolved in water. This was confirmed in a study in Denmark (18), where no monensin was detected in waters draining agricultural soils.

When on agricultural soil, monensin undergoes both biotic and abiotic degradation, with biotic degradation being the prevalent (17). Organic matter and soil moisture appear to be the most important factors influencing monensin degradation in soil (17, 19). The soil half-lives of monensin reported in literature are highly variable (Table 2). According to Sassman and Lee (17) the half-life of monensin in soils under laboratory conditions at 23°C was approximately 2 days, while Donoho (4) obtained a half-life of 13 days in field conditions. In another laboratory experiment, Yoshida et al. (19) determined a half-life of 22.7 days in a soil with 1.9% organic carbon (OC) and 4.2 days in a soil with 4.69% OC, while no degradation was observed in air-dried soil. There was a linear relationship between soil moisture and the rate of monensin degradation. EFSA (13) reports soil half-life values of 18, 13

and 15 days for sandy loam, silty loam and clay loam soils, respectively. However, much shorter dissipation half-lives – between 2.3 and 4 days – were reported in another EFSA study (7).

Toxicity of monensin to non-target soil organisms

After knowing the potential environmental concentrations of monensin to which non-target soil organisms would be exposed, the calculation of its potential environmental risk in agricultural soil requires the data on the toxicity of monensin to soil organisms.

In spite of extensive use of monensin for more than 40 years and its potential presence in the environment, there is only little published information concerning its effects on non-target organisms. Jensen et al. (20) have found that the survival of the adult springtail *Folsomia fimetaria* was not affected by monensin at concentrations up to 800 mg/kg dry soil, whereas its EC_{50} for reproduction was 591 mg/kg. Enchytraeids (*Enchytraeus crypticus*) showed similar responses (LC_{50} >800 and EC_{50} for reproduction = 356 mg/kg dry soil). Isopods (*Porcellio scaber*) also exhibited low sensitivity to monensin in soil or food (6); LC50 was >849 mg/g. Earthworms (*Eisenia sp.*), however, are much more susceptible to monensin. Median lethal concentrations of 56 mg/kg and 49.3 mg/kg have been reported (6, 7), whereas EC_{50} for earthworm reproduction was 12.7 mg/kg (6). Some research has also been published on the effects of monensin on plants (7), which are also sensitive to its presence in soil. Wheat, mustard and red clover were tested for emergence and seedling growth. Mustard was shown to be the most sensitive of the three species and had an LC_{50} of 17 mg/kg and EC_{50} for growth of 4 mg/kg.

Recommendations for storage and use of monensin-contaminated manure

According to the EU Technical Guidance Document (21), the potential risk of a substance to the environment is calculated as

Table 1: Literature data on monensin half-lives in manure

Half-life (days)	Method	Reference
17	Wet chicken manure, no treatment	(7)
21	Chicken manure ground and dried at 70°C	(7)
5	Chicken manure, method not described	(7)
3-4	Wet chicken manure at 37°C	(13)
22	Turkey manure in a pile, no treatment	(15)
19	Turkey manure with weekly mixing and water adjustment	(15)
11	Composted turkey manure	(15)

Table 2: Literature data on monensin half-lives in soil

Half-life (days)	Method, soil properties	Reference
13	Field experiment	(4)
18	Laboratory experiment, sandy loam	(13)
13	Laboratory experiment, silty loam	(13)
15	Laboratory experiment, clay loam	(13)
2.3	Laboratory experiment, sandy loam	(7)
4.0	Laboratory experiment, clay loam	(7)
2.5	Laboratory experiment, silty clay loam	(7)
2.0	Laboratory experiment, sandy soil, 0.87% OC	(17)
1.3	Laboratory experiment, clay loam, 2.2% OC	(17)
22.7	Laboratory experiment, loam, 1.9% OC	(19)
4.2	Laboratory experiment, clay loam, 4.69% OC	(19)

a quotient between the predicted environmental concentrations of the substance and the predicted no-effect concentration (PNEC), which is derived from the available data on the toxicity of the substance to non-target organisms. If the obtained risk quotient exceeds 1, there is potential risk involved in its use and suitable measures need to be taken to mitigate the risk.

As described earlier, the predicted environmental concentration of monensin in Slovenia, calculated on the basis of actual monensin levels measured in broiler manure and the legislation regulating the amount of manure that can be used on arable land, we can expect monensin concentrations of up to 50 µg/g soil at the time of manure application.

When toxicity data are available for a producer (photosynthesizing plants), a consumer and/or a decomposer, the PNEC in soil is calculated using assessment factors (21). The lowest measured no-effect concentration (NOEC) is divided by an appropriate factor. If NOEC data are available from only one long-term toxicity test (e.g. plant emergence and growth, or earthworm reproduction), the NOEC is divided by a factor of 100.

In the case of available data on monensin toxicity, several long-term toxicity tests have been performed on consumers (6, 7, 20) and on producers (7), but no data has been provided in the EFSA report (7) on the NOEC for plant emergence and growth. Furthermore, most of the reported results (7, 20) are based on nominal and not measured values of monensin and are therefore unreliable (6). The only known NOEC from a long-term toxicity test with measured monensin concentrations is the value for earthworm reproduction (6), which was 3.5 mg/kg dry soil. This value divided by an assessment factor of 100 gives 35 µg/g soil. The PEC/PNEC ratio is thus 1.43, which indicates that using contaminated broiler manure with no prior aging could be detrimental to the environment. With the very high variability of the available data on its dissipation in soil, it is also uncertain for how long the harmful effects of monensin would persist. One half-life would be sufficient for the risk quotient to fall below 1 (a drop in the soil concentration from 50 to 25 µg/g). However, the measured half-lives vary from just over one day to more than three weeks (Table 2) and are highly dependent on soil type, organic content, moisture and temperature.

The practice of using manure with no storage should therefore be avoided, not only because of monensin contamination, but also because use of manure without adequate storage poses a risk of spreading potential diseases and environmental contamination, as well as considerable loss of nutrients. Manure should be stored long enough to reduce monensin concentrations by at least one half. With the high variability of reports on monensin half-life in manure (Table 1), there is considerable uncertainty regarding the appropriate storage time. The longest half-lives reported were 22 days in manure with no treatment (mixing, water addition or composting). We could therefore say that one month of manure storage should be sufficient to render the manure safe for use on agricultural soil. Composting (addition of plant material to increase the carbon/nitrogen ratio, aeration and water adjustments) was shown to be more efficient in reducing monensin levels, since aerobic microbial processes are the main monensin degradation pathway. If possible, composting should thus be preferred to the traditional way of aging manure with no additional treatment.

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MONENZIN – PREGLED DEJAVNIKOV, KI VPLIVAJO NA VSEBNOSTI V OKOLJU TER PRIPOROČILA ZA VARNO SHRANJEVANJE IN RABO GNOJA, KI VSEBUJE MONENZIN

S. Žižek, M. Gombač, T. Švara, M. Pogačnik

Povzetek: Monenzin je polietrni karboksilni antibiotik, ki ga v veterinarski medicini uporabljamo za preventivo in zdravljenje kokcidioze pri perutnini. Je najpogosteje uporabljan kokcidiostatik pri nas. V prebavnem traktu tretiranih živali se le delno metabolizira, večina pa se ga izloči v aktivni obliki. Z gnojenjem kmetijskih površin monenzin vnašamo v okolje, kjer lahko škoduje talnim organizmom in tako zmanjša proizvodne potenciale in trajnostno rabo kmetijskih zemljišč. O gnojenju s kontaminiranim gnojem pri nas ni pravilnikov, veliko pa je tudi negotovosti o razgradnji in učinkih monenzina v okolju. Pregledali smo dostopno literaturo o učinkih monenzina na netarčne organizme ter njegove pričakovane koncentracije v gnoju in pognojeni zemlji z namenom, da bi ugotovili, kakšni ukrepi bi bili potrebni, da bi omilili morebitne škodljive posledice prisotnosti monenzina v okolju. Po najbolj realističnem scenariju, če gnoja z monezinom zdravljenih živali ne bi starali, bi pričakovane koncentracije monenzina v okolju presegale ravni, ki nimajo učinka na talne nevretenčarje. V tem primeru bi gnoj z monenzinom predstavljal tveganje za okolje. Gnoj je potrebno shranjevati ne samo zaradi nevarnosti kontaminacije z monenzinom, temveč tudi zato, ker uporaba nestaranega gnoja predstavlja možnost širjenja bolezni ter onesnaženja in izgube hranil.
Že enomesečno obdobje staranja gnoja pomeni znižanje koncentracije monenzina na raven, ki ne predstavlja nevarnosti za talne organizme. Kompostiranje je boljše od staranja gnoja v kupu, saj je razgradnja monenzina v tem primeru hitrejša.

Ključne besede: monenzin; gnojenje; ocena tveganja za okolje

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Original Scientific Article

INFLUENCE OF PROLONGED TREATMENT PROTOCOLS ON MAXIMUM RESIDUE LEVELS OF AMOXICILLIN CONCENTRATIONS IN BOVINE MILK

Ožbalt Podpečan[1], Andrej Pengov[2], Petra Zrimšek[4], Pavle Sekulovski[5], Andrej Kirbiš[3]*

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Summary: The aim of this study was to evaluate two off-label protocols for the treatment of bovine mastitis with regard to antibiotic residues in milk and length of withdrawal periods. Forty-eight udder quarters infected with *Staphylococcus aureus*, from 19 dairy cows were included in the study. The animals were divided into two groups (A and B); Group A received intra-mammary and systemic intramuscular treatment with amoxicillin while Group B received intra-mammary treatment only. The impact of the treatment regime on antibiotic concentrations in milk was evaluated. All cows received six intra-mammary injections with cattle-labelled injectors in 12-h intervals, whereas cows in Group A were additionally treated intra-muscularly twice in 24-h intervals. Milk samples were taken in 12-h intervals up to 120 hours after the beginning of treatment, which was considered to be a cut-off value for the withdrawal period. Amoxicillin concentrations were measured using a Waters Xevo Triple Quad mass spectrometer (QQQ) and a Waters UPLC. Amoxicillin concentrations were significantly higher in Group A than in Group B in all measured intervals. Antibiotic concentrations significantly decreased at 72 h (Group B) and 84 h (Group A) after the beginning of treatment. At the end of the producer-suggested withdrawal period, concentrations of amoxicillin were above MRL in 65% of udder quarters in Group B and in 40% of udder quarters in Group A.

Key words: amoxicillin; milk; *Staphylococcus aureus*; mass spectrometry

Introduction

Mastitis is one of the most significant and costly diseases in the dairy industry (1, 2). Over 100 different microorganisms can cause inflammation of the mammary gland; in the majority of cases, staphylococci, streptococci and coliform bacteria are involved. Mastitis pathogens are usually categorized as contagious, such as *Streptococcus agalactiae* and *Staphylococcus aureus,* environmental, such as *Escherichia coli*, or skin flora opportunists, such as coagulase-negative species of staphylococci (3).

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Staphylococcus aureus (S.aureus) is contagious mastitis pathogen and one of the most prevalent major mastitis pathogens in the United States and Europe (4).

In dairy herds, *S. aureus* mastitis can be usually successfully controlled and even eradicated using adequate preventive measures; however, these procedures are time consuming and costly (5). Therefore, in recent years more emphasis has been placed on the treatment of infected udder quarters. Despite that, cure rates concerning the treatment of clinical and especially subclinical *S. aureus* mastitis are still disappointing, and the selection of animals for therapy is crucial in order to achieve reasonable results (6). Reported cure rates for *Staph. aureus* mastitis range considerably; for example, cure

rates for subclinical *Staph. aureus* mastitis range from 4 to 92% (7, 8, 9). The probability of cure depends on the cow, the pathogen and treatment factors. Cure rates decrease with the increasing age of the cow, increasing somatic cell counts, the increasing duration of infection, increasing bacterial colony counts in milk before treatment, and the increasing number of quarters infected (4). Antibiotic preparations for mastitis treatment introduced in the previous 25 years have not been a significant improvement over products that had been in use for a longer period. Moreover, in recent years, no new antibiotics have been released on the market. In order to improve cure rates, treatment regimens have been extended, and a combination of intra-mammary and systemic treatment has been employed (10).

It is well documented that extended therapy regimes improve the bacteriological cure rates of *S. aureus* mastitis (10, 11, 12). Due to the course of the inflammation process in the infected mammary gland and the formation of scar tissue and micro-abscesses (13, 14), the common 1- to 2-day intra-mammary therapy may, in the majority of cases, be too short. Therefore, at least one initial systemic injection should be administered in combination with a 3-day intra-mammary treatment. Benefits of extended treatment protocols, such as higher proportions of cure, resulting in decreasing somatic cell counts, reduced risk of transmission and improved marketability of milk, must be weighed against several drawbacks, including the price of the antibiotic, loss of milk due to withdrawal, increased risk for residues in the milk, and the potential of infecting the mammary gland through repeated infusions via the teat canal (4, 15, 16). Moreover, it is also emphasized that antibiotic treatment will not control new mammary gland infections with *S. aureus*; therefore, only adequate preventive measures will provide lasting results.

Additionally, an extension of the therapy regime prescribed by the producer means that the withdrawal periods must also be prolonged. Within the European Union, veterinarians are allowed to prescribe drugs in an off-label manner, regarding animal species, route of administration or dosage, but are then obligated to ensure that residues do not enter the food chain. Residues are of concern due to possible allergic reactions in people, potential buildup of antibiotic-resistant organisms in people, and the inhibition of starter cultures used in production of cultured milk products, such as yogurt and cheeses (17). According to the provisions in the directive of the European Parliament, 2001/82/EC, in case of off-label treatment the withdrawal period for milk "shall not be less than 7 days" (18).

