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Εκδότης
Ελληνική Κτηνιατρική Εταιρεία
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Tel.: 210.8642284
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Cheques and remittances
D.Vourvidis
P.O. Box 3546, 10210 Athens, Greece

Επιταγές και εμβάσματα
Δ.Βουρβίδης
Τ.Θ. 3546, 10210 Αθήνα

Mailing address
J Hellenic Vet Med Soc (M. Zafiropoulou)
Hellenic Veterinary Medical Society
Patission 158, 11257 Athens, Greece

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ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

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2	ECAWBM	European College of Animal Welfare and Behavioural Medicine	1
3	ECAAH	European College of Aquatic Animal Health	2
4	ECBHM	European College of Bovine Health Management	3
5	ECEIM	European College of Equine Internal Medicine	0
6	ECLAM	European College of Laboratory Animal Medicine	0
7	ECPHM	European College of Porcine Health Management	3
8	EPVS	European College of Poultry Veterinary Science	3
9	ECSRHM	European College of Small Ruminant Health Management	10
10	ECVAA	European College of Veterinary Anaesthesia and Analgesia	1
11	ECVCN	European College of Veterinary Comparative Nutrition	0
12	ECVCP	European College of Veterinary Clinical Pathology	1
13	ECVD	European College of Veterinary Dermatology	3
14	ECVDI	European College of Veterinary Diagnostic Imaging	1
15	ECVECC	European College of Veterinary Emergency and Critical Care	0
16	ECVIM-ca	European College of Veterinary Internal Medicine--companion animals	0
17	ECVN	European College of Veterinary Neurology	0
18	ECVO	European College of Veterinary Ophthalmology	0
19	ECVP	European College of Veterinary Pathology	0
20	ECVPH	European College of Veterinary Public Health	7
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ΕΛΛΗΝΙΚΗ ΚΤΗΝΙΑΤΡΙΚΗ ΕΤΑΙΡΕΙΑ (ΕΚΕ)
HELLENIC VETERINARY MEDICAL SOCIATY (HVMS)

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Περιεχόμενα

History of the Hellenic Veterinary Medical Society

The Hellenic Veterinary Medical Society (HVMS) is one of the oldest Scientific Societies in our Country. It was founded in 1924 and its first scientific journal was published in 1926. Prompter, Founder and Animating Spirit of HVMS was the General Ioannis D. Petridis (1870-1947), first President and for many years Honorary President of the HVMS. Among the 49 founding members of the HVMS there was also the memorable professor Konstantinos Livadas, the founder of the Veterinary School of the Aristotelian University in Thessaloniki. In spite of the disagreements, the HVMS contributed greatly to the foundation of Veterinary School.

During that time there was only one Scientific Society in Greece, the Medical Society of Athens, which was founded in 1835 and published its first scientific journal in 1922. The HVMS dealt not only with scientific but also with professional topics, like the establishment of the invoices for the veterinarians' payment, taxes, insurance etc. Also, at that period, the accession of the Veterinary Branch in the Hygienists' Pension and Self Insurance Treasury (TSAY) was achieved.

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the Veterinary Microbiological Institute of Votanicos, from where all members of the Governing Board and the Editorial Board of the Journal of the HVMS, were coming from. There, the first «nucleus» of the Library of the HVMS, has been created. That is the reason, this second period of the HVMS successor of the «Petridis period», used to be called «Votanikos period, 1944-1965».

Because HVMS's income was very small, it will remain homeless for many years. Looking for a meeting place the HVMS will find positive response from several services and societies (State Veterinary Offices, Greek Chemical Society, Hellenic Agricultural Society, Medical Society of Athens, Institute of Agricultural Studies, State Veterinary Service of Athens, National Organization of Greek Handwork), which during the following years are going to offer its premises, while in the mid 1958 and for a short period, depending on its financing capabilities, the HVMS will rent its own room.

In 1944, the HVMS writes down its first post-war Member Book and in 1948 has already acquired its first 74 regular members. Also, HVMS is actively working with scientific subjects during regular meetings and public seminars, analyzing current veterinary issues, members' proposals and so on. On 29th May 1947 Mr Petridis presented in the Academy of Athens an issue for veterinary science and its contribution to the progress of the agricultural production and safeguard of Public Health. Also, it should be pointed out, that because there was no professional body, the HVMS is also dealing with issues related to the execution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veterinary legislation, on the organization of the Veterinary Service in the Ministry of Agriculture as well as on livestock topics. In the decade of 30s the Supreme Veterinary Advisory Council was created mainly dealing with scientific issues and other aims like promotion, publicity and consolidation of the veterinary science and the veterinary profession in our country and internationally.

The Hellenic Veterinary Medical Society publishes a quarterly scientific journal called Journal of the Hellenic Veterinary Medical Society (J Hellenic Vet Med Soc), as well as other scientific publications, organizes Congresses, Symposiums, Meetings, Lectures etc and generally and almost exclusively it has undertaken for life the Continuing Education of the Greek veterinarians and the students of the two Veterinary Schools.

Nowadays, the Hellenic Veterinary Medical Society is governed by a 9 member Governing Board which is elected every 3 years and has 3 branches:

- **Branch of Companion Animals**
- **Branch of Food Hygiene and Public Health**
- **Branch for Farm Animals**

The HVMS collaborates with the Supreme Educational Foundations, the Technological Educational Institutes, the Veterinary Services, and the Veterinary Associations as well as with Scientific Societies and the Greek and Foreign Chambers.

- **The HVMS is member of the:**
- **Worldwide Veterinary Society**
- **Worldwide Veterinary Society for Companion Animals**
- **Federation of European Veterinary Societies for Companion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (founding member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Professors, Researchers), in the Public Administration, in the Army as well as in the Professional Veterinary Societies and Chambers, in Greece and abroad.

Since 29 May 2001, having signed the contract and since 15 December 2002 the date on which the official opening celebration took place, the Hellenic Veterinary Medical Society is housed in its private premises in a beautiful and majestic one-floor apartment, on the 7th floor of a building in the centre of Athens at 158, Patission street, of 265m2 area, including main lobby (14m^2), secretary (13m^2), lecture room (91m^2), the President's office (22m^2), the Governing Board meeting room & library (44m^2), the kitchen (18m^2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President	: Theodoros Cl. Ananiadis†
Vice-President	: Veniamin Albalas
General Secretary	: Athanassios E. Tyrpenou
Spec. Secretary	: Konstantinos Chandras
Treasurer	: Olga Sabatakou
Member	: Emmanuel Archontakis
Member	: Apostolos Rantsios

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Review article
Ανασκόπηση

Diagnostic approach of anemia in ruminants

E.G. Katsogiannou[1], L.V. Athanasiou[1], G. Christodoulopoulos[1], Z.S. Polizopoulou[2]

[1] Department of Medicine, Faculty of Veterinary Science, University of Thessaly
[2] Diagnostic Laboratory, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

Διαγνωστική προσέγγιση της αναιμίας στα μηρυκαστικά

Ε.Γ. Κατσόγιαννου[1], Λ.Β. Αθανασίου[1], Γ. Χριστοδουλόπουλος[1], Ζ.Σ. Πολυζοπούλου[2]

[1] Παθολογική Κλινική, Τμήμα Κτηνιατρικής, Πανεπιστήμιο Θεσσαλίας
[2] Διαγνωστικό Εργαστήριο, Τμήμα Κτηνιατρικής, Σχολή Επιστημών Υγείας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

ABSTRACT. Anemia in ruminants is an abnormal condition characterized by the decrease of the hematocrit (Packed Cell Volume, PCV), the mass of erythrocytes (Red Blood Cells, RBCs) and/or hemoglobin. Anemia is classified as hemolytic, hemorrhagic or anemia caused by the decreased production of erythrocytes; the first two categories are characterized by a regenerative response. Hemorrhagic anemia can be caused by ectoparasites or parasites of the gastrointestinal system, hemorrhagic bowel syndrome, abomasal ulcers, vena cava thrombosis as well as from the genitourinary tract. In addition, primary and secondary hemostatic disorders can be accompanied by hemorrhagic anemia. Hemoparasites, toxins produced from *Clostridium perfringens* type D and *Clostridium hemolyticum* and leptospirosis are some of the causes of hemolytic anemia. Furthermore, certain plants, drugs or heavy metals and lack of certain trace elements can cause hemolysis. Immune-mediated hemolytic anemia has also been reported in ruminants. The reduced production of erythrocytes can be caused by deficiency of vitamin B12 or iron, as well as by chronic diseases. Pathologic conditions of bone marrow like inflammatory or neoplastic cells filtration and hypoplasia or aplasia of bone marrow are related to reduced production of erythrocytes. After laboratory confirmation by complete blood count analysis, history taking, clinical examination

Corresponding Author:
L.V. Athanasiou
224 Trikalon str., 43100 Karditsa, Greece
E-mail: lathan@vet.uth.gr

Αλληλογραφία:
Αθανασίου Λ.Β.
Τρικάλων 224, Τ.Θ. 43100 Καρδίτσα
E-mail: lathan@vet.uth.gr

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of the animal and specific test depending on the case, are required for the diagnostic approach of anemia and especially for etiological diagnosis. Tachycardia, tachypnea, icterus, mucosal pallor as well as specific symptoms of the underlying disease are observed during the clinical examination of the animal. FAMACHA technique is widely used for the clinical diagnosis and the assessment of the severity of anemia. With respect to complete blood count, apart from the hematocrit, hemoglobin concentration, erythrocytes indices as Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC) are contributing to the classification of anemia. The size and the shape of the erythrocytes, the appearance of inclusions and reticulocytes, which are indicative of regenerative anemia, are evaluated in blood smear. Rarely, examination of bone marrow is carried out, for the differentiation of anemia as regenerative or non-regenerative. In particular, the confirmation of immune- mediate anemia is based on Coomb's test for the detection of autoagglutination. Except from hemorrhage, blood loss is detected at urinalysis or feces microscopy for the presence of blood and/or parasites. Finally, serological and molecular techniques for the detection of infectious agents, as well as specific toxicological analysis are performed in various biological materials.

Keywords: anemia, ruminants, diagnostic approach

ΠΕΡΙΛΗΨΗ. Η αναιμία στα μηρυκαστικά είναι παθολογική κατάσταση, η οποία χαρακτηρίζεται από τη μείωση του αιματοκρίτη (Packed Cell Volume, PCV), του αριθμού των ερυθροκυττάρων (Red Blood Cells, RBCs) ή/και της αιμοσφαιρίνης. Η αναιμία χαρακτηρίζεται ως αιμολυτική, αιμορραγική ή αναιμία που οφείλεται στη μειωμένη παραγωγή των ερυθροκυττάρων, με τις δύο πρώτες κατηγορίες να χαρακτηρίζονται από αναγεννητικότητα. Η αναιμία λόγω απώλειας αίματος μπορεί να οφείλεται σε εξωπαρασίτωση ή ενδοπαρασίτωση του γαστρεντερικού σωλήνα, στο σύνδρομο του αιμορραγικού εντέρου, σε έλκη του ηνύστρου, σε έμφραξη της οπίσθιας κοίλης φλέβας καθώς και σε αιμορραγία από το ουρογεννητικό σύστημα. Ακόμη, πρωτογενείς και δευτερογενείς διαταραχές της αιμόστασης μπορεί να συνοδεύονται από αιμορραγική αναιμία. Στα αίτια της αιμολυτικής αναιμίας περιλαμβάνονται τα αιμοπαράσιτα, οι τοξίνες που παράγονται από τα *Clostridium perfringens* τύπου D και *Clostridium hemolyticum* και η λεπτοσπείρωση. Επιπλέον, ορισμένα φυτά, φάρμακα ή βαρέα μέταλλα μπορούν να προκαλέσουν αιμόλυση των ερυθροκυττάρων καθώς και η έλλειψη ιχνοστοιχείων. Η ανοσολογικής αιτιολογίας αιμολυτική αναιμία έχει επίσης αναφερθεί στα μηρυκαστικά. Η μειωμένη παραγωγή ερυθροκυττάρων οφείλεται σε έλλειψη της βιταμίνης Β12 ή του σιδήρου, καθώς και σε χρόνια νοσήματα. Παθολογικές καταστάσεις του μυελού των οστών όπως η διήθηση από φλεγμονικά ή νεοπλασματικά κύτταρα όπως και η υποπλασία ή απλασία του μυελού των οστών σχετίζονται με μειωμένη παραγωγή ερυθροκυττάρων. Για τη διαγνωστική προσέγγιση της αναιμίας μετά την εργαστηριακή επιβεβαίωση με τη γενική εξέταση του αίματος και κυρίως για την αιτιολογική διάγνωση απαιτείται η λήψη ιστορικού, η κλινική εξέταση του ζώου, και ειδικές κατά περίπτωση εξετάσεις. Κατά την κλινική εξέταση του ζώου παρατηρούνται ταχυκαρδία, ταχύπνοια, ίκτερος, ωχροί βλεννογόνοι αλλά και τα ειδικότερα συμπτώματα του υποκείμενου νοσήματος. Η τεχνική FAMACHA έχει ευρέως χρησιμοποιηθεί για την κλινική διάγνωση και τον προσδιορισμό της βαρύτητας της αναιμίας. Από τις παραμέτρους της αιματολογικής εξέτασης, πέρα από τον αιματοκρίτη, η συγκέντρωση της αιμοσφαιρίνης, οι δείκτες των ερυθροκυττάρων μέσος όγκος ερυθροκυττάρων (Mean Corpuscular Volume, MCV), και μέση συγκέντρωση αιμοσφαιρίνης (Mean Corpuscular Hemoglobin Concentration, MCHC) συμβάλλουν στην ταξινόμηση της αναιμίας. Στο επίχρισμα του αίματος αξιολογείται το σχήμα και το μέγεθος των ερυθροκυττάρων, η παρουσία εγκλείστων, καθώς και η παρουσία δικτυοερυθροκυττάρων ενδεικτικών αναγεννητικής αναιμίας. Για τη διαφοροποίηση της αναιμίας σε αναγεννητική ή μη σπανιότερα γίνεται εξέταση μυελού των οστών. Ειδικότερα, η επιβεβαίωση ανοσολογικής αιτιολογίας αναιμίας στηρίζεται στην εξέταση Coomb's για την διαπίστωση αυτοαιμοσυγκόλλησης. Η απώλεια αίματος πέραν της αιμορραγίας διαπιστώνεται επίσης με την ανάλυση του ούρου και την κοπρανολογική εξέταση για την παρουσία αίματος ή/και παρασίτων. Τέλος στα διάφορα βιολογικά υλικά πραγματοποιούνται ορολογικές και μοριακές τεχνικές για την ανίχνευση παθογόνων παραγόντων καθώς και ειδικές εξετάσεις για την ανίχνευση τοξινών.

Λέξεις ευρετηρίασης: αναιμία, μηρυκαστικά, διαγνωστική προσέγγιση.

INTRODUCTION

Anemia, by definition, corresponds to the reduction of blood oxygen and it is characterized by a decrease in hematocrit, erythrocyte mass and/or hemoglobin concentration leading to tissue hypoxia. In practice anemia is defined as the concentration of hemoglobin below the lower reference limit.

Anemia can be classified either based on the ensuing pathogenetic mechanisms thus characterized as hemorrhagic, hemolytic and anemia caused by the decreased production of erythrocytes or based on the regenerative response of bone marrow and consequently characterized as regenerative or non-regenerative anemia.

The presence of hypoxia in renal erythropoietin producing cells, as a consequence of anemia, dynamically alters the concentration of circulating erythropoietin. Erythropoietin is an early identified humoral factor, an erythropoietic glycoprotein hormone that induces proliferation and differentiation of erythroid progenitors, leading to red blood cell production (Souma et al., 2015).

At first, anemia is always non-regenerative because it takes some days for mature erythrocytes to be released to the peripheral blood. Therefore, evidence of regeneration should become apparent within 4-5 days.

Regeneration is mainly observed in acute blood loss or in hemolytic anemia while it is rather unlikely in cases where anemia is related to a chronic disease or by impaired erythrocyte production by the bone marrow (Ogilvie, 1998).

ETIOLOGY

Hemorrhagic anemia

Anemia due to blood loss can be caused by intense ectoparasitism (Stromberagn and Gyillot, 1987; Shemanchuk et al., 1960). Similarly, parasitisism of the gastrointestinal track is a common cause of blood loss anemia in ruminants as in other species. Different parasitic phyla have been identified as causative agents of anemia such as nematodes in small ruminants (Baldissera et al., 2015; Bordoloi et al., 2012; Vatta et al., 2002) and in cattle (Favero et al., 2016; Van Aken et al., 1997) with *Haemonchus contortus* referred as the most common species causing blood loss anemia as well as trematodes including *Fasciola hepatica* in small ruminants (Saleh, 2008; Maltinez- Moreno et al., 1997; Knight, 1980) and in cattle (Lotfollahzadeh et al., 2008; Ross et al., 1966) and *Paramphistomum cervi,* as well (Dorny et al., 2011). In severe cases of winter dysentery, a disease with unconfirmed causative agent, fresh blood clots have been reported to be evidenced in the feces of affected calves (Divers and Peek, 2008). Other clinical conditions of the gastrointestinal tract associated with blood loss anemia are the hemorrhagic bowel syndrome caused by *Clostridium perfringens* type A (Braun et al., 2010) and abomasal ulcers (Ok et al., 2001; Vatn and Ulvund, 2000).

Vena cava thrombosis in adult dairy cattle or in feedlot cattle fed a high-carbohydrate diet is also clinically manifested by pale mucous membranes, hemoptysis, and epistaxis (Breeze et al., 1976). Blood loss is also evident from the genitourinary tract presented as hematuria as in the case of enzootic hematuria caused by urinary bladder tumors secondary to chronic bracken fern toxicity (McKenzie, 1978) as well as in pyelonephritis caused by *Corynebacterium renale* and *E.coli* in cows (Braun et al., 2008). Moreover, acute blood loss, sometimes severe, can result from injuries caused by obstetrical manipulations more often in cows that have been in labor for several hours and when the birth canal is dry and non-lubricated (Radostits et al., 2006). In addition, anemia can be caused by tumors (Stock et al., 2011). Rupture of an ovarian granulosa cell tumor followed by hemoperitoneum or spleen rupture after infiltration by lymphosarcoma (Divers and Peek, 2008), is a rather uncommon condition in cows (Masseau et al., 2004). Body cavity effusions usually result from hemostatic disorders (Radostits et al., 2006). Internal hemorrhages caused by massive thrombocytopenia have been attributed to Bovine Virus Diarrhea- Mucosal Disease (BVD- MD) virus (Dabak et al., 2007). Secondary hemostatic disorders such as rodenticide intoxication are very rarely reported in ruminants. A case of rodenticide toxicity in lambs that accidentally gain access to baits for rodent control was reported as sudden death followed epistaxis, respiratory distress and edemas in different body parts (Del Piero and Poppenga, 2006). Experimentally dosing of carbon tetrachloride, in goats resulted in hepatic damage and decreased of blood clotting activity (Smith and Sherman, 2009).

The most frequent causes of blood loss anemia per ruminant species are presented in Table 1.

Table 1. Causes of blood loss anemia in sheep, goats and cattle

	Sheep	Goats	Cattle
Ectoparasites			
Linognathus spp			A, Y
Gastrointestinal tract			
Haemonchus concortus	A, Y	A	Y
Mecistocirrus spp			Y
Fasciola hepatica	A, Y	A	A, Y
Paramphistomum cervi			A, Y
Abomasal ulceration	Y		
Hemorrhagic bowel syndrome			A
Winter dysentery			Y
Respiratory tract			
Caudal vena cava thrombosis and pulmonary embolism			A
Genitourinary tract disease			
Enzoonotic hematuria			A
Others			
Injuries			A
Tumor			A, Y
Hemostatic disorders	Y		A
Carbon tetrachloride		A	

*Sheep: Young (Y) < 12 months, Adult (A) >12 months; Goats: Young (Y) < 12 months, Adult (A) >12 months; Cattle: Young (Y) < 12 months, Adult (A) >12 months

Hemolytic anemia

Anemia due to hemolysis can be caused by parasites implicated in tick born fever including piroplasms that invade red blood cells like *Babesia spp* in small (Esmaeilnejad et al., 2012; Yeruham et al., 1998) and large ruminants (Bal et al., 2016; Trueman and Blight, 1978) and *Theileria spp* in lambs (Alani and Herbert, 1988) and cattle (Omer et al., 2002; Moll et al., 1986) and rickettsiae, mainly *Anaplasma spp* that infect granulocytes up to 90% in the peak of bacteremia as it is referred in sheep (Yasini et al., 2012), goats (Gokce and Woldehiwet, 1999) and cattles (Ashuma et al., 2013; Henniger et al., 2013). Furthermore, protozoa like *Trypanosoma* (Biryomumaisho et al., 2013; Katunguka- Rwakishaya et al., 1997; Anosa et al., 1992) and certain *Mycoplasma* species (Genova et al., 2011; Suzuki et al., 2011) can also be found in plasma and/or in erythrocytes. In addition, microfilaria, the larval stage of stephanofilarial worm, the causative agent of the bovine stephanofilarial dermatitis, enters peripheral blood circulation causing anemia by increasing erythrocytic fragility (Singh et al., 2011).

Hemolysis also results from toxins produced by *Clostridium perfringens* type D causing yellow lamb disease (Gianniti et al., 2014) and *Clostridium hemolyticum* that causes bacillary hemoglobinuria in sheep and cows (Tagaki et al., 2009; Randhawa et al., 1995).

The primary lesions of the pathogenic *Leptospira spp* circulating in the bloodstream, concern the endothelium of small blood vessels, which leads to localized ischemia in organs mainly kidney and liver. Apart from vasculitis, *Leptospira spp* hemolysins have been implicated in the pathogenesis of anemia; in calves *Leptospira spp* hemolysin has been reported as responsible for yielding holes in the erythrocyte cellular membrane (Lee et al., 2002).

Toxicity from grazing plants *Allium spp* including onion and garlic (Heidarpour et al., 2013; Aslani et al., 2005; Rae, 1999), *Brassica spp* with the most known the broccoli and the cabbage (Xu, 1992) and *Ipomoea carnea*, a flowery plant (Tartour et al., 1973), have been associated with hemolytic anemia in ruminants. Although, long acting oxytetracycline compounds have been widely used in ruminant medicine only

experimentally induced toxicosis has been reported. It seems that oxytetracycline may penetrate erythrocytes interacting with hemoglobin and thereby significantly reducing the erythrocyte count and hemoglobin concentration (Chi et al., 2010).

Moreover, natural occurring cases of anemia associated with toxicosis by heavy metals such as copper (Cregar et al., 2012; Bundza et al., 1982; Stogdale, 1978), zinc (Allen et al., 1983) and arsenic (Keshavarzi et al., 2015; Rana et al., 2010) have been reported in ruminants. Although, anemia has been reported in natural cases of lead poisoning in cows (Schlerka et al., 2004), it has not been observed in experimentally lead poisoned sheep were low doses of lead where administered (Polizopoulou, 1991). In addition, poisoning by nitrate resulted in methemoglobinemia with methemoglobinuria, in sheep (Hindson and Winter, 1996). On the other hand, nutritional deficiency in copper and selenium has been associated with formation of Heinz bodies indicative of erythrocyte oxidative damage in lambs. Moreover, copious exercise seems to further reveal the hematological impact of these trace element deficiencies (Draksler et al., 2002; Suttle et al., 1987). Similarly, anemia with Heinz body presence has been reported in hypophosphatemic cows (Jubb et al., 1990).

Rarely autoimmune hemolytic anemia is reported in young, as well in adult ruminants after vaccinations; a polyvalent botulism vaccine has been incriminated for such a condition in a cow (Yeruham et al., 2003). Similarly, antigens of the BVD- MD vaccine seem to elicit antibodies that bind not only to peripheral blood

Table 2. Causes of hemolytic anemia in sheep, goats and cattle

	Sheep	Goats	Cattle
Infectious Agents			
Babesia spp	A, Y	A	A, Y
Theileria spp	Y		A, Y
Anaplasma spp	A	A	A, Y
Trypanosoma spp	A, Y	A	A, Y
Mycoplasma spp	A		A
Bovine stephanofilarial dermatitis			A
Yellow lamb disease	Y		
Bacillary hemoglobinuria	A		A
Leptospira spp	Y		A
Toxic agents			
Allium spp	A	A	A, Y
Brassica spp			A
Ipomoa carnea	A		Y
Long-acting oxytetracycline	A		
Copper	Y	A	
Zinc	A		
Arsenic	A		A
Lead			A
Nutrition deficiency			
Copper	Y	Y	
Selenium	Y		
Phosphorus	Y		
Immune Mediated Conditions			
Vaccination			A, Y
Bovine colostrum fed to sheep	Y	Y	
IMHA			A, Y

*Sheep: Young (Y) < 12 months, Adult (A) >12 months; Goats: Young (Y) < 12 months, Adult (A) >12 months; Cattle: Young (Y) < 12 months, Adult (A) >12 months

cells but also to the stem cells in the bone marrow of neonates (Deutskens et al., 2011). These antigens cause opsonization of the affected cells which enhance phagocytosis by bovine macrophages. After vaccination with Lumpy Skin Disease Virus in cows, mild regenerative hypochromic macrocytic anemia has recently been reported, probably because of slight hemolysis (Katsoulos et al., 2017). Immune-mediated hemolytic anemia has been reported in juvenile lambs fed with cow colostrum (Winter and Clarkson, 1992) while idiopathic immune mediate anemia has been diagnosed in cases where hemolysis could not be attributed to any of the above-mentioned agents (Nassiri et al., 2011).