The objective of the present study was to evaluate the impact of two different extended off-label treatment protocols (one using a combination of intra-mammary and systemic applications and the other intra-mammary applications only) on antibiotic concentrations in milk and the length of the withdrawal period. This study also addresses the validity of the EU directive requiring a 7-day withdrawal period.

Materials and methods

Selection of animals

Nineteen lactating dairy cows (Holstein Friesian) and 48 udder quarters infected with *S. aureus* were included in the study between January and March 2011. The animals were in different phases of lactation and had no perceptible signs of inflammation of the mammary gland; subclinical *S. aureus* mastitis was established during routine milk sampling. None of the cows received any antibiotic preparations two months prior the start of the study. Standard lactation (SL), days in milk (DIM), daily milk yield (DY), body condition score (BCS), and pregnancy status were calculated from farm data records on a cow level (Tables 1 and 2). The study was conducted on a base of routine milk sampling, diagnostics and treatments in accordance to the Slovenian Veterinary Ethical Code. None of the animals was additionally exposed to any unapproved matter.

In Group A, 23 quarters were treated belonging to 9 cows, and in Group B 25 quarters belonging to 10 cows. On average, 2.6 ± 1.0 and 2.5 ± 0.8 quarters per cow were treated in Groups A and B, respectively. In both groups, the number of treated quarters varied from one to four (Table 1, Table 2).

Milk sampling for bacteriological determination

In a yearly routine, i.e. milk sampling for the microbiological status of the herd, milk samples were taken from individual udder quarters of

cows and collected into autoclaved glass tubes following teat disinfection with 70% alcohol, after discarding first three streams of milk. Collected samples were kept cool and sent to a lab the same day for bacteriological determination.

Milk samples were later streaked with a sterile swab within 24 h on quarter plates of washed blood agar with 5% sheep blood and incubated at 37°C. After 48 h, the plates were examined for aerobic bacterial growth. Gram-positive cocci were considered to be *Staphylococcus* or *Micrococcus* species if they were catalase positive. Differences in hemolysin production were classified visually by an experienced observer as either β-hemolysin positive or negative. The slide coagulase test was performed as described by the manufacturer of the rabbit plasma (Biokar diagnostics, France). Later, the API-Staph test (Biomerieux, Macy I´Etolle, France) was used for the final determination of *S. aureus*.

Treatment protocol

Cattle-labeled products for intramuscular (i.m.) and intra-mammary (i.mm) applications containing amoxicillin and clavulanic acid were used. Animals were randomly divided in two groups (Table 1 and 2) and treated by two off-label protocols. Cows in Group A were treated with a combination of systemic and intra-mammary treatment, twice in 24-h intervals with 7 mg amoxicillin-trihydrate and 1.75 mg of clavulanic acid in the form of potassium clavulanate per kilo body weight intramuscularly (Synulox® RTU, Pfizer Luxembourg SARL, Luxembourg), in addition to six intra-mammary injections (Synulox® LC, Pfizer Luxembourg SARL, Luxembourg) of 200 mg amoxicillin-trihydrate, 50 mg of clavulanic acid in the form of potassium clavulanate and 10 mg prednisolone in 12-h intervals. Cows in Group B were treated exclusively with intra-mammary (i.mm.) injections of 200g amoxicillin-trihydrate, 50 mg of clavulanic acid in the form of potassium clavulanate and 10 mg prednisolone six times in 12-h intervals (Table 3).

Milk sampling

To establish the excretion rate of the tested antibiotic product, milk samples of foremilk from treated udder quarters were collected in 12-hour intervals from zero to 120 hours after the start of treatment, which was considered as a cut-off value for withdrawal period. Additional samples were taken 240 h post treatment. Samples were kept deeply frozen at -20°C until analysis.

Sample preparation

1 mL of milk precipitated with acetonitrile (ACN) was mixed in vortex, followed by 10-min centrifugation step at 4000 RPM. The supernatant was filtrated through a 0.45 µl filter. The 10 µl filtrate were injected onto the LC/MS/MS.

LC/MS/MS instrumentation

LC conditions for chromatographic separation were as follows: column, HP ZORBAX Eclipse plus C18 column with dimensions 2.1 x 100 mm i.d., dp 1.8 µm; column temperature at 30°C; mobile phase, (A) 5% ACN and 95% H_20 with 0.1% HCOOH and (B) ACN with 0.1% HCOOH; flow rate, 0.5 mL min^{-1}. Gradient elution was performed (initial gradient: 100% A, 0.5 min: 100% A, 0.51 min: 50% A, 1.7 min: 50% A, 1.71 min: 100% A, 2.2 min: 100% A).

MS conditions

The Waters Xevo Triple Quadropole mass spectrometer was used in the ESI+ mode. The following tune parameters were used: capillary, 2.5 kV; cone, 20 V; source temperature, 150°C; desolvation temperature, 500°C. The instrument was operated in the multiple reaction mode, using the following transitions: m/z = 366.2>113.9 (quantification trace) at collision energy 18 eV, m/z = 366.2>208 (confirmation trace) at collision energy 12 eV. Amoxicilin retention time is 0.58 min. Recovery on MRL value is 85%; the relative standard deviation is 25% on the same concentration level, and the correlation coefficient is 0.89.

Statistical evaluation of results

Data concerning standard lactation (SL), days in milk (DIM), daily milk yield (DY) and body condition score (BCS) were first tested for normality of distribution using a Kolmogorov-Smirnov test. In case that data were normally

Table 1: Animal data of lactation number, standard lactation (305 days), day in milk, number of infected quarters, body condition score and pregnancy status. Group A

Cow No	Lactation No	SL (L)	DIM (L)	DY	No of quarters infected	BCS	Pregnancy status
1	2	9809	358	22	2	3.50	+
2	2	8144	396	15	1	4.00	+
3	1	9153	150	30	3	3.00	+
4	1	8132	186	26	4	3.50	+
5	3	5893	137	26	2	3.75	-
6	1	8210	213	22	3	3.25	+
7	2	8266	183	30	4	3.25	+
8	6	9105	135	29	2	3.00	-
9	2	9231	143	31	2	2.75	-
Mean	2.2	8438.1	211.2	25.7	2.6	3.33	
SD	1.6	1129.3	98.0	5.2	1.0	0.40	
Median	2.0	8266.0	183.0	26,0	2.0	3.25	

Table 2: Animal data of lactation number, standard lactation (305 days), day in milk, number of infected quarters, body condition score and pregnancy status. Group B

Cow No	Lactation No	SL (L)	DIM (L)	DY (l)	No of quarters infected	BCS	Pregnancy status
1	7	6782	211	12	3	3.75	+
2	5	6115	153	21	3	3.50	+
3	3	8491	294	28	2	3.75	+
4	3	9523	334	18	1	3.75	+
5	4	9293	127	33	3	3.25	-
6	4	6824	113	24	4	3.50	+
7	1	9178	122	34	2	2.75	-
8	4	9321	116	40	3	2.75	-
9	3	7322	185	24	2	3.75	+
10	3	10657	99	52	2	2.50	-
Mean	3.7	8350.6	175.4	28.6	2.5	3.33	
SD	1.6	1493.5	81.3	11.6	0.8	0.49	
Median	3.5	8834.5	140.0	26.0	2.5	3.50	

Table 3: A timeline diagram of drug administration in infected quarters in Groups A and B

	0 hour	12 hours	24 hours	36 hours	48 hours	60 hours
Group A	i.mm. i.m.	i.mm.	i.mm. i.m	i.mm.	i.mm.	i.mm.
Group B	i.mm.	i.mm.	i.mm.	i.mm.	i.mm.	i.mm.

distributed, a parametrical t-test test was used to test the differences between Groups A and B, whereas a Mann-Whitney Rank Sum Test was used if data were not normally distributed.

Regarding amoxicillin concentrations, the Kolmogorov-Smirnov test showed that data were not normally distributed; therefore, a $\log_{10}$-transformation of the amoxicillin concentrations was performed to normalize the data. Antibiotic concentrations ($\log_{10}$) at different time samplings in each group were tested using a One-Way Repeated Measures Analysis of Variance. In case of a significant difference among sampling, a pairwise comparison was performed with the Holm-Sidak method using Bonferroni correction. Amoxicillin concentrations ($\log_{10}$) between Groups A and B at each time sampling was compared using t-test.

Values of $P<0.05$ were considered significant for all analyses. SigmaStat 3.5 (SYSTAT Software Inc.) software was used for the statistical evaluation of the results.

Results

Cows in Groups A and B did not differ according to standard lactation, days in milk, daily milk yield and body condition score ($P>0.05$).

Figure 1 shows $\log_{10}$ of amoxicillin concentration in milk in quarters of animals in Groups A and B. Concentrations of amoxicillin in milk differ between Groups A and B from 12 to 84 h after the first treatment ($P<0.05$). Antibiotic concentration significantly decreased 48 and 72 h after the beginning of treatment in Groups A and B, respectively (Figure 1). From data of Table 4, it is evident that after 120 h from the beginning of treatment 65.2% of quarters in Group A and 40.0% in Group B show the concentration of amoxicillin above MRL (MRL = 4µg(kg); $\log_{10}$MRL = 0.6). According to cow level, 66.7 and 20.0% cows in Groups A and B, respectively, show concentration of amoxicillin above MRL. After 240 h, in three quarters from Group A (2 cows), amoxicillin concentration was above zero, but below MRL, whereas no detectable amoxicillin concentration was observed in any of assayed quarters from Group B (Table 4, Figure 1).

Discussion

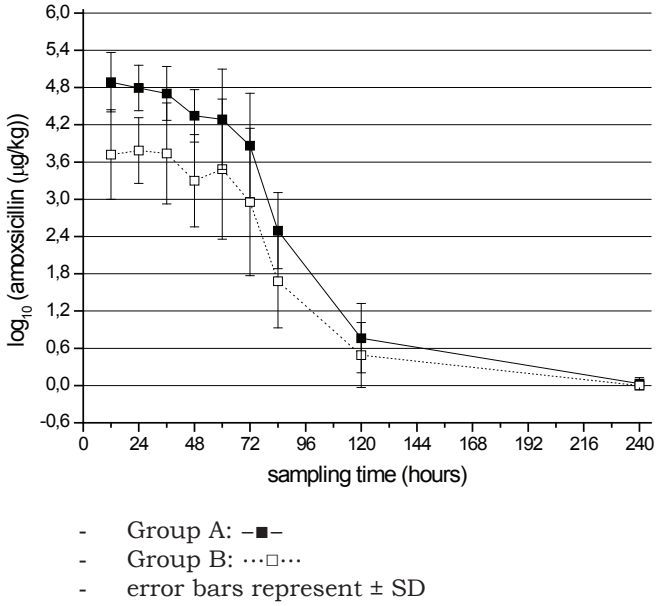
The aim of this study was to evaluate the influence of two mastitis treatment protocols on the presence of amoxicillin residues in bovine milk. Amoxicillin and clavulanic acid are considered to be the treatment of choice in case of penicillin-resistant *S. aureus* strains (4, 19). The treatment protocols tested in the present study are extended protocols based on the results of several studies that have shown that prolonged regimes are the most efficient and cost-effective in the treatment of *S. aureus* mastitis (9, 10, 11). Gillespie *et al.* (20) also found considerably higher cure rates in five- to eight-day therapies compared to two-day therapy. Sol *et al.* (12) observed 2.3-times higher rates of bacteriological cure using extended treatment protocols compared to standard treatments. In contrast, cattle-label antimicrobials for intra-mammary treatment are usually registered for between one and three administrations in 12- to 24-hour intervals. This may not allow drug concentrations at the site of action to exceed the minimal inhibitory concentration for a sufficient period, which potentially influences cure rates, as drug distribution to the site of infection is especially important in the case of *S. aureus* with which micro-abscess and intracellular habitation are common.

According to the manufacturer, the withdrawal periods for one intramuscular and one intra-mammary administration of amoxicillin used in our study were 24 h and 72 h, respectively. Amoxicillin concentrations were significantly higher in Group A (combined intra-mammary and systemic treatment, Figure 1) than in Group B (intra-mammary treatment only, Figure 1) in all measured intervals, except at 120 h and 240 h, where concentrations did not differ significantly between groups. This means that irrespective of treatment duration, higher antibiotic concentrations in milk were achieved by combining intra-mammary and systemic applications. With this protocol, amoxicillin concentrations progressively decreased but remained high until 72 h after the beginning of treatment. Amoxicillin concentrations in Group B, however, differed between sampling intervals and showed overall lower levels. Concentration of amoxicillin decreased significantly after 72 h in Group A and after 84 h in Group B. The sampling interval at 120 h was calculated as the end of the withdrawal period according to drug manufacturer (72 h after last intra-mammary administration). The sampling interval at 240 h followed withdrawal periods defined in the directive of the European

Table 4: Comparison of amoxicillin concentrations in milk quarters of animals at all sampling times according to treatment in Groups A and B

Amoxicillin concentration (μg kg^{-1})	Sampling time (h)								
	12	24	36	48	60	72	84	120	240
Group A (cows N= 9; quarter N=23)									
Mean	126608.9	93358.2	85561.2	35849.3	47930.6	23952.3	750.9	13.2	0.3
SD	130062.4	100986.7	80734.4	33481.2	51602.7	25021.0	990.6	16.5	0.9
Median	76123.0	50936.0	63490.0	21648.5	33684.0	13926.0	403.0	9.0	0.0
Min	9397.0	11863.0	6767.0	3725.0	79.0	190.0	6.1	0.0	0.0
Max	529042.0	414367.0	304143.0	135189.0	198146.0	68597.0	3724.0	70.0	3.2
Quarters above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	65.2	0.0
Cows above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	66.7	0.0
Group B (cows N= 10; quarters N=25)									
Mean	16064.2	12973.0	17621.1	7081.3	21056.5	7150.0	143.3	5.8	0.0
SD	23835.3	20318.6	24654.7	13810.3	33788.9	12599.3	222.4	8.0	0.0
Median	4295.0	5408.5	6924.0	1841.0	4135.0	1541.0	72.0	2.4	0.0
Min	177.0	475.0	144.0	55.0	35.7	4.8	0.0	0.0	0.0
Max	79182.0	84699.0	94773.0	60358.0	150014.0	45915.0	854.0	28.0	0.0
Quarters above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	92.0	40.0	0.0
Cows above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	20.0	0.0

*: MRL = 4 μg/kg



- Group A: –■–
- Group B: ···□···
- error bars represent ± SD

Figure 1: Amoxicillin concentration (log10) in milk in quarters of animals treated in Group A and B

Parliament, 2001/82/EC (18), regarding off-label use of a veterinary medical product (168 h after last intra-mammary administration). At 120 h, amoxicillin concentration was above the MRL in 65.2% of quarters in Group A and in 40.0% in Group B (Table 4). At 240 h, MRL was exceeded in none of the analyzed samples. Considering the results of Stockler *et al.* (21), amoxicillin concentrations measured in the foremilk samples in our study could be higher than concentrations potentially measured in a quarter's bulk milk. We are also aware that concentrations measured at the end of the withdrawal period could be lower on a cow level due to the dilution effect. Regardless of the treatment protocols and levels of antibiotic concentrations, our results also indicate that the duration of therapy significantly influences withdrawal periods. In our previous study (22), the clearance of water-soluble benzylpenicillin procaine from milk was much faster compared to the oil-soluble preparation used in this study; therefore, the withdrawal period may depend not only on the type of beta-lactam, but also on the

supplements, such as mineral oils and Ca-Na-Aluminosilicate in injectors registered for intramammary use.