The causes of hemolytic anemia in ruminants are presented in Table 2.

Anemia due decreased production of RBCs

Decrease of RBCs production is metabolically relative to essential nutrients such as cobalt which is converted by ruminal microflora to vitamin B12. Vitamin B12 acts as an essential cofactor of several enzyme systems promoting red blood cell synthesis. Thus, a vitamin B12 deficiency results to anemia. In case of sheep, it has been reported (Ulvund, 1990) that white liver disease attributed either to B12 deficiency or to hepatotoxic disease in deficient lambs was associated with a decrease in hemoglobin concentration. Similar findings have been reported in cobalt deficient goats (Al- Habsy et al., 2007). Iron deficiency is usually observed in young animals, because of their rapid growth and the large amount of iron needed for the production of hemoglobin, myoglobin and other iron-containing compounds (Green et al., 1997) as well as in ewes after parturition (Cihan et al., 2016). The most frequent cause of iron deficiency is endo- and ectoparasitism (Singh et al., 2014).

However, iron is also implicated in the pathogenesis of anemia of the chronic disease. Although, iron stores are adequate for erythropoiesis, they are sequestrated making iron unavailable for hematopoiesis leading to normocytic, normochromic non regenerative anemia. (Radostits et al., 2006). Anemia of chronic disease has been reported in cattle affected by lumpy skin disease due to the chronic inflammatory response to *Capripoxvirus* infection, that disturbs iron metabolism. Therefore, anemia is usually mild and slowly progressive and the main hematological abnormalities concern the indexes Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) (Abutarbush, 2015). Similarly, ovine paratuberculosis is associated with macrocytic normochromic anemia due to the chronic inflammation (Hemalatha et al., 2013), while in goats, anemia is non-regenerative (Smith and Sherman, 2009). Although, anemia is not a common finding in Bluetongue infected sheep, few cases have been reported in sheep attributed either in vasculitis caused by the virus or chronic disease (Vasileiou et al., 2016). Apart from inflammation, neoplasia has been reported to cause anemia of chronic disease. Bracken

Table 3. Causes of anemia of decreased erythrocyte production in ruminants

	Sheep	Goats	Cattle
Nutrition deficiency anemia			
Cobalt/ folate	Y	A	
Iron	A, Y		A, Y
Anemia of chronic disease	A		A
Lumpy skin disease			A
Mycoplasma paratuberculosis	A		
Chronic toxicity			A, Y
Bluetongue	A		
Anemia secondary to bone marrow dysfunction or dysplasia			
Neoplasia			A
Bovine neonatal pancytopenia			Y

* Sheep: Young (Y) < 12 months, Adult (A) >12 months; Goats: Young (Y) < 12 months, Adult (A) >12 months; Cattle: Young (Y) < 12 months, Adult (A) >12 months

Table 4. Infectious agents that have been associated with anemia * in ruminants in Greece

Infectious agents	Species	References
Haemonchus contortus	sheep, goats	Kouam et al., 2014
Besnoitia besnoiti	cattle	Papadopoulos et al., 2014
Fasciola hepatica	sheep, goats	Kantzoura et al., 2011; Katsoulos et al., 2011
Anaplasma spp	sheep, cattle	Giadinis et al., 2011; Giadinis et al., 2015
Babesia spp	sheep, goats, cattle	Papadopoulos et al., 1996
Theileria spp	sheep, cattle	Papadopoulos et al., 1996
Mycobacterium paratuberculosis	goats	Angelidou et al., 2014
Leptospira spp	sheep, cattle	Burriel et al., 2003
Yellow lamb disease	sheep	Gkiourtzidis et al., 2001
BVDV	cattle	Billinis et al., 2005
Bluetongue	sheep	Vasileiou et al., 2016
Lumpy skin disease	cattle	Tasioudi et al., 2016 Katsoulos et al., 2017

* Although the documentation of a cause- effect relationship between infection agent and anemia was not the main objective of all these publications, anemia has been observed in the affected ruminants.

fern toxicity mostly in cattle but also in sheep results to the development of tumors and consequently to this type of anemia. Bone marrow examination revealed aplasia of the hematopoietic tissues, in these animals (Prakash et al., 1996). Neoplastic tissue seems to infiltrate bone marrow as in a case of hemophagocytic histiocytic sarcoma in a cow where neoplastic histiocytes primarily proliferated in the spleen and bone marrow (Matsuda et al., 2010). A recently observed syndrome in calves is the bovine neonatal pancytopenia in which bone marrow is aplastic and hemopoietic tissues were replaced by fat cells (Fukunaka et al., 2010).

Conditions associated with anemia of reduced erythrocyte production are presented in Table 3, while the most common condition associated with anemia in ruminants in Greece are presented in Table 4.

DIAGNOSTIC APPROACH

Anemia is mostly a diagnosis confirmed by laboratory testing. However, diagnostic approach should include history and environmental assessment. Demographic data, such as age and breed, the prevalence of the causative agents in each geographic area along with nutrition and breeding conditions including vaccinations and antiparasitic control should be taken into consideration (Deutskens et al., 2011; Dabak et al., 2007; Aslani et al., 2005). Apart from the clinical suspicion of anemia, clinical manifestations of anemia as well as other laboratory findings are essential in order to reach etiological diagnosis. Furthermore, classification of anemia offers clinicians a diagnostic algorithm to compile a list of differentials with the ultimate goal to reach a definite diagnosis of the underlying cause (Figure 1).

Clinical manifestations and severity

Clinical signs derive from inadequate oxygen supply when compensatory mechanisms fail or even because of these mechanisms. Among them, mucous membrane pallor, exercise intolerance, tachypnea and/or dyspnea are the most commonly signs observed in anemic ruminants. Heart auscultation reveals an increased heart rate as well as functional murmurs due to blood cell turbulence disturbances (Radostits et al., 2006).

Symptoms of the underlying disease are also present and they can be either non-specific such as anorexia, fever and weight loss or specific to the certain disease.

The severity of clinical signs is negatively related to the chronicity of anemia and positively to the amount of blood loss. Therefore, chronic diseases as well as bone marrow insults are manifested by mild symptoms such as weight loss in adults and decrease of weight gain in lambs, reduction of foraging or

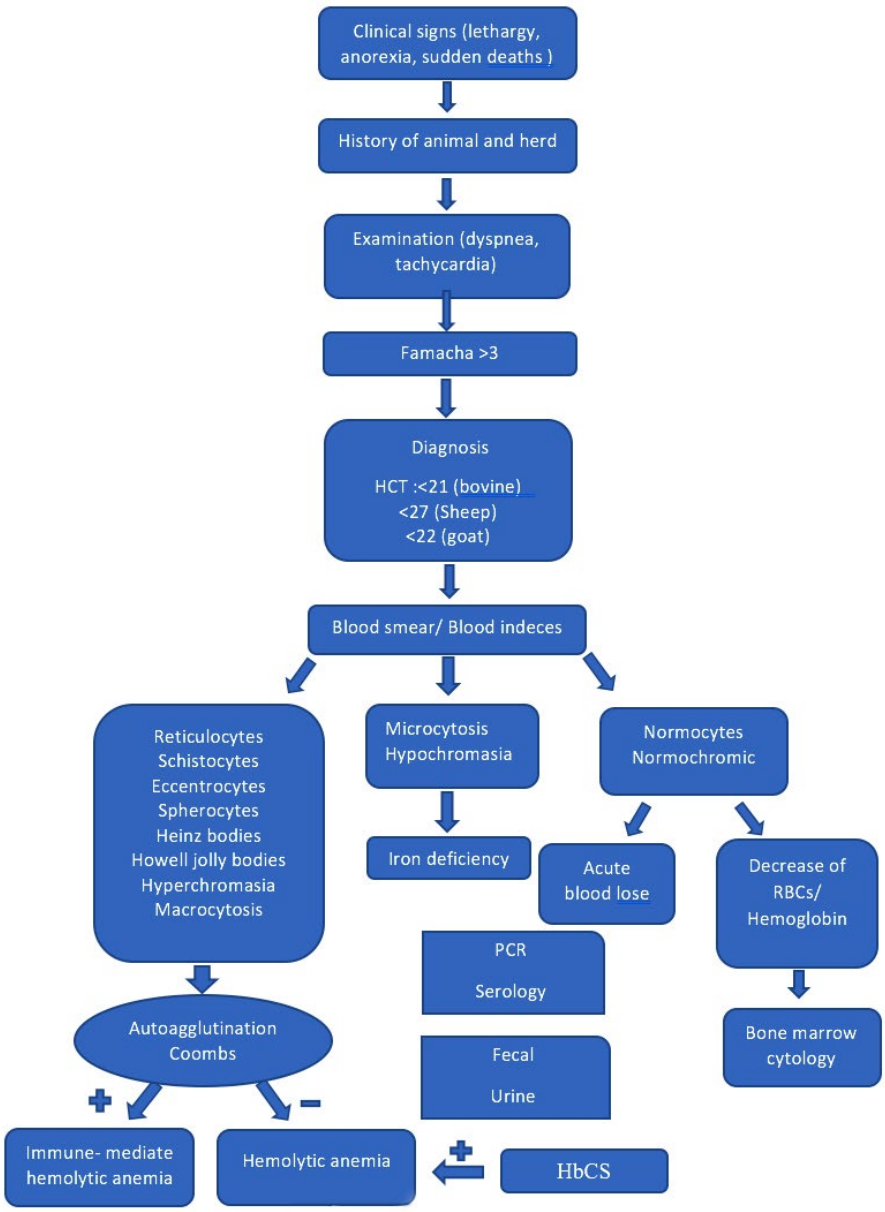

Figure 1. Algorithm for investigation of anemia in ruminants

grazing, reduced exercise tolerance and subcutaneous edema (Gianniti et al., 2014).

To the contrary severe acute hemorrhage or blood destruction is usually presented with a more severe clinical picture, even sudden deaths, depending on the cause of anemia.

Hemorrhage is evidenced during physical examination as epistaxis, hematuria and melena (Ok et al., 2001; Xu, 1992) or denoted by the presence of ectoparasites. Hemolysis is manifested by icterus, splenomegaly and abnormal urine color (Giannitti et al., 2014; Ashuma et al., 2013; Braun et al., 2008) due to hemoglobinuria or bilirubinuria. Moreover, in case of hemostatic disorders, petechiae and ecchymoses or body cavity effusions are observed (Dabak et al., 2007).

Scoring systems of the severity of clinical patent anemia as manifested by mucous membrane pallor have been developed in ruminants. The most widely used system is called FAMACHA (FAO, 1998), an acronym of the name of the originator of the system. This system had been initially developed to identify small ruminants with anemia caused by haemonchonsis, in order to selectively administer antiparasitic

compounds only in the infected and not all animals of an infected farm. This system is an easy to perform and of low cost method.

FAMACHA is based on the examination of the lower eyelid mucous membrane color against a laminated color chart presenting 5 color categories where 1 is the normal mucous membrane color and 5 is the white color. The score 3 is the borderline for starting medical treatment. The system has been validated using PCV as gold standard. The sensitivity was found to be higher in sheep than in goats negatively related to the cut off value of the PCV that was used to differentiate anemic from non-anemic animals (Sotomaior et al., 2012). Furthermore, conjunctiva color varies in different breeds therefore; modifications of the color in the chart based on breed specific colors are suggested (Moors and Gauly, 2009)

In bovine, FAMACHA was used to score anemia predominantly caused by *Trypanosoma congolense.* The system was found adequate to identify moderate and severely anemic cattle with a FAMACHA score of 4 and 5, respectively (Grace et al., 2007).