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VPLIV PODALJŠANIH TERAPEVTSKIH PROTOKOLOV NA OSTANKE AMOKSICILINA V KRAVJEM MLEKU

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Povzetek: Namen študije je bil ovrednotiti vpliv dveh terapevtskih protokolov z izredno uporabo zdravil pri vnetju mlečne žleze na pojav ostankov zdravil v mleku in dolžino karence. V raziskavo je bilo vključenih oseminštirideset mlečnih četrti, okuženih z bakterijo *Staphylococcus aureus*, 19 krav molznic. Živali so bile razdeljene v dve skupini (A in B), skupina A je bila zdravljena intramamarno in intramuskularno, skupina B pa le intramamarno. Ovrednoten je bil vpliv terapevtskega protokola na koncentracije antibiotika v mleku. Vse krave so bile zdravljene s 6 injektorji za intramamarno uporabo tri dni na 12 ur, medtem ko so bile krave v skupini A dodatno intramuskularno zdravljene še z enakim antibiotikom, registriranim za parenteralno uporabo dvakrat v razmaku 24 ur. Mlečni vzorci so bili odvzeti v 12-urnih intervalih do 120 ur po zdravljenju, ki je predstavljalo točko preloma za določitev dolžine karence. Koncentracije amoksicilina so bile zmerjene na masnem spektrometru Waters Xevo Triple Quad (QQQ) in Waters UPLC. Koncentracije amoksicilina v mleku so bile značilno višje pri skupini A v primerjavi s skupino B v vseh meritvenih intervalih. Koncentracije antibiotika so se pri skupini B značilno znižale 72 ur, pri skupini A pa 84 ur po začetku zdravljenja. Ob izteku karence, določene pri proizvajalcu, je bilo v skupini A 65 % vimenskih četrti nad dovoljeim MRL, pri skupini B pa 40 %.

Klučne besede: koncentracije amoksicilina; kravje mleko; *Staphylococcus aureus*; masna spektrometrija

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Original Scientific Article

COMPARISON OF THE CHEMICAL COMPOSITION AND NUTRITIONAL VALUES OF FRESH AND FROZEN RAINBOW TROUT

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Summary: The aim of this work was a comparison of the chemical compositions (including water, carbohydrate, protein, fat, ash content, mineral substances and fatty acids profiles and nutritional value) of fresh refrigerated and frozen rainbow trout (*Oncorhynchus mykiss*). Fillets of rainbow trout (without skin) obtained from aquaculture, and divided into two groups, trout cooled and stored at 0–4 °C and trout frozen and stored at –18 °C, were used. By providing the basic chemical composition of rainbow trout fillets, we determined that the water content was 73 %, protein content 18.5 %, fat content 2.8 %, and ash content 1.15 %; there were no statistically significant differences when comparing fresh and frozen trout. Based on the profile of fatty acids, the most representative n-3 polyunsaturated fatty acids (PUFAs) in rainbow trout were docosahexaenoic acid (DHA) 12.7 % and eicosapentaenoic acid (EPA) 4.5 %. PUFAs n-6 consisted primarily of linoleic acid (14.5 %), mono-unsaturated fatty acids of oleic acid (21.4 %), and saturated fatty acids palmitic acid (13 %). The sum of total PUFAs represents 42.9 %, when n-3 PUFAs represent 22.55 % and n-6 PUFAs 20.36 %. Comparing the nutritional value of rainbow trout fillets calculated from the basic chemical composition, statistically significant differences between fresh (106.34 kcal) and frozen (106.11 kcal) fillets were not found, which means that these different ways of extending shelf life should not affect the nutritional value.

Key words: chemical composition; fatty acids; freezing; nutritional value; trout

Introduction

In 2008, fishing and aquaculture produced worldwide approximately 142 million tons of fish, of which 115 million tons were used for human consumption with an estimated conversion of about 17 kg (body weight) per capita. Aquaculture accounted for 46 % of total fish supply for consumption in 2008, which is an increase from 43 % in 2006. Aquaculture continues to be the fastest growing sector of food-producing animals (1). In 2008, diadromous fish production was dominated by salmon farming (1.5 million tons), milkfish (0.68 million tons), rainbow trout (0.58 million tons) and eel (0.26 million tons) (2). Aquaculture is of increasing importance for the supply of fish such as trout and salmon to industrialised countries. Highly effective production has been attained through the establishment of breeding programmes, the optimisation of feeds, and improved disease treatment (3). The quality of farmed fish and of products made from farmed fish strongly depends on the rearing conditions and, in particular, on the feed composition (4).

The chemical compositions of fish species show differences according to season, migratory

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behaviour, sexual cycle and feeding cycles. These factors are mainly observed in wild fish. Farmed fish in aquaculture may also exhibit differences in chemical composition, but in such cases there factors are monitored so that the chemical composition can be predicted. High-quality dietary fish meat is a result of its higher proportion of simple protein, favourable fat composition, high lipophilic vitamins content, the softness of muscle fibres, the absence of elastin in connective tissue and relatively high mineral content. The composition of fish meat is dependent on the type of fish, season, sexual cycle, aquatic environment, age and sex, when water content varies in the range of 50 to 80 %, protein content 15 to 25 %, fat content 0.1 to 35 % and mineral substances 0.8 to 2 % (5).

Lipids are vital components of fish diets due to their role in providing energy sources, the essential role of some fatty acids, carriers of fat soluble vitamins and resource of polar lipid plus sterols, which are essential structural compounds of cell membranes. Polyunsaturated fatty acids (PUFA) and their derivatives, eicosanoids, are highly biologically active substances of a lipid nature. They are divided according to the position of the first double bond nearest the methyl end of the n-3, n-6, n-9 unsaturated fatty acids. Mammalian bodies are able to synthesize double bonds of n-9 position toward the carboxyl end of fatty acids. However, they are not able to form double bonds to position n-3 and n-6; these fatty acids must be obtained from the diet and, therefore, are considered to be essential (6). Fatty fish, such are tuna, mackerel, herring, anchovy and salmon, and fish oils contain essential n-3 polyunsaturated fatty acids known as eicosapentaenoic acid (EPA) (C20:5 n-3) and docosahexaenoic acid (DHA) (C22:6 n-3) (7).

The aim of this work was the determination of the chemical composition, including water, protein, fat, ash content, mineral substances profile and fatty acids profiles, and the calculation of nutritional value of fresh refrigerated and frozen trout.

Materials and methods

Twenty-four rainbow trout (*Oncorhynchus mykkis*) were raised at the Rybarstvo Požehy farm. Fish with an average body weight of 300 g were eviscerated, washed, cooled, and vacuum packaged to avoid surface dehydration, and subjected to two different treatments. The first group (n = 12) was placed directly into a cold room (0 to 2 °C) and stored for five days. The second group (n = 12) was individually quick frozen (IQF) until the temperature of the fish cores had reached –18 °C. The frozen trout were immediately stored in a freezing room (–18 °C) for six months for further analyses. Subsequently, the samples were thawed in cold conditions (0–2 °C) until the fish cores had reached –1 °C.

The basic chemical parameters, including profiles of mineral substances and fatty acids of twelve fresh rainbow trout, were performed immediately after cooling. The basic chemical parameters (water, carbohydrate, protein and fat contents) were examined in fresh vacuum-packed trout after five days of storage and in frozen samples after six months of storage and subsequent thawing, and nutritional values were calculated.

Determination of chemical composition

The basic chemical composition (water, carbohydrate, protein, fat and ash content) was determined according to AOAC Official methods of analysis (8). Samples for the determination of the profile of mineral substances were prepared using microwave degradation in an MLS-1200 MEGA (fy Milestone) digestion system; analysis of minerals (Ca, Mg, Na, K, Cu, Zn, Mn, Fe) was carried out using Flame Atomic Absorption Spectroscopy (FAAS) Solar 939 (fy Unicam). Phosphorus content was analysed via spectrophotometry.

Determination of profile of fatty acids

Total lipids in samples were isolated using chloroform/methanol according to Čertík et al. (9). Methylesters of fatty acids were measured in rainbow trout oil by gas chromatography using GC-6890 N (Agilent Technologies) according to Čertík et al. (10). On the column DB-23 (50 %-cyanopropyl-methylpolysiloxan, length 60 m, diameter 0.25 mm, width of film 0.25 μm), 1 μl of fatty acids methylesters was automatically injected, and then the samples were analysed under following conditions: carrier gas: hydrogen (44 cm/s at 130 °C), temperature of injection: 220 °C, split: 1:50, FID detector (250 °C, flow of hydrogen: 30 ml/min, flow of oxygen: 500 ml/

min), temperature regime: 130 °C - 1 min, 130–170 °C – 6.5 °C/min, 170–215 °C – 2.7 °C/min, 215 °C - 7 min, 220–240 °C – 20 °C/min, 240 °C - 2 min). Records were evaluated using ChemStation B0103 (Agilent Technologies) and quantified on the basis of retention times of known standards of fatty acids C4–C24 (Sigma, USA).

Calculation of nutrition value

The energy value to be declared in rainbow trout was calculated using the following conversion factors (11): carbohydrate 4 kcal/g and 17 kJ/g; protein 4 kcal/g and 17 kJ/g and fat 9 kcal/g and 37 kJ/g.

Statistical evaluation

The mean values and standard deviations were calculated by using column statistics with processing of six values for each analysed group. Statistically significant differences between groups were calculated using t-test in the program GraphPad Prism 5 (2007). Differences were evaluated as statistically significant when this P value was < 0.05.

Results

In fresh rainbow trout fillets before vacuum packing, the basic chemical composition (water, protein, fat, ash content and profile of mineral substances) listed in Table 1 and the profile of the fatty acids found in fish oil (Table 2, Figure 1) were determined.

By providing the basic chemical composition of rainbow trout fillets, we determined that the average water content was 73 %, protein content 18.5 %, fat content 2.8 %, and ash content 1.15 %. Based on the determination of profile of mineral substances, these are listed by order of concentrations in Table 1.

Based on the profile of fatty acids, the most representative polyunsaturated fatty acids (PUFA) n-3 series found in oil of rainbow trout were docosahexaenoic acid (DHA) at 12.7 % and eicosapentaenoic acid (EPA) at 4.5 %. The PUFA n-6 series consisted primarily of linoleic acid (14.5 %), monounsaturated fatty acids (MUFA) oleic acid (21.4 %), and saturated fatty acids (SFA), palmitic acid (13 %). The amount of total PUFA represents 42.9 % of the n-3 PUFA are 22.554 % and n-6 PUFA 20.361 %.

The basic chemical compositions (water content, protein content, fat content) in the fresh chilled and frozen trout fillets, vacuum packed, were determined (Table 3).

In comparing the chemical composition of both groups, no statistically significant differences ($P>0.05$) were found.

Based on the chemical composition, the nutritional value of chilled and frozen trout fillets was calculated using conversion factors (11) (Table 4).