Despite the subjectivity of color estimate, FAMACHA remains a convenient rough estimate of anemia in ruminants prior to laboratory testing.

Laboratory evaluation

Blood testing

Complete blood count (CBC)

Clinical suspicion of anemia is confirmed by a complete blood count, mainly the hematocrit and red blood cell count. It is worth mentioning that in neonates, hematocrit value is relatively high at birth, while, till the age of 6 months, it decreases below the lower reference interval for adults. Different reference intervals are provided in literature reflecting differences in populations and equipment used for their determination. For instance, according to Schalm's hematology textbook (Weiss and Wardrop, 2010), hematocrit reference interval is 21-30%, 27-45% and 22-38% for cow, sheep and goat, respectively.

For the classification of anemia and the assessment of regenerative response, reticulocyte count along with blood smear evaluation should be performed. In regenerative anemia, the number of reticulocytes is increased in peripheral blood therefore serial monitoring of blood parameters is required. In case of low number of reticulocytes, monitoring is essential to find out if the anemia is non-regenerative or if there is an acute regenerative anemia. Repeating of CBC analysis is important as well as in the case of high number of reticulocytes, in order to see if the anemia does no anymore exists or if the anemia is changing from regenerative to non- regenerative. Reticulocytes in sheep and goats with basophilic stippling are easily detected in EDTA anticoagulated blood stained by Giemsa. Regeneration in ruminants is also demonstrated by macrocytosis and polychromasia while anisocytosis is quite common in healthy ruminants (Weiss and Wardrop, 2010). In response to severe anemia red blood cells contain nuclear fragments, the Howell- Jolly bodies (Alani and Herbert, 1988). Macrocytosis, is determined by calculating the Mean Corpuscular Volume (MCV) (Alani and Herbert, 1988). To the contrary, microcytosis (decreased MCV) is indicative of chronic disease (Abutarbush, 2015) as well as nutrition deficiencies. Furthermore, a diagnosis of iron deficiency anemia is very likely when microcytosis coexists with a decreased mean cell hemoglobin concentration (MCHC) (Green et al., 1997). On the other hand, an increase in the MCHC is observed in hemolytic anemia because of the released of hemoglobin from the ruptured erythrocytes in blood (Radostits et al., 2006).

Apart from the detection of reticulocytes and the presence of polychromasia, blood smear evaluation is needed in order to identify morphological abnormalities such as spherocytosis in case of both immune mediated hemolytic anemia and toxicity (Bundza et al., 1982). In diseases that cause physical injury to red blood cells, usually hemoparasitoses (*Trypanosoma* spp, *Mycoplasma* spp), schistocytes are observed (Gladden et al., 2016; Anosa et al., 1992). Moreover, oxidative damage of hemoglobin associated with onion toxicosis (Heidarpour et al., 2013) is denoted by the presence of Heinz bodies in the cytoplasm of red blood cells and/or eccentrocytes, where hemoglobin has been eccentrically accumulated (Borreli et al., 2009).

Finally, parasitic inclusions such *Babesia* spp, *Theilleria* spp, *Mycoplasma* spp and *Anaplasma* spp (Gladden et al., 2016; Henniger et al., 2013; Izzo et

al., 2010) can be found.

Alterations in the number and/or morphology of white blood cells are also observed in case of anemia and they are associated with the underlying disease. Similarly, anemia caused by hemostatic disorders can affect the number and/or morphology of platelets. For instance, thrombocytopenia has been reported in anemic calves with BVD-MD (Dabak et al., 2007).

Agglutination testing

Aggregation of erythrocytes forming irregular shapes is observed either in inflammatory reactions especially in bovine known as rouleaux formation (Weiss and Wardrop, 2010) or in autoimmune diseases where red blood cell clumps are not dissolved if blood is diluted with 0.9% saline (Nassir et al., 2011). Suspected immune- mediate hemolytic anemia is confirmed by direct Coombs' test which becomes positive when anti- immunoglobulin and anti- complement antibodies are mixed with host erythrocytes (Nassir et al., 2011). Both IgG and IgM reactive antibodies have been implicated in the pathogenesis of idiopathic immune-mediated hemolytic anemia, however, IgG is much more common than IgM. They are mostly considered as warm hemagglutinins since they are reactive at body temperature. Cold hemagglutinin disease (IgM antibodies that bind to erythrocytes below body temperature) results in thrombi formation that lead to ischemic necrosis usually observed at the tips of the ears.

While immune mediated anemia is usually regenerative, it can rarely be non-regenerative when bone marrow progenitor cells are targeted instead or along with mature erythrocytes.

Scoring of anemia without using a blood count analyzer

Efforts to estimate haemoglobin without the use of laboratory equipment and consumables have been made in human medicine and the Haemoglobin Colour Scale (HbCS) has been developed (WHO, 2004). The test requires a drop of blood that is applied on a special chromatographic paper which is compared to a laminated card with different colours related to different haemoglobin concentrations. The performance of this test in cattle was reported to be good for the detection of moderate to severe anemia and less sensitive in cases of mild anemia (Grace et al., 2007).

Other biological material testing

Fecal examination

Feces of anemic ruminants should be examined for color change due to the presence of blood, consistency, amount, shape, odor and the presence of mucous (Braun et al., 2008). Laboratory examination of feces includes microscopy for the detection of parasites, presence of occult blood and microbiology including molecular techniques for the detection of causative agents of the underlying disease (Bott et al., 2009).

Urinalysis

A strong indication for urinalysis in anemic ruminants is the red or dark brown color in urine. Urine sediment microscopy reveals the presence of red blood cells (hematuria). The red color of the supernatant after urine centrifugation either due to the presence of hemoglobin or myoglobin should be further differentiated by chemical analysis (Giannitti et al., 2014; Braun et al., 2008). Certain microorganisms can also be detected in urine employing either microscopy or molecular techniques (Denipitiya et al., 2017).

Bone marrow evaluation

Bone marrow evaluation is not routinely performed in ruminant medicine as it requires sample referral to a specialized laboratory. Bone marrow cytology is used to determine the regenerative response. If there is an increased cellularity in bone marrow, anemia is regenerative, while if there are few cells with morphological abnormalities, bone marrow dysfunction and dyserythropoiesis as well as non- regenerative anemia is more likely to occur (Fukunaka et al., 2010; Steffen et al., 1992). Bone marrow samples can be used for the detection of the causative agents of the underlying diseases.

Additional tests

Serological and molecular techniques are widely used for the etiological diagnosis of diseases that are associated with anemia in ruminants (Sonawane and Tripathi, 2015; Yang et al., 2015; Grova et al., 2011).

Moreover, chemical analysis is used for the detection of heavy metals and toxins in biological material as well as in feed and water (Keshavarzi, 2015).

CONCLUSION

Anemia is a frequent abnormal condition in ruminants. It is usually associated with pathological state of other tissues or organs rather than reflecting primary defects in erythropoiesis A complete blood count is the indispensable part of the laboratory investigation of every case of anemia. However, a systematic approach includes history taking and clinical examinations followed by laboratory testing targeted to the clinical findings in order to identify possible etiologic factors.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Review article
Ανασκόπηση

Current Situation in Turkish Sericulture Sector

M. Polat[1] , Y. Cevger[2]

[1] *Department of Animal Health Economics and Management, Faculty of Veterinary Medicine, Kastamonu University*

[2] *Department of Animal Health Economics and Management, Faculty of Veterinary Medicine, Ankara University*

ABSTRACT. The purpose of this study is to assess sericulture sector according to actual data which has both cultural and economic value for Turkey and has feature of being a livestock sub-production area mostly considered as a source of additional income. Fresh cocoon, being the output of sericulture, is an important raw material for silk and silky textile industry and also has a traditional and cultural value. The situation assessment was made in terms of production level discussing the fresh cocoon data in Turkey by years. In Turkey, as of 2016, the annual cocoon production is 103 tons, and 2,001 families deal with this occupation. The organization and historical process of sericulture sector was discussed and the supports provided and changes over the years in sericulture sector were examined. The organization in sericulture dates back to old times. In 1940, the first cooperatives were founded in Bursa, Bilecik and Adapazarı in order to maintain and increase the cocoon production after the foundation of the Republic. These cooperatives were merged and Bursa Association of Agricultural Sales Cooperatives for Silk Cocoons (KOZABİRLİK) was founded on 11 May 1940. Although the support given to sericulture has changed over the years, is very important for the sector. By 2016, the purchase price of cocoons was 5 TL / kg while the price of support was 40 TL / kg. Some inferences were made about the market and foreign trade by taking into account the silky textile and silk carpet exportation, being the strength of the sector. Despite the decrease in export amount in recent years, only silk carpet exports have generated nearly 100 million dollars in income in the last 5 years. The key problems, faced in sericulture sector were determined and then to draw attention to these problems. The current situation assessment was made in the conclusion by making general inferences about the sector.

Keywords: Sericulture, Raw Silk, Fresh Cocoon, Silk Carpet, Exportation

Corresponding Author:
Polat M.
Kastamonu University, Faculty of Veterinary Medicine, Department of Animal Health Economics and Management, 37200 – Kastamonu/TURKEY
e-mail: vet.hekim.muratpolat@gmail.com, muratpolat@kastamonu.edu.tr

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INTRODUCTION

In Turkey, sericulture, which does not require too much investment, is generally carried out in small-scale operations as an additional activity alongside with other livestock activities and therefore it plays an important role in using the labor force of family members. Sericulture offers jobs and is an important income source for many people living in the rural areas mainly in the developing countries (Agatha, 2013).

Almost every region of Turkey is suitable for mulberry tree and sericulture in terms of climate, soil, topographical conditions and social structure. Sericulture is considered as advantageous with regards that the production means in sericulture are extremely simple, the product can be obtained in a short period as 35-40 days and easily turned into cash and in terms of requiring less labor than other livestock activities and also exploiting the elderly or female labor force (Berrin et al. , 2015).

Fresh cocoons create approximately 14-fold added-value until they become several silk weaving products such as silk carpets after the raw silk is obtained through reeling process. The production generates employment to a large extent through floater (silk reeling from cocoon), spinning, dyeing, textile and silk carpet weaving. Additionally, the contribution of the sericulture to the economy is important for Turkey, exporting the raw silk and finished products (Ümran, 2011).

Considering all these features, sericulture makes a great contribution directly or indirectly to the national economy as cocoon and silk sector in terms of production and employment.

CURRENT SITUATION AND PRODUCTION LEVEL OF SERICULTURE

The fresh cocoons, being the output of sericulture, is the raw material for the silk and silky textile industry and also has a traditional and cultural value. The number of villages and families, engaging in sericulture, the number of silkworm egg boxes used and fresh cocoon production values in Turkey are shown in Table 1 (TÜİK, 2017a).

When analyzing Table 1, it can be observed that the decrease in the number of villages and families, engaging in sericulture between 1991 and 2001 directly affected the production and therefore the silky textile industry of which the raw material is

Table 1. Sericulture production values in Turkey (1991-2016)

Years	Number of Villages Engaged in Sericulture	Number of Families Engaged in Sericulture	Number of Silkworm Egg Boxes Used	Fresh Cocoon (tons)
1991	1,635	29,689	50,623	1,353
1996	398	5,756	7,529	215
2001	213	1,555	2,445	47
2006	233	2,527	5,699	127
2007	212	2,274	5,273	125
2008	195	2,193	5,564	125
2009	203	2,295	5,683	136
2010	194	2,134	5,477	126
2011	295	2,623	5,808	151
2012	342	2,572	5,576	134
2013	327	2,343	5,261	121
2014	340	1,760	3,739	80
2015	474	1,956	4,674	115
2016	576	2,001	5,303	103

silk was affected negatively. The main reasons for the production decrease include; unconsciously increased use of pesticides, increase in terrorist incidents in the Southeastern Anatolia region and damping implemented on cocoon prices since 1989 by China, which has a great share in fresh cocoon, silk and silky textile trade (Esra, 2008).

When considering the geographical and social situation in Turkey, the sericulture for which all the provinces are suitable for the production activities, is carried out in 42 provinces as of 2016. The production has been realized in 35 of 42 provinces but the fresh cocoon could not be obtained in 7 of them. Fresh cocoon production by provinces for the year of 2016 in Turkey is given in Table 2 (TÜİK, 2017b).

When analyzing Table 2, it can be observed that Diyarbakır province alone constitutes 43.9% of total fresh cocoon production. The provinces of Antalya, Ankara, Bilecik, Sakarya, Muğla and Eskişehir follow Diyarbakır. While total production of these 7 provinces constitutes 86.2% of the total production, it is seen that Bursa province, which was the center of sericulture at the beginning of 1990s, has not displayed its previous activity as of 2016. The reason of this is the abandonment of production over years because of the increased industrialization and pollution in Bursa and its vicinity.

As a result of abandonment of production in sericulture in Turkey by years, the share in the World production has also gradually decreased. While 66% of 589,170 tons of fresh cocoon in the World was conducting by the People's Republic of China as of 2014, Turkey is on the 15th rank in the world with 80 tons of production and 0.014% share (FAOSTAT, 2017).

ORGANIZATION AND SUPPORT POLICIES IN SERICULTURE

Turkey has a history that goes back 1,500 years in sericulture (Keun, 1979; Gülşen and Ahmet, 2003). Sericulture was not only a commercial activity, but also affected many parts of the production area. In 1888, the first school of sericulture named Bursa Harir Darüt Talimi, which teaches the silkworm rearing from the scientific point of view (Esra, 2008). This school started education with 12 students and 5,000 students graduated from Bursa and Bilecik since the foundation of Turkish Republic (Nuran, 1996). The presence of 161 silk yarn factories operating in Gemlik, Geyve, Adapazarı, Izmit, Mudanya and Bandırma, especially in Bursa and Bilecik in early 20th century, is an indication that sericulture is an important economic activity (Ertuğrul Murat, 1996).

The organization in sericulture dates back to old times. In 1940, the first cooperatives were founded in Bursa, Bilecik and Adapazarı in order to maintain and increase the cocoon production after the foundation of Turkish Republic. These cooperatives were merged and Bursa Association of Agricultural Sales Cooperatives for Silk Cocoons (KOZABİRLİK) was founded on 11 May 1940 (Berrin, 2011).

Kozabirlik which has sought to improve nationwide after its first foundation years opened coop-

Table 2. Fresh cocoon production in tons by provinces in Turkey (2016)

Provinces	Number of Villages	Number of Families	Number of Silkworm Egg Boxes Used	Fresh Cocoon (tons)	Share %
Diyarbakır	54	640	1,682	45.1	43.9
Antalya	41	329	599	16.2	15.7
Ankara	32	113	386	8.2	8
Bilecik	26	83	250	6.9	6.7
Sakarya	23	77	249	4.7	4.6
Muğla	47	133	283	4.3	4.2
Eskişehir	23	48	125	3.3	3.2
Total (42 Provinces)	**576**	**2,001**	**5,302**	**103**	**100**

eratives in Edirne in 1944, Mihalgazi in 1951 and Alanya in 1984. Today, Kozabirlik continues its activities both in cooperative regions through its cooperatives in Bursa, Bilecik, Adapazarı, Mihalgazi and Alanya regions and in other regions where the production activities are carried out and which has production potential (Diyarbakır, Hatay, Muğla/Köyceğiz, İzmir/Ödemiş, Batman/Sason) and has 3,344 registered partners (Kozabirlik, 2017).

Kozabirlik not only makes cocoon purchase but also provides a significant contribution to the silkworm production through its silkworm egg production facility, established in 1963. On the other hand, the Association established a modern reeling factory in order to process the silk cocoon produced through the project supported by the Ministry of Agriculture in 2008 and so it has provided the employment area and also reduced foreign dependency in raw material processing (Kozabirlik, 2017).

The supports in sericulture have had many different periods over the years and also excluded from the support scope from time to time. Notwithstanding, both the incentives and supports have increased for the development of production sector at the present time.

Despite the increase in production costs in Turkey, since the prices of dry cocoons are low in foreign markets, fresh cocoon producers have started to be supported as of 1999. The supports have continued as subsidies in 1999 and 2000, as subsidy and direct support in 2001 and as direct support after 2002. Beside this, the eggs and plants are distributed as free of charge (TZOB, 2011).

Since the supports in sericulture were covered by the Support and Price Stabilization Fund, it was required to be covered by the budget of the Ministry

Table 3. Purchase price and supports by years for fresh cocoons *

	Purchase Prices of the Association (TL/kg)			Supports (TL/kg)			Total Purchase Prices (TL/kg)			In Total Purchase Price	
Years	Current Price	** Real Price	Index (%)	Current Price	** Real Price	Index (%)	Current Price	** Real Price	Index (%)	Association Purchase Share (%)	Supports Share (%)
1999	0.50	4.56	100.00	1.40	12.78	100.00	1.90	17.35	100.00	26.32	73.68
2000	0.65	4.47	97.97	1.73	11.87	92.85	2.38	16.34	94.20	27.37	72.63
2001	1.00	3.65	79.91	3.50	12.77	99.89	4.50	16.41	94.63	22.22	77.78
2002	2.00	5.58	122.19	5.50	15.34	120.01	7.50	20.92	120.58	26.67	73.33
2003	3.00	7.35	160.92	7.00	17.14	134.10	10.00	24.48	141.16	30.00	70.00
2004	2.30	4.95	108.41	7.70	16.57	129.62	10.00	21.51	124.04	23.00	77.00
2005	2.50	5.15	112.72	8.50	17.49	136.87	11.00	22.64	130.52	22.73	77.27
2006	2.50	4.61	101.02	9.50	17.52	137.10	12.00	22.13	127.61	20.83	79.17
2007	3.50	6.09	133.50	9.50	16.54	129.41	13.00	22.63	130.49	26.92	73.08
2008	6.00	9.66	211.69	10.00	16.10	126.01	16.00	25.77	148.55	37.50	62.50
2009	4.00	6.08	133.23	12.00	18.24	142.74	16.00	24.32	140.24	25.00	75.00
2010	3.00	4.19	91.78	15.00	20.95	163.89	18.00	25.14	144.91	16.67	83.33
2011	3.50	4.31	94.48	20.00	24.64	192.82	23.50	28.96	166.94	14.89	85.11
2012	4.00	4.81	105.40	20.00	24.05	188.21	24.00	28.86	166.42	16.67	83.33
2013	4.50	5.06	110.84	20.00	22.49	175.94	24.50	27.55	158.81	18.37	81.63
2014	5.00	5.37	117.68	30.00	38.16	298.57	35.00	43.53	250.96	12.34	87.66
2015	5.00	5.18	113.54	30.00	33.56	262.59	35.00	38.74	223.36	13.38	86.62
2016	5.00	5.00	109.54	40.00	40.00	312.98	45.00	45.00	259.44	11.11	88.89

* Fresh cocoon purchase and support prices of the Association were obtained from the 45th Bursa Directorate of Agricultural Sales Cooperatives for Silk Cocoons; ** As of 2016.

of Agriculture in order that this situation be based on a solid foundation. The supports were included in the scope of the Decree on Livestock Supports of the Ministry of Agriculture as of 2006 through the studies conducted for this reason and the first application of these supports was started in 2006 through the fund allocated from the budge of the Ministry of Agriculture (TZOB, 2011).

The EU gives direct income support to sericulture, at € 133 per box, provided that there is no economic benefit for the Union, provided that a minimum of 20 kg of product is obtained. In Iran, the state encourages the production of silk by purchasing fresh cocoon at $ 3.5 and selling it to consumers at a lower price. In Turkey, the supports for sericulture are as the free egg distribution and the direct support payment to the producers in sales of fresh cocoons (Gülşen and Ahmet, 2003; GTB, 2017).

The purchase price and supports by years for fresh cocoon is given in Table 3 and Fig 1.

When analyzing Table 1 and Figure 1, it can be observed that the fresh cocoon purchase prices of the association, supports and total purchase price in Turkey have shown a steady increase by years. This situation corresponds to the production figures coinciding with the same years and it is seen the supports have mobilized the silkworm production. It is obvious that the government support has increased within total purchase price over the years and has a very important share. This reveals how important the government support is in sericulture.

State support in sericulture is increasing day by day. In this grant scheme, which includes rearing rooms construction, equipment and equipment purchasing, mulberry cultivation, which will be implemented between 2017 and 2019, the grant rate is set at 100 % (Resmi Gazete, 2017). Increasing support in recent years will revitalize the sector.

MARKETING AND FOREIGN TRADE IN SERICULTURE

In the world, silk is an important textile raw material and is used in luxury consumer goods. Silk has a miniscule percentage of the global textile fibre market-less than 0.2 % (Rajat and Mahesh, 2005; ITC, 2017). This figure, however, is misleading, since the actual trading value of silk and silk products is much more impressive. This is a multibillion dollar trade, with a unit price for raw silk roughly twenty times that of raw cotton (ISC, 2017).

Turkey is a country with a great silk market potential. Almost all of the raw silk obtained from fresh cocoons in Turkey is used in the silk carpet production. However, the raw silk production in Turkey is far from meeting the needs for silk carpet industry.

Annual raw silk production is 22-25 tons and the raw silk that the sector needs is provided through imports. Besides this, the silk yarn importation other than raw silk has increased in recent years. The countries such as China, India, and Uzbekistan come to the forefront in importation.

The major silk consumers of the world are; USA, Italy, Japan, India, France, China (ISC, 2017). In

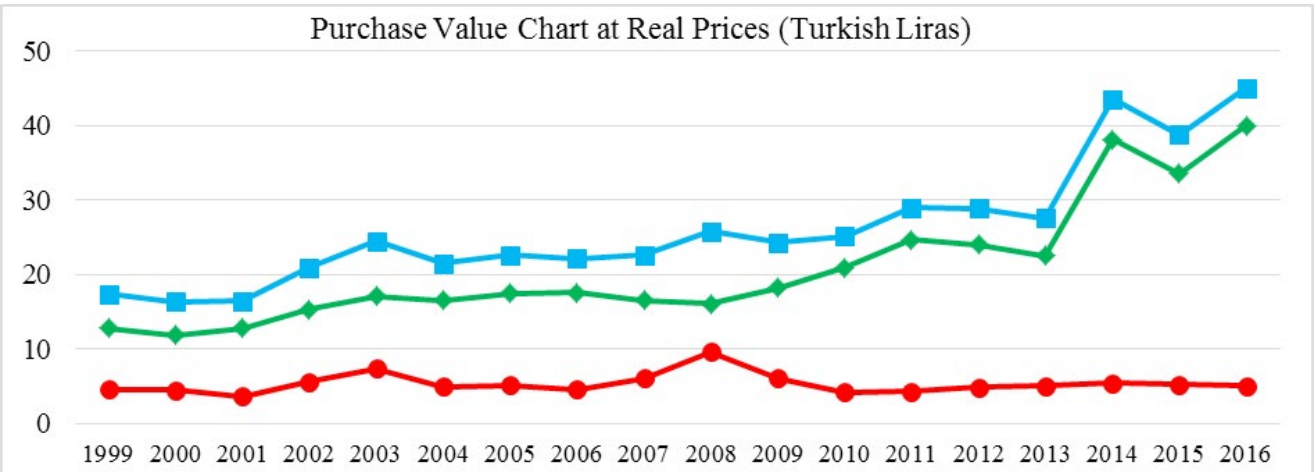

Figure 1. Association purchase prices, supports and total purchase prices (Turkish Liras per Kg)

Table 4. Turkish raw silk and silk yarn export and import values (2010-2016)

	Raw Silk				Silk Yarn			
	Export		Import		Export		Import	
Years	Kg	$	Kg	$	Kg	$	Kg	$
2010	0	0	92,442	2,205,181	21,937	388,179	89,457	4,121,647
2011	0	0	113,991	3,358,816	21,334	196,898	54,101	2,305,366
2012	0	0	47,957	1,574,124	22,446	341,166	47,067	2,015,059
2013	0	0	58,300	2,424,858	16,094	445,650	66,821	3,883,426
2014	0	0	57,947	2,291,283	22,295	446,674	69,319	4,053,657
2015	0	0	36,499	1,523,632	22,615	520,411	59,000	3,410,839
2016	0	0	26,496	1,013,688	29,229	283,506	36,263	2,063,777

Table 5. Turkish silk carpet export and import values (2010-2016)

	Silk Carpet					
	Export			Import		
Years	Kg	m^2	$	Kg	m^2	$
2010	127,786	43,369	3,880,0371	89,903	29,583	17,311,372
2011	165,169	34,156	4,463,0032	81,951	25,664	15,494,130
2012	110,305	22,204	3,342,1183	88,751	30,175	19,471,431
2013	99,077	24,353	2,869,3375	95,331	30,474	19,408,021
2014	94,788	20,432	1,957,8653	59,210	19,706	13,018,848
2015	62,046	11,932	1,012,0427	28,166	9,298	3,458,337
2016	30,248	5,990	578,1679	22,967	7,367	1,864,200

addition, China, which is the main producer of cocoon and silk in the world, promotes imports to Turkey as a result of the cheap price policy resulting from subsidies, cheap labor force and dumping sales (Matei et al. , 2006).