Table 1: Chemical composition of fillets from rainbow trout

Parameter	Value
Water g.100 g^{-1}	73.03 ± 0.66
Protein g.100 g^{-1}	18.57 ± 0.52
Fat g.100 g^{-1}	2.81 ± 0.73
Ash g.100 g^{-1}	1.15 ± 0.38
Potassium g.100 g^{-1}	0.425
Phosphorus g.100 g^{-1}	0.240
Calcium g.100 g^{-1}	0.091
Magnesium g.100 g^{-1}	0.051
Sodium g.100 g^{-1}	0.050
Iron mg.100 g^{-1}	2.840
Manganese mg.100 g^{-1}	1.560
Zinc mg.100 g^{-1}	1.020
Copper mg.100 g^{-1}	0.040

Table 2: Profile of fatty acids in rainbow trout oil

Fatty acids	Formulas	Value (%)
Myristic acid	C 14:0	3.2
Pentadecanoic acid	C 15:0	0.3
Palmitic acid	C 16:0	13.0
Palmitoleic acid	C 16:1-9c	5.1
Hexadecadienoic acid	C 16:2-9c,12c	0.8
Heptadecanoic acid	C 17:0	0.3
Stearic acid	C 18:0	3.0
Oleic acid	C 18:1-9c	21.4
Cis-vaccenic acid	C 18:1-11c	3.0
Linoleic acid n-6	C 18:2-9c,12c	14.5
Alpha linolenic acid n-3	C 18:3-6,9,12c	0.4
Gamma linolenic acid n-6	C 18:3-9,12,15c	2.0
Octadecatetraenoic acid n-3	C 18:4-6,9,12,15c	1.2
Eicosenoic acid	C 20:1-11c	4.8
Eicosadienoic acid n-6	C 20:2-11c,14c	0.8
Dihomo-linolenic acid n-6	C 20:3-8,11,14c	0.4
Arachidonic acid n-6	C 20:4-5,8,11,14c	0.8
Eicosatrienoic acid n-3	C 20:3-11,14,17c	0.2
Eicosatetraenoic acid n-3	C 20:4-8,11,14,17c	0.8
Eicosapentaenoic acid n-3	C 20:5-5,8,11,14,17c	4.5
Docosanoic acid	C 22:0	0.1
Erucic acid	C 22:1-13c	3.7
Docosadienoic acid n-6	C 22:2-13c,16c	0.1
Docosapentaenoic acid n-3	C 22:5-7,10,13,16,19c	1.7
Docosahexaenoic acid n-3	C 22:6-4,7,10,13,16,19c	12.7
Tetracosenoic acid	C 24:1	0.0

Table 3: Chemical composition of fresh and frozen fillets from rainbow trout

	Water	Protein	Fat	Carbohydrates
Fresh fillets	76.84 ± 0.58	18.77 ± 0,46	2.74 ± 0.62	1.65 ± 0.28
Frozen fillets	76.90 ± 0.81	18.35 ± 0.66	2.99 ± 1.11	1.45 ± 0.41

Table 4: Nutritional value of fresh and frozen fillets from rainbow trout

Component	Fresh fillets			Frozen fillets		
	g.100 g^{-1}	Kcal	kJ	g.100 g^{-1}	Kcal	kJ
Protein	18.77	75.08	319.09	18.35	73.4	311.95
Fat	2.74	24.66	101.38	2.99	26.91	110.63
Carbohydrates	1.65	6.6	28.05	1.45	5.8	24.65
Energy value in 100 g		106.34	448.52		106.11	447.23

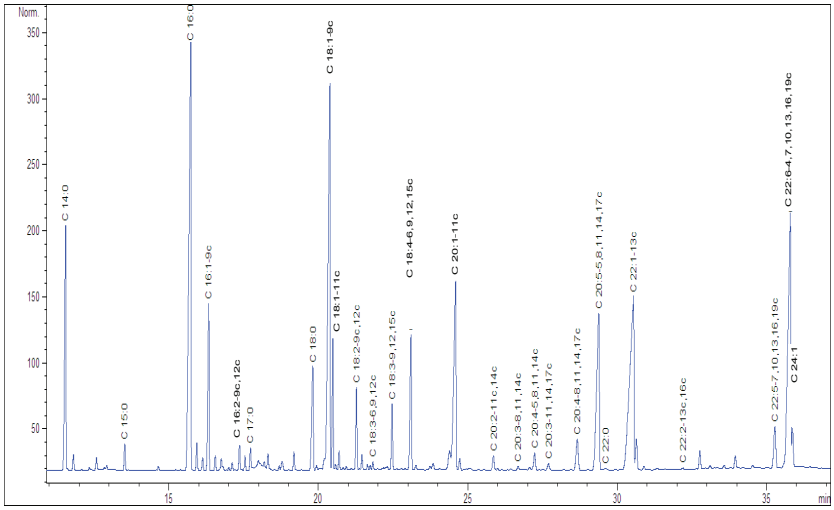

Figure 1: Chromatographic record of fatty acids profile in rainbow trout oil

Comparing the nutritional value of rainbow trout fillets calculated from the average values of basic chemical composition, no statistically significant differences (P>0.05) between fresh (106.34 kcal) and frozen (106.11 kcal) fillets were found.

Discussion

Fish meat consists of water, proteins and other nitrogenous compounds, lipids, carbohydrates, vitamins and minerals. The water content in the whole body of the trout was 70 %, and 73 % in the fillets, protein content 17.25 and 20.03 %, respectively, and the fat content 10.58 % throughout the whole body, compared with 5.18 % in the fillets. The ash content was 2.36 % in the whole fish and 1.48 % in trout fillets (3). These values are comparable to our results achieved in the determination of chemical composition in rainbow trout fillets. However, the biggest differences can be in fat content, when the content of total lipids in different species of freshwater fish can range between 0.6 to 30 %. In the various organs of fish, there were impacts on the content and composition of fatty acids and lipids fish species, sex, age, water temperature, degree of pollution, nutritional status, seasonal variations and origin of fish (12).

Lipids and fatty acids play a significant role in membrane biochemistry and have a direct impact on membrane-mediated processes, such as osmoregulation, nutrient assimilation and transport. However, the nature and quantity of these lipids in fish vary according to species and habitat (6). It is known that n-3 fatty acids are essential for neural development in infants and during the first few years of life, and have beneficial effects on hypertension, inflammation, arrhythmias, psoriasis, aggression, depression, coronary heart disease, inflammatory and auto-immune disorders and cancer (13). The fatty acid composition of fish is highly variable between and within species. It has been indicated that these variations are influenced by a number of factors, such as food availability and nutritional habits of fish, catch area, fish size, age, maturity, season and sampling tissue (14).

Based on the profile of fatty acids, the most representative polyunsaturated fatty acids (PUFA) n-3 series found in oil of rainbow trout were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA); the PUFA n-6 series consisted primarily of linoleic acid. The amount of total PUFA is 42.9 %; 22.554 % of which is n-3 PUFA and 20.361 % is n-6 PUFA. The optimal composition of fatty acids in the diet is a decisive factor that plays a role in preventing disease and improving health. Generally, in Europe, consumers are experiencing the effects of an increased intake of fats in their diet; therefore, the factor of composition of the fatty acids and their ratio is even more important. The recommended ratio of polyunsaturated fatty acids is 1 portion of n-3 PUFAs to 2 portions of n-6 PUFAs. In the current diet based on the consumption of red and white meat, PUFAs are imbalanced in the ratio from 1:10 to 1:50. In our study, ratio of polyunsaturated fatty acids in rainbow trout oil 1.1 portion of n-3 PUFAs to 1 portion of n-6 PUFAs was found.

The fatty acid (FA) composition of some tissues of *Salmo trutta labrax* in Turkey was investigated. Fatty acids profiles of muscle, liver, gonad, egg and adipose tissue were compared in terms of total and individual saturated and unsaturated fatty acids. The results of the present study have revealed that the most abundant individual FAs were palmitic, oleic and docosahexaenoic acids (DHA) in most of the tissues. In general, SFA was higher in the muscle tissue while the MUFAs were dominant in the gonads. There was also a significant difference between the PUFA profiles of the tissues. For example, total n-3 PUFAs were 4.8 to 7.7-fold higher than that of the n-6 PUFAs, and the eggs had the highest n-3 PUFA (48.09 ± 9.38) content (15).

The fatty acid composition of liver and muscle tissues of immature and mature *Oncorhynchus mykiss* fed on two different diets were determined. Fatty acid analyses were carried out using gas chromatography. Palmitic acid (C16:0), oleic acid (C18:1 n-9), linoleic acid (C18:2 n-6) and docosahexaenoic acid (C22:6 n-3) were the key components in both liver and muscle tissues of immature and mature rainbow trout of both sexes. The amounts of C22:6 n-3 were higher in the liver (29.04 ± 0.06 – 27.41 ± 0.17 %) and muscle (13.05 ± 0.40 – 11.37 ± 0.21 %) of immature fish than in mature fish, and depended on the composition of the diet. The results of this study have shown that fatty acid composition in fish tissues can vary considerably, depending on the age of fish and their diet (14).

It is well known that lipid oxidation is one of the most significant problems in fish processing and subsequent storage and shelf-life (16). Unsaturated fatty acids are more easily oxidizable than saturated acids, and thus it is assumed that the fatty fish whose higher fat content are subject to oxidative changes faster than lean fish with a standard diet (17). Due to oxidation, the organoleptic characteristics of fish are also ultimately adversely affected (7).

It is known that fish meat has high nutritional and biological value. The muscle tissue contains approximately 15–20 % protein, and fish proteins have, in contrast to mammals and poultry, favourable amino acid composition. Fish is a rich source of mineral substances; small bones softened by processing (e.g. marinated fishery products) can be eaten along with the meat and thus become a valuable source of phosphorus, calcium, iodine and selenium. However, by comparing the nutritional value of rainbow trout fillets calculated from the basic chemical composition, no statistically significant differences between fresh cooled (106.34 kcal) and frozen (106.11 kcal) fillets were found, which means that these different ways of extending shelf life should not affect the nutritional value.

In conclusion, the beneficial effects of a diet rich with n-3 PUFA for humans are a reason for the regular consumption of fish and encourage aquaculturists to find alternatives to increase fish production. Fish can make a significant contribution toward reducing fat in people's diet. Not only is fish low in fat, but it is also a tasty, highly nutritious and wholesome food that can offer an endless variety to menus. Rainbow trout is an excellent meat group choice because it is lower in fat and calories than some foods from the meat group and is also an excellent source of many essential nutrients. The presented fish species with its high n-3 PUFA content verified in this research could be a potential healthy food fish in terms of the positive effects of EPA and DHA in the diet.

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PRIMERJAVA KEMIČNE SESTAVE IN HRANILNE VREDNOSTI SVEŽE IN ZAMRZNJENE ŠARENKE

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Povzetek: Namen našega dela je bil primerjati kemično sestavo (vsebnost vode, ogljikovih hidratov, beljakovin, maščob, pepela, mineralnih snovi in maščobnih kislin) in hranilno vrednost sveže oz. ohlajene in zamrznjene šarenke (*Oncorhynchus mykiss*). Fileti iz šarenk (brez kože), pridobljeni iz ribogojnic, so bili razdeljeni v dve skupini, v ohlajene in shranjene pri 0-4 °C ter v zamrznjene in shranjene pri -18 °C. S kemično analizo smo ugotovili, da vsebujejo 73 % vode, 18,5 % beljakovin, 2,8 % maščob in 1,15 % pepela. Ugotovili smo, da ni statistično značilnih razlik v kemični sestavi filetov iz šarenk med načinoma shranjevanja. Da bi ugotovili vpliv načina shranjevanja na njihovo hranilno vrednost, smo v njih analizirali tudi vsebnost maščobnih kislin. Fileti šarenke vsebujejo največji delež dokozaheksaenojske kisline (DHA) - 12,7 % in eikozapentaenojske kisline (EPA) - 4.5 % izmed n-3 polinenasičenih maščobnih kislin. Med n-6 polinenasičenimi maščobnimi kislinami je najvišji delež linolne kisline (14,5 %), med mononenasičenimi maščobnimi kislinami delež oleinske kisline (21,4 %) in med nasičenimi maščobnimi kislinami delež palmitinske kisline (13 %). Polinenasičene maščobne kisline predstavljajo 42,9 % , med temi n- 3 22,55 % in n-6 20,36 %. Na osnovi kemijčne analize smo primerjali hranilno vrednost filetov šarenke in ugotovili, da ni statistično značilnih razlik med svežimi (106,34 kcal) in zamrznjenimi (106,11 kcal), kar kaže, da različna načina podaljšanja roka uporabnosti ne vplivata na prehransko vrednost.

Ključne besede: kemična sestava; maščobne kisline; zamrzovanje; hranilna vrednost; postrv

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Original Scientific Article

A RETROSPECTIVE STUDY OF CANINE TESTICULAR TUMOURS IN SLOVENIA

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Summary: The aim of this study was to provide up-to-date information on the incidence of and some epidemiological data on the testicular tumours of dogs in Slovenia. Amongst the 1975 tumours of male dogs submitted to the Institute of Pathology, Forensic and Administrative Veterinary Medicine at the Veterinary Faculty of the University of Ljubljana between January 1995 and January 2012, testicular tumours were diagnosed in 206 dogs (the mean age 9.9 ± 2.7 years). The detection rate of testicular tumours was 10.4% (206/1975) in all tumours in male dogs.

Altogether, 301 testicular tumours were diagnosed in our study: 144 seminomas (SEMs) (47.8%), 86 interstitial cell tumours (ICTs) (28.6%), 59 Sertoli cell tumours (SCTs) (19.6%) and 12 mixed germ cell-sex cord stromal tumours (MGCSCTs) (4%). Single unilateral testicular tumours were detected in 141 dogs (68.4%), whereas 37 dogs (18%) exhibited multiple unilateral tumours. Twenty-eight dogs (13.6%) had bilateral tumours: 12 dogs (5.8%) had one tumour in each testicle, and 16 dogs (7.8%) had multiple bilateral tumours. Foci of neoplastic cells were found in the lymph or/and blood vessels of six (50%) MGCSCTs, 20 (40.8%) SCTs and 34 (28.1%) SEMs. The frequency of blood/lymphatic vessel invasion was higher in SEMs with a diffuse growth pattern ($P<0.05$) and in SCTs with diffuse/tubular growth, but the difference was near the limit of significance ($P=0.07$). No metastases were noted in ICTs.

The highest prevalence of testicular tumours was detected in mixed breed dogs (20.4%), followed by German Shepherds, Collies, Poodles, Golden Retrievers, Cocker Spaniels, German Boxers, Labrador Retrievers and Samoyeds.

Key words: pathology; testicular tumours; dog; Slovenia

Introduction

According to the World Health Organization's (WHO) classification of tumours of domestic animals (1), the major types of testicular tumours are sex cord-stromal (gonadostromal) tumours, germ cell tumours and mixed germ cell-sex cord stromal tumours. Interstitial (Leydig) cell tumours (ICT) and Sertoli (sustentacular) cell tumours (SCT) are the most common types of testicular tumours, derived from the sex cord-stromal tumours, whereas seminoma (SEM) is the most common type is derived from the germ cells (1).

Testicular tumours are common in dogs, and their prevalence is increasing: from 1960 to 1992 the prevalence ranged from 0.91 to 16% (2, 3, 4, 5), and from 2000 to 2009 was from 5.4% to 27% (6, 7, 8, 9). In men, the worldwide incidence of testicular cancer has doubled since 1970s (10, 11).

This paper reports on the first retrospective study on testicular tumours of dogs in Slovenia since 1995, providing information on their incidence, as well as their histopathological, clinical and epidemiological characteristics.

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Materials in methods

A retrospective study was done on the paraffin-embedded archive samples of testicular tumours collected at the Institute of Pathology, Forensic and Administrative Veterinary Medicine at the Veterinary Faculty of the University of Ljubljana. The largest proportion of samples is represented by surgical biopsy samples sent from veterinary clinics, while the rest were samples taken during necropsies performed at the institute.

Formalin-fixed, paraffin embedded, 4 µm-thick tissue sections stained with haematoxylin and eosin, collected over 17 years, from January 1995 to January 2012, were reviewed. Tumours were classified according to the latest WHO classification of tumours of the genital system of domestic animals (1). If more than one tumour was diagnosed in one testicle, the tumour was classified in the category of multiple testicular tumours. If tumours were found in both testicles, they were classified as bilateral testicular tumours.

Furthermore, data regarding age and breed of the dogs and the location of the tumours were obtained from the necropsy records.

A Chi-square test was used to evaluate the relationship between the tumour type and the age of dogs, tumour type and tumours with metastases, between tumour types and tumour location, and between the age of dogs and the number of tumours.

Results

Detection rate of testicular tumours

From January 1995 to January 2012, different tumour types were histopathologically diagnosed in 1975 samples of various tissues and organs of male dogs. Amongst them, testicular tumours were diagnosed in 206 dogs. The detection rate of testicular tumours was 10.4% (206/1975) of all tumours in male dogs.

The tumour types and their characteristics

In total, 301 testicular tumours were diagnosed in 206 dogs involved in this study.

Unilateral tumours were detected in 178 dogs (86.4%): in 141 dogs (68.4%) only single testicular tumours were detected, and 37 dogs (18%) exhibited multiple unilateral tumours. The most common multiple unilateral testicular tumours were SEM-ICT (12/37 dogs=32.4%) and multiple SEMs (12/37 dogs=32.4%). Combination SEM-SCT tumours were found in nine dogs (24.3%) and SCT-ICT in two (5.4%). In two dogs (5.4%), three tumour types: SEM, SCT and ICT were detected in one testicle.

Bilateral tumours were found in 28 dogs (13.6%). Twelve dogs (5.8%) with bilateral tumours had one tumour in each testicle, but 16 dogs (7.8%) had multiple tumours in one or both testicles. The most common amongst the first group were bilateral SEMs (5 dogs= 2.4%), followed by bilateral SCTs (3 dogs= 1.5%). Bilateral ICT, SEM-ICT, SEM-MGCSCT and SCT-MGCSCT were found in one dog each.

Data regarding the group with multiple bilateral tumours are shown in Table 1.

Amongst 301 testicular tumours, there were 144 SEMs (47.8%), 86 ICTs (28.6%), 59 SCT (19.6%) and 12 mixed germ cell-sex cord stromal tumours (MGCSCT) (3.99%).

Seminomas

The total number of SEMs was 144 (47.8%); 121 tumours were available for histopathological re-evaluation. Histologically, 47 tumours (38.8%) had a diffuse growth, 62 tumours (51.2%) showed intratubular growth, whereas both types of growth were observed in 12 tumours (9.9%).

Blood/lymphatic vessel invasion was found in 34 (28.1%) of 121 SEMs. A significant relationship was found between the tumour type and the frequency of blood/lymphatic vessel invasion ($P<0.05$). The frequency of blood/lymphatic vessel invasion was the highest in SEMs with a diffuse growth pattern (57.5%) and much lower in a combination of diffuse and intratubular and intratubular growth patterns (Table 2) ($P<0.05$).

In only two of 1043 dissected male dogs (0.19% of dissected dogs) were metastases of SEM found; in both cases, they were in the iliac lymph nodes, and distant metastases were found in the lungs of one dog.

Interstitial cell tumours

The total number of ICTs was 86 (28.6%), and 81 tumours were available for histopathological

Table 1: Combinations of testicular tumour types in 16 dogs with multiple bilateral tumours. Legend: SEM, seminoma; SCT, Sertoli cell tumour; ICT, interstitial cell tumour; MGCSCT, mixed germ cell-sex cord stromal tumour

Tumour combination	Number of dogs	%
SEM/ICT-SEM/ICT	4	25
multiple SEMs-multiple SEMs	4	25
ICT-ICT/SEM	4	25
multiple ICT-multiple ICT	2	12.5
SEM-SEM/ICT	2	12.5
Together	16	25

Table 2: The frequency of blood/lymphatic vessel invasion in different growth patterns of seminomas

Tumour types	Number of tumours	Number of tumours with blood/lymphatic vessel invasion	Frequency of blood/lymphatic vessel invasion (%)
Diffuse growth pattern	47	27	57.4
Intratubular growth pattern	62	6	9.7
Diffuse and intratubular growth pattern	12	1	8.3
SEM – together	121	34	28.1

Table 3: The frequency of blood/lymphatic vessel invasion in different growth patterns of Sertoli cell tumours. Legend: SCT, Sertoli cell tumour

Tumour types	Number of tumours	Number of tumours with blood/lymphatic vessel invasion	Frequency of blood/lymphatic vessel invasion (%)
Diffuse/tubular SCT	22	12	54.5
Tubular SCT	27	8	29.6
SCT – together	49	20	40.8

Table 4: Location of testicular tumour types. Legend: SEM, seminoma; SCT, Sertoli cell tumour; ICT, interstitial cell tumour; MGCSCT, mixed germ cell-sex cord stromal tumour.

Tumour type	N	Left testicle	Right testicle	Unknown location
SEM	144	39	31	74
ICT	86	22	23	41
SCT	59	12	11	36
MGCSCT	12	0	3	9
Total	301 (100.0%)	73 (24.2%)	68 (22.6%)	160 (53.2%)

re-examination. Histologically, two growth patterns were observed: a solid-diffuse pattern and a cystic-vascular growth one. No metastases were noted in ICTs.

Furthermore, in nine dogs, microscopic proliferations of interstitial cells, considered to be nodular hyperplasia of interstitial cells, were detected.

Sertoli cell tumours

The total number of SCTs was 59 (19.6%). Paraffin tissue blocks of 49 tumours were available for histopathological examination. Histologically, two growth patterns were found: diffuse/tubular and tubular. Both had a similar frequency, while SCTs with pure diffuse growth were not detected.

Blood/lymphatic vessel invasion was found in 20 of 49 SCTs (40.8%). The frequency of blood/lymphatic vessel invasion was 54.6% in SCTs with diffuse/tubular growth patterns; however, in SCTs with the tubular pattern, the frequency of invasion was almost two times lower (29.6%), but the difference was near the limit of significance (P=0.07) (Table 3).

In only one out of 1043 dissected male dogs (0.10% of dissected dogs) were metastases found in the iliac lymph nodes and the liver.

Mixed germ cell-sex cord stromal tumours

The total number of detected MGCSCTs was 12 (4%), in which a combination of neoplastic germ and Sertoli cells was found. Foci of neoplastic cells were found in lymph and/or blood vessels in six tumours (50%).

Tumour locations

The data on the tumour locations were available only for 141 tumours. Seventy-three tumours (24.2%) developed in the left testicle and 68 (22.6%) in the right (Table 4). The tumour location was unknown in 160 (53.2%) cases. There was no relationship between tumour type and tumour location (P=0.283).

The age of dogs

Data about the age of dogs with testicular tumours was available for 191 dogs. Their age varied from 2 to 17 years. Most of the testicular tumours were diagnosed between 9 and 12 years (38.8%), less frequently between 6 and 9 years (22.3%) and between 12 and 15 years (21.4%). The mean age of dogs with testicular tumours was 9.9 ± 2.7 years (Fig. 1). A significant relationship was found between the tumour type and the age of dogs (P<0.05).

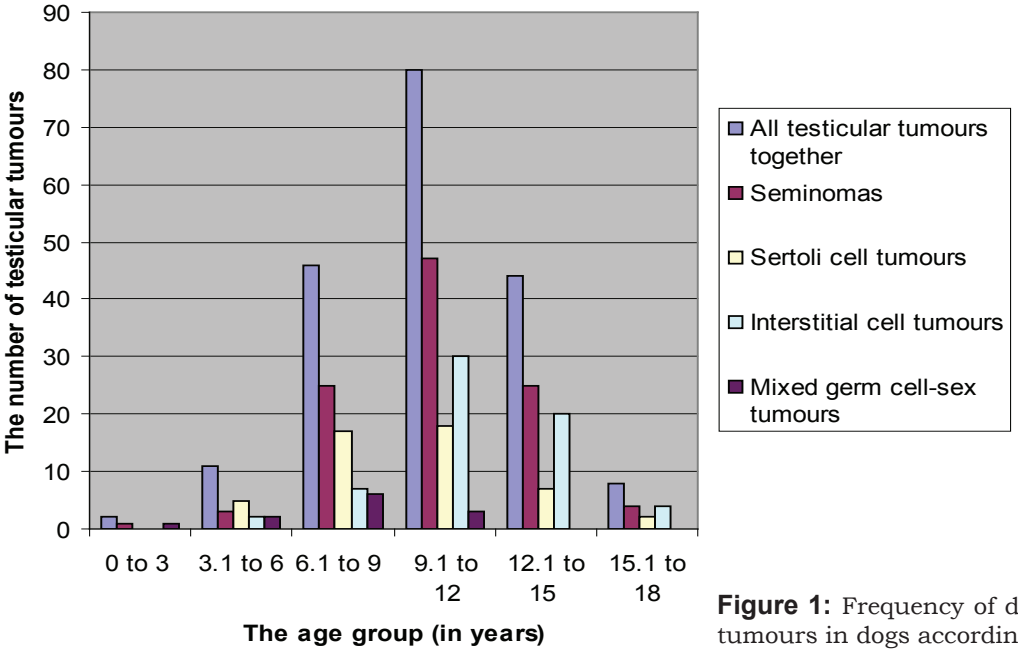

Figure 1: Frequency of different types of testicular tumours in dogs according to age.

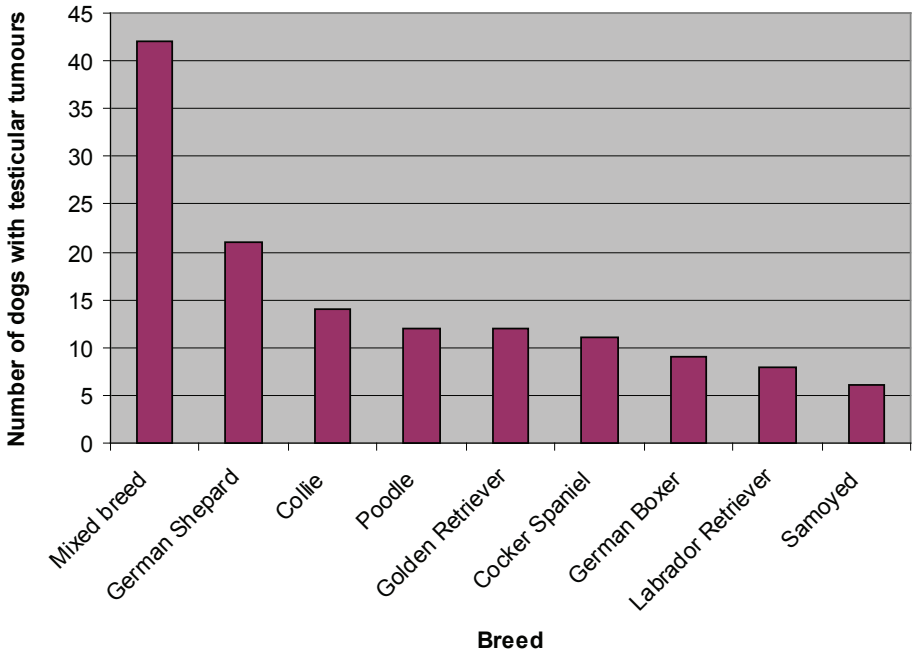

Figure 2: Frequency of different types of testicular tumours in dogs according to breed

The age distribution of dogs with SEMs, SCTs and ICTs was similar to the one listed above; they were most often diagnosed between 9 and 12 years.

Mixed germ cell-sex cord stromal tumours were diagnosed in dogs younger than 12 years, with the highest frequency between 6 and 9 years of age (50%) (Fig.1 and 2).

Breeds of dogs

Data on dog breeds were available for 200 dogs. The highest prevalence of testicular tumours was found in mixed breed dogs (41 dogs=20.4%), followed by German Shepherds (21 dogs= 11%), Collies (14 dogs=7.3%), Poodles and Golden Retrievers (12 dogs=6.3%), Cocker Spaniels (11 dogs=5.8%), Boxers (9 dogs=4.7%), Labrador Retrievers (8 dogs=4.2%) and Samoyeds (6 dogs=3.1%). Cases of testicular tumours were found in single dogs of other breeds (Fig. 2).