The raw silk, silk yarn and silk carpet export and import are conducted intensively in sericulture in Turkey. Among these, the silk carpet export is placed on the top. Turkish raw silk and silk yarn export and import values are given in Table 4 (TÜİK, 2017c, 2017d).

When analyzing Table 4, it is seen that Turkey does not export raw silk and provides its domestic market's needs for raw silk and silk yarn by import. Any customs duty is not applied to the raw silk and silk yarn waste and this can be shown as its factor. In addition to this, while the silk yarn exports are rapidly declining, imports continue at an equal rate.

The production of silk carpet, being the silk weaving product, is the product offering the highest added-value in this sector in Turkey. Hand weaving further increases the economic value of silk carpet. Turkish silk carpet export and import values are given in Table 5 (TÜİK, 2017e).

When analyzing Table 5, it is observed that there has been a gradual decrease in the export of silk carpets in recent years. Notwithstanding, the decrease in import makes the balance on the side of export. The political problems, faced in the countries which are among the silk carpet markets of Turkey, internal disturbances and economic crises influence the decrease in export over the years.

PROBLEMS EXPERIENCED IN SERICULTURE

Although sericulture sector is an additional field

of activity, it has some problems in itself. These problems includes: the losses due to the poisoning occurred as a result of contamination of mulberry leaves used in silkworm breeding, the losses occurred as a result of breeding at home by not creating special breeding places because it can be done once a year and for a short time.

Other problems of sericulture sector are incomes of alternative livestock and agricultural activities are more than sericulture income and exemption of customs on raw silk imports reduce the competitive power of the domestic market.

CONCLUSION

Although sericulture, which has national, historical, cultural and economic values for Turkey, had some periods with ups and downs, it maintains the distinction of being a traditional product. Besides, it is important to create an extra income source for the enterprises since it is a livestock sub-production area mostly conducted as a secondary occupation.

Despite all the problems experienced and declines in production, the silkworm production, re-mobilizing through the government supports is an important production branch that can be evaluated in rural area. Since both the cost and labor requirements are low, it has advantages in terms of profitability.

Considering the domestic market, the raw material needs are not met by import (by removing customs exemption) but on the contrary can be met through the increased production and quality by supporting the producers. This also indicates that sericulture is an up-and-coming production branch.

Consequently, Turkey can have an important role in revitalization of interest in sericulture by years and fulfillment of silk and silk goods needs of the European Union countries together with the increased production. It is necessary to protect sericulture that is our national value, to increase fresh cocoon production and quality by making silkworm egg production at a more technical level. As a result of this, the producers will generate more income and the number of producers will be increased and accordingly silk production, then silk carpet production and export will be increased. Eventually all these developments will make significant contributions to the national economy.

CONFLICT OF INTEREST STATEMENT

I have no conflict of interest to declare.

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Research article
Ερευνητικό άρθρο

Evaluation of Anti-Coccidial Activity of Different Extraction Products of *Allium sativum* (Garlic) in Broilers

M. Waqas[1], R. Akhtar*[2], H. Akbar[1], M. Lateef[1], I. Rashid[1], M. Ijaz[3]

[1]*Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[2]*Department of Pathology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[3]*Department of CMS, University of Veterinary and Animal Sciences, Lahore, Pakistan*

ABSTRACT. The present study was performed with objective to evaluate the anti-coccidial effect of three different forms of *Allium sativum* (garlic) in broiler birds. A total of 90 broiler chicks (day-old) were divided into nine equal groups. The mixed *Eimeria* species obtained from gut samples (*E. tenella* and E. *necatrix*) collected from different commercial poultry shops in Tolinton Market Lahore, Pakistan. These guts were checked in Parasitology laboratory in Department of Parasitology, University of Veterinary and Animal Sciences, Lahore. The positive cases were separated for extraction, sporulation and identification of oocyst(s). The oocysts counts per gram of droppings were determined by McMaster technique on day 0, 3, 7 and 10 of treatment. Each bird was challenged with 50,000 sporulated oocysts of *Eimeria* at 17th day of age. Three different forms of *Allium sativum* (garlic) including aqueous extract, methanol extract and powder form at dose rate 2 and 4 gm/kg body weight were used in challenged birds In all the forms and doses of the *Allium sativum* the oocyst per gram count was decreased but the best result was obtained with aqueous form at dose rate of 4gm/kg BW from day 7 to 10. The present study concluded that *Allium sativum* (garlic) can be used as natural anti-coccidial component to ameliorate the side effects and resistance of commercial anticoccidials in practice.

Keywords: *Allium sativum*, Aqueous, Broilers, *Eimeria*, Extracts, Methanol

Corresponding Author:
Dr. Raheela Akhtar,
Department of Pathology, Faculty of Veterinary Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan.54000.
E-mail: raheela.akhtar@uvas.edu.pk

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INTRODUCTION

Coccidiosis is an enteric disease of poultry caused by protozoan parasite of genus *Eimeria*. It causes huge production losses due to increased morbidity and mortality while its control mainly depends upon the use of anti-coccidial drugs. The preventive use of anticoccidial drugs is not only costly but also leads to serious issue of drug resistance in poultry and humans (Cervantes, 2015). Therefore this increasing resistance due to anti-coccidial drugs has created the need to look for new ways to control the disease. As the herbal products have always been effectively used for the control and treatment of various diseases in poultry and the use of *Allium sativum* (garlic) has become popular in fish farming due to its immune-protective and growth promoting activities. Moreover, garlic (*Allium sativum*) has been known as an herbal remedy to prevent and treat a variety of heart diseases and metabolic diseases, such as atherosclerosis, thrombosis, hypertension, dementia, cancer, and diabetes (Elbanna, 2012). Therefore the idea behind the present research was to use different forms and doses of *Allium sativum* to determine its anti-coccidial effects in broilers. Previous studies report wide usage of *Allium sativum* due to its pharmacological activities particularly the antiparasitic activities (Gaafar, 2012) but there are limited studies to evaluate its anticoccidial effect in broilers (Kim et al., 2013; Alnassan et al., 2015).Moreover, there has been no studies to compare the effects of different forms of *Allium sativum* in broiler chicks still far. Therefore the present study was first attempt towards the determination of the anticoccidial activity of different forms of *Allium sativum* in broiler chicks.

MATERIALS AND METHODS

The present study was carried out according to the legal requirements of ethical review committee of University of veterinary and Animal Sciences, Lahore, Pakistan. *Allium sativum* (Garlic) was collected from local market of Lahore, identified and authenticated from Department of Botany, University of the Punjab, Lahore, Pakistan. *Allium sativum* powdered (100 gm) was mixed with 500 mL distilled water and was boiled for 1.5 hours, cool down to 40°C and filtered through Whatman filter paper No. 1. The filtrate was concentrated in rotary evaporator and extract was stored at 4°C till further use (Onyeyili et al., 2001). Similarly methanol extract was prepared in Soxhlet apparatus and was stored at 4°C until used (Asuzu & Onu, 1994). A total of 90 broiler chicks (day-old) were divided into nine equal groups. Group 1 was negative control (non infected), group 2 was positive control infected with *Eimeria tenella*, group 3 was treated control of amprolium (2mg/Kg of feed). Group 4 and 5 were aqueous extracts, group 6 & 7 were methanol extracts and group 8 & 9 were powder form of *Allium sativum* at the rate of 2 and 4 mg/kg body weight respectively.The *Allium sativum* was administered in water once per Os in 500 ml drinking water to whole group.

The birds were offered experimental starter feed without anti-coccidial feed additives (Crescent Feed Kot Radha Kishan Pakistan) *ad libitum*.

Vaccination was done as per following schedule: ND+IB at day 1, IBD at day 8 and then ND at day 14.

To obtain the mixed *Eimeria* species (*E. tenella* and E. *necatrix*), guts were collected from different commercial poultry shops in Tolinton Market Lahore, Pakistan. These guts were checked in Parasitology laboratory in Department of Parasitology, University of Veterinary and Animal Sciences, Lahore. The positive cases were separated for extraction, sporulation and identification of oocyst(s). The oocyst(s) were preserved in 2.5% potassium dichromate solution and fil-

Table 1: Description of different treatments in all groups

Groups	Treatments
Group 1	Negative control without infection
Group 2	Positive control with coccidiosis infection
Group 3	Treated control of amprolium (2gm/ Kg of feed)
Group 4	Aqueous extract of Allium sativum (2gm/Kg body weight).
Group 5	Aqueous extract of Allium sativum (4gm/Kg body weight).
Group 6	Methanol extract of Allium sativum (2gm/Kg body weight).
Group 7	Methanol extract of Allium sativum (4gm/Kg body weight).
Group 8	Allium sativum powder form (2gm/kg body weight).
Group 9	Allium sativum powder form (4gm/kg body weight).

tered through muslin cloth. The filtrate was centrifugation at 1500 rpm for two minutes and the sediment was re-suspended in 2% potassium dichromate solution for the sporulation of oocysts. McMaster technique was used on day 0 (before treatment) and on day 3rd, 7th and 10th post treatment(s) for oocyst counting on pooled faecal samples from each group. Each bird was challenged with 50,000 sporulated oocysts of *Eimeria* at 16th day of age. The oocyst count was performed at day 21st, 25th and 28th. The efficacy percentage was determined by following formula:

$$\text{Efficacy (\%)} = \{(\text{Pre-treatment egg count/g - post treatment egg count/g})/ \text{Pre-treatment egg count/g}\} \times 100$$

Data was analyzed using two way ANOVA by SPSS version 21.00.

RESULTS AND DISCUSSION

There have been few studies on effects of garlic against coccidiosis (Kim et al., 2013; Alnassan et al., 2015) however, we first time compared the effect of different forms of garlic (*Allium sativum*) against coccidiosis in broilers. Our results revealed minimum oocyst per gram count (OPG) in group 5 followed by group 3, 7 and 9 at day 3, 7 and 10. However, the OPG of these three groups did not differ significantly (*P>0.05*) but were significantly higher *(P<0.001)* in comparison to group 5. Group 4 and 6 also showed non-significant differences *(P>0.05)* among them while the OPG of these groups was also significantly greater *(P<0.001)* from group 5 (Table 2). The lower OPG in aqueous extract may be due to more active phenolic compounds, vitamins and trace elements (Selenium and Germanium) in aqueous extract of *Allium sativum* that interact with cytoplasmic membranes of *Eimeria* leading to the death of coccidia cells (Sikkema et al., 1995). It may be possible that these compounds are harmed or inhibited by methanol but remain active in aqueous extract. Our results are also comparable to the previous studies of Gull et al. (2012) who found more antibacterial activity of aqueous extract of garlic than methanol extract but are in contrast to Mousavi et al. (2009) who found equal antifungal activity of aqueous and methanol extract of garlic. Although our results are in line with the findings of Arczewska-Włosek and Swiątkiewicz (2012) who reported that *Allium sativum* dry extract (powder) can reduce OPG in coccidiosis infected broiler chickens but they did not evaluated the different extraction products of garlic as we did in our study. The minimal OPG was found on day 10 (2018.52± 595.68) that was significantly different (*P<0.001)* from day 0 and day 3, while this data was significantly comparable (*P<0.001)* from data at day 7 (Table 3). As group 2 (positive control)

Table 2: OPG difference in various treatment groups

Groups	Mean
Group 1	0.00 ± 0.00^{a}
Group 2	9045.83 ± 1157.65^{b}
Group 3	716.67 ± 107.95^{cd}
Group 4	816.67 ± 118.82^{ed}
Group 5	470.83 ± 74.08^{c}
Group 6	895.83 ± 135.06^{ed}
Group 7	650.00 ± 101.62^{ed}
Group 8	1016.67 ± 151.52^{e}
Group 9	782.61 ± 112.05^{de}

Table 3. OPG on day 0, 3, 7 and 10 post treatment

Days	Mean OPG + standard error
Day 0	0.00 ± 0.00^{a}
Day 3	2250.00 ± 397.14^{b}
Day 7	2114.81 ± 474.80^{bc}
Day 10	2018.52 ± 595.68^{c}

Different superscripts in column show significant difference (P<0.001) between different days (before and after challenge)

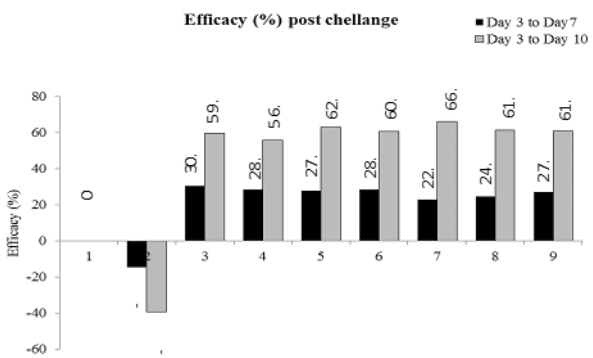

Figure 1: Efficacy of different treatments on OPG between different days (Day 3 to Day 7 and Day 3 to Day 10).

showed increasing trend of OPG so the efficacy in the graph showed negative values, while all other treatment showed positive values. The efficacy was greatest from day 3 to day 10 as compared to day 3 to day 7 because OPG decreased in decreasing order from day 3 to day 7 and day 7 to day 10 (Figure 1). This is in contrast to the studies of Pourali et al. (2013) who observed an increase in OPG at day 5 to 6 and the peak level of OPG was observed at days 8 to 9, sharply decreasing at day 10 after use of *Allium sativum*. This may be due to difference in infection age of birds as in our study the birds were infected at day 18 of age whereas Pourali et al. (2013) infected the birds in their study at day 34 of the age.