Discussion

In this study, the detection rate of testicular tumours was 10.4% of all tumours of male dogs. The incidence reported in the literature since 2000 varied from 9.45% to 27% (6, 7, 8, 9). Differences in the reported prevalence are partly due to different methodologies used; some authors (7, 8) determined the relative incidence after histological examination of both testes collected at the necropsy of each dog, although macroscopically testes were without visible alterations, while others (6, 9) determined the detection rate on biopsy and necropsy samples collected due to visible testicular tumours or other gross pathological testicular lesions. Earlier studies were based mostly on biopsy samples (2, 4, 5) or tumours detected at routine necropsies (3). The real prevalence of testicular tumours in dogs from Slovenia is presumably higher than calculated in this study, since many samples of testicular tumours are not sent for histopathological examination (personal communication with clinicians). In the year 2010, when clinicians were encouraged to submit all detected testicular tumours, the detection rate rose to 18.8%.

Almost a third of the dogs (65 dogs=31.6%) included in this study had more than one testicular tumour, either multiple uni- or bilateral or one tumour in each testicle. This frequency is similar to the 31% incidence reported by Grieco et al. (2008), and slightly lower than the 38.29% incidence reported by Santos et al. (2000). An earlier study (12) stated that 8.45% of dogs had more than one testicular tumour. In dogs from Slovenia, the most common tumour type detected in animals with more than one testicular tumour was SEM. Solely or in other combinations, it was found in 56 dogs (86.2%) with more than one testicular tumour.

SEMs were also the most common tumours found in this study (47.8%). SEMs were also reported as the most frequent tumour type by authors of recent studies (7, 9, 13), while some other authors described ICTs as the most common tumours (6, 8) or reported that both tumour types occur at equal frequencies (12, 14, 15). However, the highest prevalence of SEMs in the most recent studies is interesting, as SEMs in men are the prevailing testicular type and their incidence is on the increase (10, 11).

Twelve MGCSCTs (3.99%) were also diagnosed; this is a group of tumours in which Sertoli and germ cell elements are intimately combined within a single tumour (16). This type of testicular tumour was first described in 1981 (17) and was later detected with a prevalence from 4.76% (7) to 22.9% (9).

Blood/lymphatic vessel invasion was found in 28.1% of SEMs, 40.8% of SCTs and 50% of MGCSCTs. In SEMs, foci of neoplastic cells in lymph/blood vessels were significantly related to diffuse type of growth. In SCTs in which the pure diffuse type was not detected, blood/lymphatic vessel invasion was connected with diffuse/tubular growth. According to the literature, in SEMs and SCTs the diffuse histological pattern is more likely to be associated with malignant behaviour (1, 16). Regional and distant metastases were reported in less than 15% of dogs diagnosed with either SCTs or SEMs (18). Metastasis occurs in the adjacent lymph nodes of the sublumbar and pelvic region and to internal organs (16). The metastatic rate was assessed mainly in older retrospective studies (3, 12, 19). Single case reports of metastatic disease are described in newer references (20, 21, 22, 23).

The data on the location were available for less than half of the tumours. Statistical analysis did not show any relationship between tumour type and tumour location (P=0.283). According to the literature, in the descended testes there is no predilection for cancer development in either the right or the left testis (18). However, the abdominal and inguinal location of the testis is known to be one of several predisposing factors for the development of testicular tumours, with the right testis more often being retained (18) and, therefore, predisposed to tumorogenesis (7, 14, 18, 24). The occurrence of SCTs and SEMs in cryptorchid testes is much higher than in scrotal testes (1, 18, 24). In the records of this study, the data regarding cryptorchidism were sparse and were, therefore, not included in our study.

The largest number of testicular tumours (38.8%) was diagnosed at the age between 9 and 12 years (the mean age was 9.9 ± 2.7 years). The mean age of dogs with testicular tumours in other studies was between 9.5 years (25) and 11.9 years (13). Dogs older than six years had a 21.5 times higher risk of developing testicular tumours then dogs under two years of age and 4.2 times higher risk than 3- to 5-year-old dogs (7).

Of the studied dogs with testicular tumours, 20.4 % were mixed breeds, followed by German Shepherds, Collies, Poodles, Golden Retrievers, Cocker Spaniels, Boxers, Labrador Retrievers and Samoyeds. Testicular tumours in other breeds were found only sporadically. Several authors wrote about increased risk of the development of primary testicular tumours in Boxers (12, 25), German Shepherds (8), Afghan Hounds (26), Weimaraners (26), Shetland Sheepdogs (15, 24), cairn terriers (25), border and Shetland collies (15, 25), Pekingeses (25) and Maltese dogs (9). According to statistical data, German Shepherds and mixed breeds are most common amongst dogs in Slovenia, together constituting 55% of the canine population (27). Similarly, other authors state that some of the breeds with a high incidence of testicular tumours are also dominant in the population of dogs in their countries (8, 9) and, therefore, this may not represent the true breed predisposition.

This study provides the first information on the incidence, histopathological, and some clinical and epidemiological characteristics of testicular tumours of dogs in Slovenia from 1995 to 2012. Further studies would be required to elucidate the role of other risk factors, such as body condition, diet, physical activity, testicular trauma, etc., in the pathogenesis of canine testicular tumours.

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RETROSPEKTIVNA ŠTUDIJA TUMORJEV MOD PRI PSIH V SLOVENIJI

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Povzetek: Retrospektivno študijo tumorjev mod pri psih v Sloveniji smo opravili, da bi pridobili najnovejše podatke o njihovi incidenci in nekaterih epidemioloških značilnostih. Med 1975 tumorji, ki smo jih na Inštitutu za patologijo, sodno in upravno veterinarstvo Veterinarske fakultete Univerze v Ljubljani diagnosticirali pri psih med leti 1995 in 2012, smo tumorje mod ugotovili pri 206 psih, kar predstavlja 10,4 % pojavnost (206/1975). Skupno smo diagnosticirali 301 tumor mod: 144 seminomov (SEM) (47,8 %), 86 tumorjev intersticijskih celic (ICT) (28,6 %), 59 tumorjev sertolijevih celic (SCT) (19,6 %) in 12 mešanih tumorjev semenskih trakov in germinalnih celic (angl. mixed germ cell-sex cord stromal tumours, MGCSCT) (4 %). Po en tumor na enem od mod smo ugotovili pri 141 psih (68,4 %), 37 psov (18 %) pa je imelo na enem modu multiple tumorje. Osemindvajset psov (13,6 %) je imelo bilateralne tumorje mod, od teh jih je 12 (5,8 %) imelo po en tumor na vsakem modu, 16 (7,8 %) pa multiple tumorje na obeh modih. Skupine tumorskih celic smo v limfnih in/ali krvnih žilah ugotovili pri 6 (50 %) MGCSCT, 20 SCT (40,8 %) in 34 (28,1 %) SEM. Metastaziranje je bilo bolj pogosto pri SEM z difuzno obliko rasti ($P<0,05$) in SCT z difuzno/tubularno rastjo. Pri slednjih je bila razlika blizu meje signifikance ($P=0,07$). Pri ICT nismo ugotovili metastaziranja.
Povprečna starost psov s tumorji mod je bila 9,9 ± 2,7 let. Tumorje smo najpogosteje ugotovili pri mešancih (20,4 %) in nemških ovčarjih (11 %), škotskih ovčarjih (7,3 %), manj pogosti pa so bili pri kodrih in zlatih prinašalcih (6,3 %), koker španjelih (5,8 %), nemških bokserjih (4,7 %), labradorcih (4,2 %) in samojedih (3,1 %).

Ključne besede: patologija; tumorji mod; psi; Slovenija

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Original Scientific Article

EFFECT OF DIETARY EXTRUDED LINSEED, VERBASCOSIDE AND VITAMIN E SUPPLEMENTS ON SELECTED SERUM BIOCHEMICAL PARAMETERS AND PLASMA OXIDATIVE STATUS IN LACAUNE EWES

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Summary: Selected serum biochemical parameters and plasma oxidative status were evaluated in Lacaune ewes on a diet supplemented with extruded linseed, verbascoside and vitamin E. A 98 day-trial was conducted on 44 ewes and started 40 ± 2 days post partum. The animals were divided into four homogeneous groups of eleven animals each; one control group (CON) with a basal diet, and the other three experimental groups supplemented with extruded linseed (L group), extruded linseed-verbascoside (LVB group) and extruded linseed-verbascoside-vitamin E (LVBE group). All animals individually received an isoproteic and isoenergetic diet, consisting of 700g of concentrated feed and meadow hay *ad libitum*. Blood sampling of the ewes was performed three times: at the beginning (0 d), midway (49 d) and end of the trial (98 d). The following parameters were determined: triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, bilirubin, alanine aminotransferase, aspartate aminotransferase, thiobarbituric acid reactive substances (TBARS), reactive oxygen metabolites (ROMs), vitamin A and vitamin E. Dietary integration in the LVB and LVBE groups resulted in an improvement in the blood profile, characterized by a significant (P=0.001) increase in HDL cholesterol and a marked decrease in triglycerides (P=0.001), total cholesterol (P=0.017), LDL cholesterol (P=0.001) and bilirubin (P=0.035), due to the verbascoside supplementation. In the same groups, there was an increase in blood vitamin A (P=0.002) and vitamin E (P=0.001), and a reduction (P=0.001) in ROMs and TBARS, with an improved plasma oxidative status. The dietary vitamin E integration did not produce a significant improvement in the parameters studied, with no statistical differences between LVB and LVBE groups. Animal feed containing extruded flaxseed, might therefore benefit from the addition of a verbascoside supplement, because of the positive effect showed by this molecule on the blood parameters.

Key words: extruded linseed; serum biochemical parameters; verbascoside; vitamin E; Lacaune ewe

Introduction

The integration of feed with extruded linseed is a strategy used in animal feed for milk production in order to improve its acidic profile (1, 2, 3, 4, 5,). Sheep milk fat is characterized by a low concentration of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and a high content of saturated fatty acids (SFAs), in particular myristic and palmitic acid, with a hypercholesterolemia effect (6, 7). Dietary extruded flaxseed increases the level of MUFA, PUFA and conjugated linoleic acid (CLA) and improves the health benefits of milk and dairy products (5, 8). However, in animals this dietary strategy can lead to an increase in MUFA and PUFA contents in blood, in subcutaneous and intramuscular lipids, thus increasing the susceptibility of these tissues to lipid peroxidation (9, 10).

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The use of dietary antioxidants is recommended to limit lipid peroxidation and preserve animal health and product quality (11). Vitamin E is a synthetic antioxidant commonly used in animal nutrition due to its ability to inhibit lipid peroxidation (12) through the inactivation of peroxyl radicals and by capturing radical electrons (13); but its bioefficiency is limited when n-3 PUFA intake is increased (14).

Dietary antioxidant molecules integrated with vitamin E could be more effective for preventing plasma lipid peroxidation (15), as also found by Gladine et al. (10), in dairy cows supplemented with flaxseed oil. This effect was probably because of the hydrophilic properties of plant extracts rich in polyphenols which exhibit affinity for those parts of cells that are complementary to the lipophilic vitamin E (16). Studies on chickens (17) have shown that plant extracts obtained from oregano prevented lipid peroxidation in muscle tissue and may be complementary to vitamin E (17).

Researchers have therefore, focused on characterizing plant extracts, and on isolating and identifying the constituents responsible for antioxidant activities (18, 19). *Lippia citriodora*, a plant species of the Verbenaceae family, is characterized by the presence of different flavonoids and phenolic compounds, including verbascoside (also known as acteoside), and luteolin derivatives (20). Verbascoside is a phenylpropanoid glycoside with anti-inflammatory (21), anti-cancer (22), antioxidant (23) and cardioprotective activities (24). In our previous studies (25, 26, 27, 28), on different animal species, sheep, rabbits and hare, after dietary integration of verbascoside we found a significant improvement in blood parameters, with a decrease in triglycerides, total cholesterol, LDL cholesterol, bilirubin, ROMs and TBARS and an increase in HDL cholesterol, vitamin A and vitamin E. Corino et al. (29) also found a positive effect on plasma oxidation and content of IgA by feeding weaned pigs an extract of *Lippia Citriodora* rich in verbascoside.

The aim of this study was to assess the effects of dietary antioxidant substances, verbascoside and vitamin E, on serum biochemical parameters and plasma oxidative status in milking Lacaune ewes, fed a diet containing extruded flaxseed.

Material and methods

Diet and animals

The trial lasted 98 days and was conducted on 44 Lacaune ewes. At the beginning of the test (40 ± 2 days post partum) the animals were divided into four groups of 11 ewes each, homogeneous by age (4-6 years), body weight (54.06 ± 2.85 kg), parity (III-V) and body condition score (BCS, 2.32 ± 0.11). One group was the control (CON), without the addition of extruded linseed, or feed supplements; while three experimental groups received a dietary supplementation in the feed, of only extruded linseed (L group); extruded linseed and verbascoside (LVB group); and extruded linseed, verbascoside and vitamin E (LVBE group). All ewes were reared in single boxes (size 1.5 x 3.0 m) and all experimental procedures involving animals were in accordance with European Community guidelines and approved by the Italian Ministry of Health.

Each animal received a daily isoproteic and isoenergetic diet as follows:

-CON group: 700 g of basal concentrated pellets, without extruded linseed, and meadow hay *ad libitum*;

-L group: 700 g of concentrated pellets, containing extruded flaxseed 200 g/kg feed, and meadow hay *ad libitum*;

-LVB group: 700 g of concentrated pellets, our formulation, containing extruded flaxseed 200 g/kg feed plus verbascoside 2.86 g/kg feed, and meadow hay *ad libitum*;

-LVBE group: the same dietary treatment as LVB group plus vitamin E 14.29 g/kg feed.

The feed ratio was commensurate with the physiological and productive requirements of milking ewes, according to Nutrient Requirements of Sheep (NRC, 30).

The chemical composition of the feed and meadow hay is reported in Table 1.

The antioxidant supplement contained a water-soluble extract of Verbenaceae (*Lippia* spp.) leaves, prepared on an industrial scale by a standardized procedure which includes ultrasonic extraction with 60% aqueous ethyl alcohol (EtOH) followed by spray drying with maltodextrins as an excipient.

The phenylpropanoid glycoside and benzoic acid content of the feed supplement are reported in Table 2, according to a certificate of analysis provided by the manufacturer. The quantitative analysis of the phenolic compounds was performed by HPLC-UV-DAD (Rastrelli, personal communication) according to Piccinelli et al. (31). To avoid oxidation in the feed, the supplement is microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray-cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

The dietary vitamin microencapsulated supplement contained 20% of D, L-α-tocopherol acetate, covered with a protective film consisting of a mixture of vegetal fatty acids, and stabilized with a natural antioxidant provided by IZA company (Forlì, Italy).

Blood sampling and analyses

The ewes' blood was blood sampled three times: at the beginning (0 d), midway (49 d) and at the end of the trial (98 d). Blood samples were taken with a vacutainer (Venosafe, Terumo Europe N.V., Leuven, Belgium) from the external jugular vein, on fasted animals for at least 10 hours, with the use of two tubes: the first, with a gel separator, for the production of serum and the second, with lithium heparin for the plasma production. The blood was centrifuged for 15 min at 3000 rpm and the serum immediately tested using an automatic clinical chemistry analyzer, model ARCO (Biotecnica Instruments S.p.A., Rome, Italy) for the following parameters: triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Thiobarbituric acid reactive substance (TBARS) levels of reactive oxygen metabolites (ROMs) and the concentrations of vitamins A and E were determined in the plasma. TBARS was determined in the plasma according to Esterbauer and Zollner (32). Briefly, a standard curve was generated using 1,1,3,3-tetramethoxypropane (Sigma Aldrich, St. Louis, USA). Trichloroacetic acid 10% (v/v) was added to the plasma samples, promoting the precipitation of proteins. The resulting mixture was incubated for 15 min on ice. After centrifugation at 2200 rpm at 4 °C for 15 min, 0.67% thiobarbituric acid was added to the supernatant. The mixture was incubated in a water bath at 90 °C for 10 min, after which the absorbance was read at 532 nm in a spectrophotometer. The results were expressed as µmol of thiobarbituric acid per litre of plasma.

The concentration of ROMs in plasma was determined by a spectrophotometer and a colorimetric method, as proposed by Diacron (Diacron International srl, Grosseto, Italy), using a commercial kit at a wavelength of 505 nm (33). The results were expressed in Carr units (1 Carr unit equals 0.024 mmol/l of H_2O_2).

Vitamins A and E were extracted from the plasma samples with chloroform (34) and analyzed on an HPLC system (Kontron Instruments, Milan, Italy) consisting of an autosampler (HPLC Autosampler 360, Kontron Instruments, Milan, Italy) with a 20 ml loop, a high pressure mixing pump and a 5 µm, 250 x 4.60 mm C18 column (Phenomenex, Torrance, CA, USA). The mobile phase was 100% methanol at a flow rate of 1.0 ml/min. A fluorimeter detector (SFM) and computer with Kroma System 2000 software were used. The vitamin A and E concentrations were determined using an internal standard and the elution time of pure standards.

Statistical analysis

After assessing the frequency distribution, all variables were subjected to analysis of variance using the GLM procedure of the statistical package SPSS (35). Analysis included between-subjects main effect (D) of dietary supplementation (CON, L, LVB and LVBE), within-subjects main effect of sampling time (T) and interaction of dietary supplementation x sampling time (D x T). An individual ewe was the experimental unit. The differences between means were considered significant for $P<0.05$ using the Sheffè test.

Results

Selected serum biochemical parameters

The dietary antioxidant treatment carried out in the LVBE and LVB groups, significantly affected ($P<0.01$) the lipid profile parameters, such as triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol (Table 3).

Table 1: Chemical composition and fatty acid profile of diets

		Concentrates[1]		Hay
		TypeA[2] (CON group)	**Type B[3]** (L, LVB, LVBE groups)	
Chemical composition	**%**			
Dry matter		923.0	929.9	92.20
Crude protein		178.0	180.2	7.80
Ether extract		33.6	125.1	1.80
Ash		70.0	81.9	7.50
Neutral detergent fiber (NDF)		169.9	249.1	57.60
Acid detergent fiber (ADF)		47.8	74.9	39.50
Lignin		10.9	22.7	5.50
Calcium		12.6	13.8	1.10
Phosphorus		3.3	4.0	0.25
Fatty acid	**g100·g^{-1} FA**			
C 16:0		15.21	9.60	19.00
C 18:0		1.99	4.14	3.20
C 18:1 n-9		22.98	19.20	6.55
C 18:2 n-6		54.15	21.92	26.95
C 18:3 n-3		3.52	43.95	36.90
Others fatty acid		2.15	1.19	7.40
Metabolizable energy	**Mcal·kg^{-1}**	3260	3300	1355

[1]**Ingredients of concentrate pellets**: barley, soybean flour, corn flour, extruded linseed (for experimental groups only) wheat bran, wheat flour, molasses, di-calcium phosphate, calcium carbonate, sodium chloride, sodium bicarbonate, magnesium oxide. Minerals and vitamins supplement /kg of concentrate: iron ($FeSO_4$) 100 mg; iodine ($Ca(IO_3)_2$) 5.00 mg; cobalt ($CaSO_4$) 3.00 mg; zinc (ZnO) 100 mg; selenium (Na_2SeO_3) 0.30 mg. Vitamins: A 40,000 U.I; D3 4,000 U.I; E acetate 40 mg; B_1 3.0 mg; B_2 2.0 mg; B_6 0.40 mg; B_{12} 0.010 mg.

[2]**Type A**: - control concentrate without extruded linseed and dietary supplements (*CON group*)
[3]**Type B**: - experimental concentrate with 200 g of extruded linseed·kg^{-1} of concentrate (*L group*)
- experimental concentrate with 200 g of extruded linseed + 2.86 g of verbascoside supplement·kg^{-1} of concentrate *(LVB group)*
- concentrate with 200 g of extruded linseed + 2.86 g of verbascoside supplement + 14.29 g of microencapsulated vitamin E supplement (D, L-α tocoferil acetate)·kg^{-1} of concentrate (*LVBE group)*

Table 2: Phenylpropanoid glycosides and benzoic acid content of feed supplement

Components	**g·kg^{-1} of supplement**
Gallic acid	1.755 ± 0.07
3.4-dihydroxybenzoic acid	0.450 ± 0.04
Methyl gallate	1.955 ± 0.09
Isoverbascoside	0.455 ± 0.04
Verbascoside	4.470 ± 0.08

Table 3: Serum biochemical parameters in Lacaune ewes

Parameters		Groups†				SEM§	P-value‡		
		CON	L	LVB	LVBE		D	T	D x T
Ewes	**n.**	11	11	11	11				
Triglycerides	**mg·dl^{-1}**								
0 d		19.87^{a}	20.14^{a}	21.30^{a}	22.90^{a}	0.569			
49 d		22.75	23.66	19.58	21.63	0.619			
98 d		25.86^{1b}	26.76^{1b}	18.20^{2b}	19.40^{2b}	0.709	0.001	0.035	0.001
Total cholesterol	**mg·dl^{-1}**								
0 d		72.85	73.76	74.62^{a}	73.24^{a}	0.479			
49 d		74.02	74.93	72.23	71.19	0.461			
98 d		73.95^{1}	74.86^{1}	70.51^{2b}	69.89^{2b}	0.579	0.017	0.021	0.047
HDL cholesterol	**mg·dl^{-1}**								
0 d		26.76	26.40	27.42^{a}	26.93^{a}	0.368			
49 d		26.93^{1}	26.75^{1}	29.35^{2}	29.60^{2}	0.379			
98 d		26.12^{1}	26.31^{1}	33.69^{2b}	32.90^{2b}	0.680	0.001	0.001	0.001
LDL cholesterol	**mg·dl^{-1}**								
0 d		41.71	42.62	39.14^{a}	38.36^{a}	0.613			
49 d		43.97^{1}	39.14^{1}	36.32^{2}	36.34^{2}	0.895			
98 d		45.03^{1}	45.58^{1}	34.89^{2b}	34.72^{2b}	0.944	0.001	0.017	0.116
ALT	**UI·l^{-1}**								
0 d		36.19	35.65	38.04^{a}	38.70^{a}	0.390			
49 d		38.88	37.98	37.14	38.09	1.150			
98 d		37.90	37.00	34.03^{b}	35.82^{b}	0.480	0.484	0.015	0.308
AST	**UI·l^{-1}**								
0 d		74.50	74.05	74.92	72.80	1.300			
49 d		73.98	73.53	72.01	71.78	0.890			
98 d		74.71	74.17	72.39	72.24	2.550	0.140	0.373	0.839
Bilirubin	**mg·dl^{-1}**								
0 d		0.534	0.536	0.532^{a}	0.540^{a}	0.018			
49 d		0.547	0.543	0.517	0.522	0.011			
98 d		0.548^{1}	0.553^{1}	0.505^{2b}	0.488^{2b}	0.025	0.035	0.029	0.011

Table 4: Plasma oxidative status marker in Lacaune ewes

Item		Groups†				SEM§	P-value‡		
		CON	L	LVB	LVBE		D	T	D x T
Ewes	**n.**	11	11	11	11				
ROMs	**U·Carr^{-1}**								
0 d		158.69^{a}	163.28^{a}	160.17^{a}	161.65^{a}	2.74			
49 d		261.92^{1b}	266.44^{1b}	133.58^{2b}	138.28^{2b}	10.41			
98 d		289.33^{1c}	298.42^{1c}	119.23^{2c}	116.62^{2c}	15.73	0.001	0.001	0.001
TBARS	**µmol·l^{-1}**								
0 d		0.212^{a}	0.202^{a}	0.194^{a}	0.208^{a}	0.029			
49 d		0.296^{1b}	0.323^{1b}	0.176^{2}	0.182^{2}	0.022			
98 d		0.481^{1c}	0.535^{1c}	0.138^{2b}	0.140^{2b}	0.031	0.001	0.003	0.001
Vitamin E	**µmol·l^{-1}**								
0 d		0.165	0.164	0.163^{a}	0.160^{a}	0.008			
49 d		0.157^{1}	0.166^{1}	0.334^{2b}	0.311^{2b}	0.020			
98 d		0.162^{1}	0.168^{1}	0.541^{2c}	0.478^{2c}	0.042	0.001	0.001	0.001
Vitamin A	**µg·ml^{-1}**								
0 d		0.145	0.148	0.143^{a}	0.150^{a}	0.008			
49 d		0.146^{1}	0.153^{1}	0.185^{2b}	0.201^{2b}	0.010			
98 d		0.147^{1}	0.152^{1}	0.221^{2b}	0.264^{2b}	0.012	0.002	0.001	0.006

† CON- control diet; L- 200g of extruded linseed·kg^{-1} of concentrate; LVB-200 g of extruded linseed + 2.86 g of verbascoside supplement based·kg^{-1} of concentrate; LVBE- 200 g of extruded linseed + 2.86 g of verbascoside supplement + 14.29 g of micro-encapsulated vitamin E·kg^{-1} of concentrate; § SEM= Standard error of mean; ‡ D= fixed effect of dietary supplementation; T=fixed effect of time; D x T= interaction dietary supplementation x time; ^{1,2} within a row, means without a common superscript differ (P<0.05); $^{a, b}$ within a column, means without a common superscript differ (P<0.05).

Triglycerides, at the end of the trial (98d), significantly decreased (P<0.01) in LVB and LVBE groups by 29.6% and 25.0% compared to the CON group and by 32.0% and 27.5% compared to the L group, respectively. No statistical difference was found between the LVB and LVBE groups. In addition, from the first to the third sampling, an effect of dietary treatment was observed with a significant triglycerides reduction (P<0.05) in the LVB and LVBE groups; while, a significant increase in values (P<0.05) was reported in the CON and L groups in the same period.

Total cholesterol was influenced by the experimental treatment (P<0.05) at the last sampling, with a decrease of 4.6% and 5.5% in LVB and LVBE groups compared to the CON group, and by 5.8% and 6.6% compared to the L group, respectively. No statistical difference between the LVB and LVBE groups was found. The dietary time effect on the serum concentration of total cholesterol was significant, from the first to the third sampling, which showed a significant decrease (P<0.05) in the LVB and LVBE groups. In the same period of time, the CON and the L group did not present significant variations.

At the end of the test, the dietary treatment led, to a significant increase in HDL cholesterol (P<0.01) in the LVB and LVBE groups, by 29.0% and 26.0% compared to the CON group, and by 28.0% and 25.0% compared to the L group, respectively. No statistical difference between the LVB and LVBE groups was found. From the first to the third sampling the time of treatment highlighted a significant increase (P<0.01) of HDL cholesterol values in the LVB and LVBE groups In the same period of time, the CON and the L group did not show any significant variations.

LDL cholesterol was statistically affected (P<0.01) by dietary treatment, at the end of the test (98d), with a reduction of values by 22.5% and 22.9% in the LVB and LVBE groups compared to the CON group, and by 23.5% and 23.8% compared to the L group, respectively. No difference between the LVB and LVBE groups was found. From the beginning to the end of the trial, the dietary time effect determined a significant reduction (P<0.05) in LDL cholesterol in the LVB and LVBE groups, while values in the CON group and L group remained essentially unchanged.