The present study concluded that the anticoccidial activity of *Allium sativum* may vary with different forms and doses and it was maximum with aqueous extract at dose rate of 4gm/kg BW. Moreover, its addition in poultry feed can be suggested to reduce the burden of high cost and progressively increasing drug resistance due to *Eimeria* species.

CONFLICT OF INTEREST STATEMENT

I have no conflict of interest to declare.

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Research article
Ερευνητικό άρθρο

New approach for medial patellar ligament splitting for treatment of upward fixation of patella

M.W. El Sherif

Department of Animal Surgery, Faculty of Veterinary Medicine, Assiut University, New Valley, Egypt.

ABSTRACT. A new medial patellar ligament splitting technique in cattle and donkeys is presented. It has been successfully applied on thirteen alive animals affected with permanent upward fixation of patella (UFP). The technique is simple, quick and easily applicable. In contrast to other medial patellar desmotomy techniques the present approach is less invasive, the skin at the surgical site is not incised but punctured, the pericapsular fat and joint capsule are not invaded. Minimal tissue invasiveness limits the infection of surgical site, minimizes bleeding and decreases their related postoperative consequences.

Keywords: Upward fixation, patella, desmotomy.

Corresponding Author:
Mohamed W. El Sherif
E-mail: drmwt@hotmail.com

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INTRODUCTION

Upward fixation of patella (UFP) has been reported with a higher incidence rate in equine than in cattle (Hanson and Peyton, 1987). This condition is believed to be due to poor conformation of the hind limb (Stick, 2006). The condition may occur temporarily which may spontaneously recover or permanently requiring surgical correction (Lacorix, 2005). Intermittent UFP is when the patella releases spontaneously, but locks again in variable intervals in forward movement (Brooks, 2007). Signs may disappear and normal steps are noticed after exercise (Curtis, 1961). Permanent dislocation of the patella leaves the limb in caudal extension and may even be dragged behind (Greenough, 2016). Affected animals are unable to protract the leg forward, or flex the affected limb causing the involved hind leg to drag behind. Jerky movement is observed at the walk when the MPL and its fibrocartilage disengage from the femoral trochlear ridge (Brooks, 2007). The condition is economically important as it reduces market value of the affected animal especially in riding animals like donkeys. Conservative treatments (Stashak, 2002; Brooks, 2007 and Peitzmeier et al, 2015) were reported. Surgical treatments including medial patellar ligament desmotomy (MPLD) and ultrasound-guided medial patellar ligament splitting (MPLS) were presented (Turner and Mcllwraith, 1989; Stashak, 2002; Tnibar, 2002; Brooks, 2007; Madhu1 et al., 2012 and Singh et al., 2015). MPLD is usually performed while the animal is standing and under the effect of sedation and local anesthesia. MPLS required general anesthesia and animal positioned in dorsal recumbency. MPLD and MPLS are relatively invasive surgical approaches that require skin incision. Short and long term post-operative complications associated with these surgical treatments include swelling, pain, sever bleeding, wound infection and persistent low-grade lameness were reported (RamaKrishna, 1972; Sharma, 1980; Ali and Hashim, 1984 and Tnibar, 2002). The present study presents a new surgical technique for splitting of the MPL as a surgical choice for the treatment of irresponsive upward fixation of patella in cattle and donkeys which is thought to be minimal invasive, easy and more suitable for field conditions.

MATERIALS AND METHODS

The study was approved by the animal welfare committee of the faculty of veterinary medicine, New Valley, Egypt. Ref. No. 1/2016.

Five donkeys (four males and one female, aging 7±2 years and weighing 220±30 kg) and eight cows (aging 4±2 years and weighting 400±50 kg) were admitted to the mobile surgery clinic of the faculty of veterinary medicine, New valley, Egypt, (2014-2016). They were diagnosed with permanent UFP. The donkeys and cows were sedated with xylazine HCl (Xylaject, ADWIA pharma, Egypt), (1mg/kg and 0.1mg/kg) administered intravenously.

The donkeys were secured with tying the front feet with ropes and kept in standing position while cows were positioned in lateral recumbency with the affected limb up. The stifle region of the operated limb was clipped and disinfected with absolute alcohol wipes followed by application of povidone iodine 10% solution (Betadine, Mondio, Switzerland). Ten milliliters of lidocaine HCl 2% (Depocaine, Depiky pharma, Egypt) was injected underneath the skin and deep between the middle and medial patellar ligaments, about 2 cm above the tibial tuberosity. Further disinfection was performed with alcohol wipes and povidone iodine solution. The medial patellar ligament was located with its insertion to the tibial tuberosity then held with the tips of thumb and index fingers. Instrument needed are half circle reverse cutting needle # 3, needle driver and silk strand (USP 1) in an appropriate length. Using aseptic technique, a sterile strand of silk mounted on a half circle reverse cutting needle is inserted through skin 2-3cm above the tibial crest at the medial aspect of the medial patellar ligament (at the tip of finger) and directed underneath the ligament to emerge on the opposite side (at the tip of the other finger) between the middle and medial patellar ligaments. The two ends of the silk strand were grasped with both hands and were used to transect the ligament with sawing action movements. The sawing action was discontinued when a "POP" sound was noted, indicating completion of the desmotomy. The silk strand was then removed and surgical site disinfected again with povidone iodine solution (Figure 1). Daily wound care with application of 10% povidone iodine antiseptic solution to the site of the procedure was performed for five successive days after surgery. Stable rest for 6 weeks was recommended. The surgical time (the time between the needle insertion and removal of silk strand from the

Fig 1: The surgical procedure in donkeys in standing position on the top; insertion of needle, A and splitting of the ligament, B, and in cattle in lateral recumbency with the affected limb above, at the bottom, C.

surgical site) for each procedure was recorded. Post-operative assessment was based on daily examination of animals for lameness signs, presence of gross signs of inflammation such as "swelling, hotness, pain and redness".

RESULTS

A pilot anatomical study performed on 10 donkey cadavers and bovine carcasses revealed the optimal site for the needle insertion was at the cranial aspect of the stifle, three fingers (2-3cm) proximal to tibial tuberosity. This site was found to be safe due to presence of periarticular fat while, proximal to this point, it is possible to damage the superficial branch of the saphenous nerve or to penetrate the medial extension of the femoropatellar joint capsule.

The surgical procedure was technically easy to perform and no intra-operative complications were recorded. The mean surgery time was 3 ±1 minutes.

Minor bleeding was noted at the points of needle insertion and exit point which stopped spontaneously. "POP" sound was heard in all cases and indicated complete transection of tensed medial patellar ligament. All treated animals regain normal posture and were able to walk normally immediately after the procedure.

No post-operative complications were recorded over two months after the procedures in any of the treated animals. Daily examination of the animals in rest and motion revealed no signs of lameness. Minor, unnoticed scar points were present at the points of insertion and exit of the surgical needle. Signs of mild inflammation "mild swelling and redness" was noticed in one cow which subsides at the second day of the operation. Stifle of the treated animals was normal on manual palpation except for limited tissue movability which may indicate fibrosis.

DISCUSSION

The procedure presented here is a new minimal MPLS technique. It was performed successively and described in cattle and donkeys. Open and closed MPLD techniques described (RamaKrishna, 1972; Sharma, 1980; Ali and Hashim, 1984; Hanson and Peyton, 1987; Turner and Mcllwraith, 1989 and Naveen et al., 2013) are the most common performed techniques for treatment of UFP condition in large animals. Both techniques involve skin incision and stifle tissue invasion. The advantage of the present approach is the absence of skin incision, minimal tissue invasion, limited amount of surgical instrumentation needed and speed of execution.

Another technique which is the MPL splitting described by (Tnibar, 2002 and Andersen and Tnibar, 2016) was presented to avoid invasion of the femoro-patellar joint. The technique is more sophisticated and involves performing under general anesthesia, skin incision and percutaneous splitting the MPL with Bard Parker blade under direct visualization of ultrasound. The present technique was easily performed in the field, performed in standing or lateral recumbent positions with minimal physical or chemical securing, didn't require special instruments and performed under local anesthesia. Anatomical and ultrasonographic findings reported by (Uddin et al., 2009; Kassab and Badawy, 2011) declared that the medial patellar ligament is laying directly underneath the skin, have the smaller width and thickness than the middle and lateral patellar ligaments and the gap between the middle patellar ligament and other two ligaments is more wide and distinct than the groove lies between the

middle and lateral patellar ligaments. Determination of the medial patellar ligament and holding it between the thumb and index fingers with little pressure with the tips of the fingers within the grooves makes the ligament more distinct and facilitates direct insertion of the needle directly underneath the ligament and avoids invasion of the joint. The invasion of the femoro-patellar joint is thought not to occur. If occurs, it is thought to be minimal. Further Ultrasound based study should be established to evaluate the femoro-patellar joint invasion.

Incomplete severing of the ligament is a common intra-operative complication of the blind or closed technique (RamaKrishna, 1972), which necessitates several attempts to sever the ligament completely. The results of present technique showed complete transection of the medial patellar ligament in all treated animals. Bleeding, infection and swelling are common post-operative complications referred to skin incision and tissue invasion (Hanson and Peyton, 1987; Shettko and Trostle, 2000 and Stick, 2006). The present approach was minimally tissue invasive and the subsequent bleeding, infection and swelling were not noticed. The short term gross examination of treated animals showed immediate return of stifle function and absence of post-operative inflammatory signs.

Placing the animal in lateral recumbency is a disadvantage but it was recommended in cattle in order to avoid sudden movement of the animal during the procedure and as it was easier to allocate the MPL in this position. Further long-term study is required to evaluate this technique with the aid of more advanced diagnostic methods and tools.

CONCLUSIONS

Upward fixation of patella is a common lameness producing condition with a high incidence in cattle and equine. Surgical desmotomy of the medial patellar ligament is the most reliable treatment for this condition. Several surgical techniques were presented with a potential post -operative complications. The present technique was found to be easy, time and money saving, field applicable, accurate and associated with minor post-operative complications.

CONFLICT OF INTEREST STATEMENT

All the authors declares that there is no conflict of interest for the presented case repost.