The AST and ALT values (Table 3) were not affected by dietary treatment; however there was a significant decrease in ALT values (P<0.05) over time in the LVB and LVBE groups. There were no significant changes in the CON and L group over the same period of time. Bilirubin concentration (Table 3) was not significantly affected (P<0.05) by dietary treatment, at the end of the test, with a decrease in values of 7.8% and 10.9% in the LVB and LVBE groups compared to the CON group, and 8.7% and 11.7% compared to the L group, respectively. No statistical difference by dietary treatment between the LVB and LVBE groups was observed. The treatment time effect showed a significant reduction in values (P<0.05) in the LVB and LVBE groups, while values in the CON and L group remained almost unchanged.

All the parameters in Table 3, except LDL cholesterol, ALT and AST, showed a significant interaction of experimental treatment in relation to the time of administration, highlighting significant modifications of values over the time of administration.

Plasma oxidative status markers

At the end of the test (98d), ROMs values (Table 4) showed a significant decrease (P<0.01) of 58.8% and 59.7% in the LVB and LVBE groups compared to the CON group, and 60.0% and 60.9% compared to the L group, respectively. Statistical differences were determined as early as the midway-trial sampling (49 d) and continued until the end of the trial. No statistical difference between the LVB and LVBE groups was found. The dietary time effect was also significant (P<0.01) throughout the trial, with a reduction of ROMs values in the LVB and LVBE groups; while in the same time period ROMs increased by 82.3% in the CON group and 82.7% in the L group.

At the end of the trial TBARS values (Table 4) were also significantly (P<0.01) lower by 71.3% and 70.9% in LVB and LVBE groups compared to the CON group, and by 74.2% and 73.8% compared to the L group, respectively. This difference is highlighted, from the second sampling, with a decrease in values in the LVB and LVBE groups and an increase in the CON and L groups. TBARS also did not register any significant difference between the LVB and LVBE groups. The duration of the dietary treatment, from the first to the third sampling, in the LVB and LVBE groups, showed a statistical decrease (P<0.01) of TBARS values; while in the CON and L groups in the same period, there was an increase of 126.9% values and 164.8%, respectively.

The administration of feed supplements in the LVB and LVBE groups produced a significant increase (P<0.01) in vitamin E concentration (Table 4) from sampling day 49 until 98 d where the increase was 233.9 % and 195.1%, compared to the CON group, and 222% and 184.5%, compared to the L group, respectively. No statistical difference in vitamin E concentration was recorded between the LVB and LVBE groups. The increase in vitamin E was markedly high (P<0.01) over time, and from the first to the third sampling increased by 231.9% and 198.8% in the LVB and LVBE groups; while over the same period in the CON and L groups, the vitamin E concentration remained unchanged.

At the end of the test, the Vitamin A concentration (Table 4) increased significantly (P<0.01) in the LVB and LVBE groups by 50.3% and 79.6%, compared to the CON group, and by 45.4% and 73.7%, compared to the L group, respectively. The vitamin A concentration increased in the second sampling, in both LVB and LVBE groups, while the CON and L groups values remained almost unchanged. No statistical difference between the LVB and LVBE groups was found. There was a significant (P<0.01) time effect in the LVB and LVBE groups from the first to the third sampling, while, at the same time the CON and L values remained unchanged.

Plasma oxidative status markers showed a significant interaction (P<0.01) of dietary treatment for the duration of trial was observed.

Discussion

Selected serum biochemical parameters

Ewes in the LVB and LVBE groups that were fed dietary antioxidant supplements were found to have a significant improvement in the lipid blood profile parameters, with a decrease in triglycerides, total cholesterol and LDL cholesterol and a marked increase in HDL cholesterol. This increase in HDL cholesterol was due to the exclusive action of verbascoside, in fact there were no additional effects with vitamin E supplementation in the LVBE group.

The L group on the other hand presented similar values to the CON group, in agreement with the findings reported by Bouattour (36) in Lacaune ewes, and Gobert et al (15) and Petit et al. (8) in Holstein Frisian cows fed with linseed.

It is known that the hepatic formation of cholesterol is partly dependent on the availability of HMG-CoA reductase enzymes and partly on the presence of LDL receptors. Verbascoside acts on the lipid metabolism in similar way to statins (simvastatin and pravastatin), which are used in the treatment of hypercholesterolemia to reduce cholesterol synthesis and to increase the expression of LDL receptors by acting at the level of HMG-CoA, an enzyme involved in cholesterol synthesis in the liver. Inhibition of cholesterol synthesis in the liver results in an up-regulation of liver LDL receptor expression and a concomitant decrease in plasma LDL receptor concentration (37).

Similarly, Shimoda et al. (38) attributed the decrease in blood cholesterol levels in mice, fed an extract of *Cistanche tubulosa*, to the capacity of verbascoside to inhibit the expression of the RNA messenger for enzymes involved in the synthesis of cholesterol, such as HMG-CoA reductase and mevalonate kinase, and to the increase in the transport and metabolism of cholesterol. In addition, Shimoda et al. (38) reported an increase in the expression of apolipoprotein B, the VLDL receptor and lipoprotein lipase, involved in the transport and storage of cholesterol. They also found an increase in lipin1, peroxisome proliferator-activated alpha (PPARα) receptor, cytochrome P450 and other molecules involved in cholesterol metabolism.

Nammi et al. (39) showed that the administration of ethanolic extract of ginger (*Zingiber officinalis*) in high-fat diet rats, significantly reduced blood levels of total cholesterol, LDL cholesterol and triglycerides. They also observed that ginger causes an *up-regulation* of the gene expression, coding for the LDL receptor, and a *down-regulation* of HMG-CoA reductase in target tissues of rats. Other Authors (40) reported that polyphenols activated the PPARα receptor, by modulating the expression of key proteins involved in the HDL metabolism in the liver. In studies on rats fed a leaf extract of *Gmelina arborea* (Verbenaceae family), rich in lignans, flavonoids and phenylpropanoids glycosides, Punitha et al. (41) reported a decrease in blood levels of total cholesterol, LDL cholesterol, triglycerides and an increase in HDL cholesterol, due to a reduced hepatic triglyceride synthesis and/or a reduction in lipolysis.

The lack of the effect of vitamin E on the blood lipid profile in the LVBE group is in agreement with Soliman et al. (42), who reported no significant changes in blood total lipids, in sheep and lambs fed with vitamin E and selenium. Administering α-tocopherol in growing lambs, Njeru et al. (43), found no significant increases in serum cholesterol and triglycerides. Studies conducted by Yang et al. (44) on lactating goats, reported no appreciable changes in plasmatic values of triglycerides and total cholesterol, after injection of 3000 IU D-alpha-tocopherol acetate.

In our findings, dietary treatment did not produce a significant improvement in the plasmatic concentrations of hepatic markers, such as ALT and AST, except for bilirubin, whose values significantly decreased in the LVB and LVBE groups compared to the CON and L groups. The decrease in bilirubin, is likely due to the action of verbascoside, in fact there were no statistical differences from LVB group to LVBE group. Nudda et al. (4), reported no significant variations in bilirubin and AST blood levels in goats fed with extruded flaxseed supplement. Aliyu et al. (45) showed a progressive decrease in blood bilirubin levels in rats, after administration of an aqueous extract of bark of *Boswellia dalzielii*, a plant typical of Nigeria, rich in phenolic compounds. They reported that this extract may have had a protective effect on red blood cells, which are precursors of bilirubin, and the liver. Similar results were also obtained by Onoriose et al. (46) in rats treated with CCL4 and fed with a leaf extract of the same plant.

Plasma oxidative status markers

The significant decrease in the LVB and LVBE groups regarding the plasma oxidative status markers, compared to the CON and L groups, is likely due to the verbascoside supplement because no statistical differences between the LVB and the LVBE groups were found. Verbascoside oxidizes and shrinks without becoming a highly-reactive-radical molecule, it also has a preventive function against reactive oxygen substances (ROS), with a consequent reduction in lipid peroxidation. The verbascoside activity can be attributed both to direct action (the trapping of free radicals by verbascoside due to its antioxidant activity during the propagation of chain oxidative reactions) and a blocking of the initiation phase of oxidation through the inhibition of pro-oxidant enzymes which are responsible for the production of free radicals (26).

By testing MDA, Funes et al. (47), showed how verbascoside can inhibit lipid peroxidation. The verbascoside showed a powerful antioxidant property, stronger than hydroxytyrosol and caffeic acid, and similar to quercetin. The ROS scavenger capacity of phenylpropanoid glycosides depends on the number of phenol-hydroxyl groups. In fact, the higher the number of these groups the greater the antioxidant activity, and the verbascoside antioxidant activity is due to the presence of four phenol-hydroxyl groups (48). Despite being a water-soluble compound, verbascoside can prevent lipid peroxidation, probably due to some molecular interaction with the surface of the lipid membrane, as has been proposed by Funes et al. (47) when testing other hydrophilic antioxidant substances. Liu et al. (49) reported a lower plasma value of TBARS in rabbits fed twice a day with verbascoside (0.8 mg/kg of body weight). In a study on the skeletal muscle of rats subjected to strain, whose diet (for 10 days) was supplemented with verbascoside, Liao et al. (50), observed a decrease in ROM concentrations than untreated animals. In a study on lactating cows, fed with extruded flaxseed, vitamin E and vegetable extracts rich in polyphenols, Gobert et al. (15) found that only vitamin E did not affect the plasma lipid peroxidation, while in association with other herbal extracts vitamin E prevents oxidative damage, as was highlighted in our study by the lower levels of plasma concentration of TBARS in treated groups compared to the control group.

The increased values of ROMs and TBARS in the L group are likely due to the high content of linseed α-linolenic acid and PUFA (51), which is one of the factors that can influence and increase the oxidation of tissues (52).

The increase in plasma vitamins E and A in the LVB and LVBE groups, might be due to verbascoside ability to preserve the endogenous antioxidant system, through a reduction in reactive radical species in plasma (26). The dietary supplement integration of vitamin E did not produce significant improvements in plasma vitamin A and E concentrations. The increase in plasma vitamin E could also be attributed to the ability of verbascoside to enhance the α-tocopheroxyl radical recycling, as reported for green tea polyphenols (53), or to encourage the accumulation of α-tocopherol, following a reduction in oxidative-propagation-phase activation. The increase in vitamin A on the other hand, after verbascoside integration, could be due to an improvement in the β-carotene conversion process in vitamin A, made by β-carotene 15, 15' monooxygenase enzyme (a precursor of retinol), as evidenced by Yang and Tume (54).

In conclusion, the dietary integration of extruded linseed, verbascoside and vitamin E, resulted in an improvement in the blood profile. This profile was characterized by an increase in HDL cholesterol and a significant reduction in triglycerides, total cholesterol, LDL cholesterol and bilirubin, in LVB and LVBE treated groups, due to the verbascoside supplement.

The verbascoside also resulted in improved plasma oxidative status characterized by a significant increase in plasma vitamin A and vitamin E concentrations and a significant reduction in ROMs and TBARS values. The dietary vitamin E integration did not produce a significant improvement in the studied parameters, with no statistical differences between the LVB and LVBE groups.

Animal feed containing extruded flaxseed, might therefore benefit from a verbascoside supplement, because of the positive effect shown by this molecule on biochemical parameters.

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VPLIV EKSTRUDIRANIH LANENIH SEMEN, VERBASKOZIDA IN VITAMINA E KOT PREHRANSKIH DOPOLNIL NA SERUMSKE BIOKEMIČNE PARAMETRE IN OKSIDATIVNO STANJE V PLAZMI OVC LACAUNE

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Povzetek: Določili smo izbrane biokemične parametre v serumu in oksidativno stanje v plazmi ovc Lacaune, hranjenih s krmo z dodanimi ekstrudiranimi lanenimi semeni, verbaskozidom in vitaminom E. Izvedli smo 98 dnevni prehranski poskus na 44 ovcah, ki so bile 40±2 dni po porodu. Živali so bile razdeljene v štiri homogene skupine po enajst živali v skupini. Kontrolna skupina (CON) je bila krmljena z osnovno krmo, ostalim trem skupinam pa smo obrok dopolnili z ekstrudiranimi lanenimi semeni (L), z ekstrudiranimi lanenimi semeni in verbaskozidom (LVB) ali z ekstrudiranimi lanenimi semeni, verbaskozidom in vitaminom E (LVBE). Obroki so bili usklajeni na ravni proteinov in energije, sestavljeni iz 700 g koncentrirane krme in sena *ad libitum*. Kri smo ovcam odvzeli trikrat, in sicer na začetku poskusa (0 d), na sredini (49 d) in na koncu (98 d). V serumu smo določili naslednje parametre: trigliceride, skupni holesterol, holesterol v lipoproteinih z visoko gostoto (HDL) in nizko gostoto (LDL), bilirubin, alanin-aminotransferazo, aspartat-aminotransferazo, tiobarbiturne reaktivne substance (TBARS), reaktivne kisikove metabolite (ROM) ter vitamina A in E. Naši rezultati so pokazali, da so imele živali z dodatkom verbaskozida v skupinah LVB in LVBE boljši serumski profil izmerjenih parametrov, in sicer statistično značilno višji HDL holesterol (p =0,001) in nižjo koncentracijo trigliceridov (p=0,001), celotnega holesterola (p=0,017) , LDL holesterola (p = 0,001) in bilirubina (p=0.035). Ravno tako so imele te živali v krvi več vitamina A (p=0.002) in E (p=0,001) in hkrati manj ROM in TBARS (p=0,001) ter izboljšano oksidativno stanje. Naši rezultati so pokazali, da hrani dodan vitamin E ni vplival na preiskovane parametre, saj ni bilo statističnih razlik med skupinama LVB in LVBE. Tako sklepamo, da ima dodatek verbascozida živalski krmi, ki vsebuje ekstrudirano laneno seme, pozitiven vpliv glede na preiskovane biokemične parametre in oksidativno stanje v krvi ovc.

Ključne besede: ekstrudirano laneno seme, serumski biokemični parametri, verbaskozid, vitamin E, ovce Lacaune

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