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Research article
Ερευνητικό άρθρο

Reference intervals for canine hematologic analytes using Siemens Advia 120

I.L. Oikonomidis[1], T.K. Tsouloufi[1], A. Papoutsi[2], M. Kritsepi-Konstantinou[3]

[1]Diagnostic Laboratory, School of Veterinary Medicine,
Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece
[2]Private practitioner

ABSTRACT. Hematologic investigation is essential for the evaluation of health status of companion animals. Appropriate and accurate reference intervals (RIs) are required for the interpretation of laboratory results. Thus, the primary aim of the present study was to establish canine complete blood count (CBC) RIs using Advia 120, a widely used in veterinary medicine automated hematology analyzer. Additional objectives were to evaluate sex as a partitioning factor of RIs and to investigate the effect that breed size has on CBC RIs. Reference individuals were selected by indirect sampling method from the medical records of a veterinary teaching hospital. The reference population comprised 284 adult dogs of both sexes and various breeds. The reference individuals were allocated into 3 groups based on breed size (small-sized, medium-sized and large-sized breeds). Complete blood count results from the dogs that met the inclusion criteria were used for the nonparametric calculation of RIs. Statistical and nonstatistical criteria were employed in order to decide whether sex-specific RIs are needed. Depending on the data distributions, mean or median comparisons were used to determine the effect of breed size and lifestyle on CBC results. Nine outliers were detected based on CBC results. The estimated RIs were generally comparable to those previously reported in the literature. Sex-dependent partitioning of RIs was indicated by the statistical criteria for a few analytes. From a clinicopathologic point of view though, sex-dependent partitioning of RIs is questioned and seems not to be required. Breed size appears to have an effect on CBC RIs. The RIs determined in the present study can be used as a guide for the interpretation of CBC results in dogs and can potentially be adopted by veterinary laboratories using Advia 120. Finally, based on the results of this study, breed size should probably be considered when interpreting CBC results.

Corresponding Author:
Ioannis L. Oikonomidis
The Royal (Dick) School of Veterinary Studies and The Roslin Institute,
Easter Bush Campus, EH25 9RG, Midlothian, UK
Email: economidis.john@gmail.com

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ΠΕΡΙΛΗΨΗ. Η γενική εξέταση του αίματος (ΓΕΑ) θεωρείται μείζονος σημασίας για την εκτίμηση του επιπέδου υγείας των ζώων συντροφιάς. Ωστόσο, για την ορθή ερμηνεία των εργαστηριακών αποτελεσμάτων απαιτούνται ακριβείς τιμές αναφοράς. Στο πλαίσιο αυτό, ο πρωταρχικός στόχος της παρούσας εργασίας ήταν η εξαγωγή τιμών αναφοράς για τις παραμέτρους της ΓΕΑ στον σκύλο, με τη βοήθεια του Advia 120, ενός, ευρέως χρησιμοποιούμενου στην κτηνιατρική, αυτόματου αιματολογικού αναλυτή. Δευτερευόντως, διερευνήσαμε εάν απαιτείται διαχωρισμός των τιμών αναφοράς της ΓΕΑ με βάση το φύλο των σκύλων, καθώς και αν το μέγεθος της φυλής μπορεί να επηρεάσει τις τιμές αναφοράς της ΓΕΑ. Τα ζώα επιλέχθηκαν από το ιατρικό αρχείο της πανεπιστημιακής κλινικής ζώων συντροφιάς του Τμήματος Κτηνιατρικής του Α.Π.Θ. Τον παραπάνω πληθυσμό αποτελούσαν 284 αρσενικοί και θηλυκοί σκύλοι διαφόρων φυλών. Το σύνολο των σκύλων ταξινομήθηκε σε 3 ομάδες με βάση το μέγεθος της φυλής (μικρού, μεσαίου, μεγάλου). Οι τιμές αναφοράς υπολογίστηκαν μη παραμετρικά, χρησιμοποιώντας τα αποτελέσματα της ΓΕΑ των σκύλων που πληρούσαν τα κριτήρια ένταξης στη μελέτη. Προκειμένου να αποφασιστεί εάν απαιτείται ο διαχωρισμός των τιμών αναφοράς της ΓΕΑ με βάση το φύλο, εφαρμόστηκαν τόσο στατιστικά όσο και μη στατιστικά κριτήρια. Ανάλογα με την κατανομή των δεδομένων πραγματοποιήθηκε σύγκριση μέσων ή διαμέσων, με σκοπό να ελεγχθεί η πιθανή επίδραση του μεγέθους της φυλής στις τιμές της ΓΕΑ. Εννέα ζώα αποκλείστηκαν από τη μελέτη, λόγω της ύπαρξης φανερώς ακραίων τιμών στη ΓΕΑ. Σε γενικές γραμμές, οι τιμές αναφοράς που υπολογίστηκαν στην παρούσα εργασία είναι παρόμοιες με αυτές που αναφέρονται στη διεθνή βιβλιογραφία. Επιπλέον, με βάση την εφαρμογή των στατιστικών κριτηρίων, υποδεικνύεται ότι για κάποιες παραμέτρους της ΓΕΑ απαιτούνται διαφορετικές τιμές αναφοράς για τα δύο φύλα. Παρόλα αυτά, από κλινικοεργαστηριακής απόψεως, ο διαχωρισμός των τιμών αναφοράς αμφισβητείται και πιθανώς δεν είναι αναγκαίος. Επίδραση του μεγέθους της φυλής παρατηρήθηκε στις τιμές αναφοράς της ΓΕΑ. Οι τιμές αναφοράς που παρουσιάζονται στην παρούσα εργασία μπορούν να χρησιμοποιηθούν ως οδηγός για την ερμηνεία των αποτελεσμάτων της ΓΕΑ στους σκύλους και να «υιοθετηθούν» από κτηνιατρικά εργαστήρια που χρησιμοποιούν τον αιματολογικό αναλυτή Advia 120. Τέλος, με βάση τα αποτελέσματά μας, το μέγεθος της φυλής θα πρέπει, πιθανώς, να λαμβάνεται υπ' όψιν κατά την ερμηνεία των αποτελεσμάτων της ΓΕΑ.

Keywords: complete blood count; dog; partitioning; reference values, breed size; sex

INTRODUCTION

Complete blood count (CBC) is a routinely performed blood test for the evaluation of overall health status in companion animal medicine. As for every laboratory test, interpretation of the CBC results is highly dependent on appropriate and accurate reference intervals (RIs). The latter also facilitate the implementation of external quality assurance practices. Siemens Advia 120 is a widely used in veterinary medicine automated hematology analyzer that employs laser flow cytometry technology. However, to our knowledge, there is only one study reporting CBC RIs in dogs using Advia 120 (Moritz et al., 2004). In that study, the reference population was comprised of 46 dogs. According to ASVCP Quality Assurance and Laboratory Standards (QALS) Committee guidelines for the determination of RIs in veterinary species though, the reference individuals should be ideally ≥120, in order the nonparametric method to be implemented for the calculation of RIs (Friedrichs et al., 2017). Hence, the primary objective of the present study was to establish RIs for hematologic analytes in a thus large population of adult dogs using Advia 120. The secondary objectives were: i) to investigate whether sex-specific RIs are required in dogs, similarly to humans, and ii) to further examine the effect of breed size on CBC RIs.

MATERIALS AND METHODS

Reference individuals were selected by *a posteriori* method. The medical records of the Companion

Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece between 2012-2016 were retrospectively reviewed for dogs that have been presented for a routine health examination, castration or minor dental problems. The inclusion criteria were the following: no lactation, no history of illness or medication in the near past, age÷ ≥6 months, complete vaccination and deworming, absence of abnormal findings during physical examination, CBC performed within the same day of sampling.

In our laboratory, blood samples are routinely being centrifuged after the completion of hematologic analysis and the plasma is macroscopically examined for the presence of hemolysis or lipemia. Additionally, when thrombocytopenia (defined in our laboratory as platelets count <200,000/μL) is identified, a blood film is always evaluated for the presence of platelet clumps. Thus, medical records reporting either hemolysis/lipemia or pseudothrombocytopenia were excluded from the study.

A total of 284 dogs fulfilled the defined inclusion criteria. The reference population was comprised of 110 male and 174 female dogs. The mean age of dogs was 6.0 years (range: 0.5-16.0 years), while the mean body weight was 14.9 kg (range: 1.6-52.0 kg). The exact distribution of canine breeds in the reference population of the study is presented in Table 1. The reference individuals were categorized into 3 groups based on breed size; 133 small-sized breeds, 80 medium-sized breeds and 71 large-sized breeds. Mixed breed dogs were allocated into the aforementioned categories based on their body weight, as previously described (Taylor et al., 2010): small-sized breeds: <10 kg, medium-sized breeds: 10-19.9 kg and large-sized breeds: >20 kg. Out of 284 dogs, 194 had an indoor lifestyle, whereas 90 were housed outdoors.

Blood samples were collected into K_3EDTA containing tubes (Deltalab, Barcelona, Spain). All hematologic analyses were performed on Advia 120 automated hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, USA) with the canine setting of the multispecies software. The analytes that were available in all medical records were the following: red blood cells (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell (WBC) count, differential WBC count (neutrophil, lymphocyte, monocyte, eosinophil and basophil counts), large unclassified cell (LUC)

Table 1: Distribution of canine breeds in the reference population of this study

	In reference population	
Canine breed	**Number**	**Percentage**
Akita Inu	2	0.7
Basset Hound	1	0.4
Beagle	6	2.1
Boxer	3	1.1
Canadian Shepherd	1	0.4
Cane Corso	1	0.4
Caucasian Shepherd	2	0.7
Cavalier King Charles Spaniel	1	0.4
Chow Chow	2	0.7
Cocker Spaniel	7	2.5
Collie	2	0.7
Dachshound	1	0.4
Doberman	2	0.7
Dogo Argentino	3	1.1
English Bulldog	2	0.7
Epagnuel Breton	1	0.4
French Bulldog	6	2.1
Golden Retriever	4	1.4
Greek Pointer	5	1.8
Griffon	1	0.4
German Shepherd	6	2.1
Husky	4	1.4
Jack Russel	3	1.1
Kurzhaar	1	0.4
Labrador Retriever	4	1.4
Lasa Apso	2	0.7
Malinois	1	0.4
Maltese	12	4.2
Mongrel	124	43.7
Pekingese	6	2.1
Pinscher	11	3.9
Pitbull	7	2.5
Pointer	2	0.7
Pomeranian	2	0.7
Poodle	8	2.8
Pug	2	0.7
Rottweiler	2	0.7
Schnauzer Miniature	1	0.4
Schnauzer Standard	1	0.4
Setter	5	1.8
Shih Tzu	3	1.1
Spitz	3	1.1
Tsihuahua	3	1.1
West Highland Terrier	4	1.4
Yorkshire Terrier	14	4.9
Total	284	100

count, platelet (PLT) count and mean platelet volume (MPV). At least one control (Siemens Healthcare Diagnostics, Deerfield, USA) was run daily and prior to blood sample analysis to ensure the good internal quality control.

The statistical software package SPSS 19 (SPSS Inc., Chicago, USA) was used for the calculation of descriptive statistics and comparison studies between breed-specific subgroups. Depending on the raw and transformed data distribution, mean or median comparison was used in order to determine the effect of breed size on CBC results. The RIs were determined using the statistical language R (R Foundation for Statistical Computing, Vienna, Austria) and the package *referenceIntervals*. Outliers were detected using Horn's method. The nonparametric and bootstrapping methods were used for the determination of reference and confidence intervals, respectively. Calculated RIs were at the 95th percentile, while confidence intervals were at the 90th percentile.

In order to determine whether sex-specific RIs are required in dogs, both distance and proportion statis-

Table 2: Descriptive statistics and reference intervals for hematologic analytes in dogs (n=275), using Advia 120.

Analyte	Unit	N	Mean	Median	SD	Min	Max	Data distribution	2.5th Percentile (90% CIs)	97.5th Percentile (90% CIs)
Red blood cells	10^6/μL	275	6.9	6.9	0.8	4.95	9.27	G	5.36 (5.06-5.53)	8.67 (8.40-8.80)
Hemoglobin	g/dL	275	15.8	15.8	1.9	11.5	20.8	G	12.2 (11.6-12.6)	19.4 (18.9-19.6)
Hematocrit	% L/L	275	47.5	47.4	5.7	33.1	62.2	G	37.2 (34.9-37.7)	58.7 (57.3-59.5)
MCV	fL	275	68.9	69.2	3.4	58.4	78.8	G	61.6 (59.5-62.9)	75.6 (74.5-77.4)
MCH	pg	273	22.9	23.0	1.2	18.8	26.5	NG	20.1 (19.3-20.4)	25.1 (24.9-26.5)
MCHC	g/dL	274	33.2	33.3	0.9	30.2	36.4	G	31.4 (30.5-31.7)	34.9 (34.8-35.7)
RDW	%	275	13.5	13.4	0.9	11.5	16.9	NG	12.0 (11.8-12.1)	15.7 (15.3-16.0)
White blood cells	10^3/μL 10^9/L	274	9.6	9.4	2.5	4.4	17.1	NG	5.5 (4.8-5.7)	15.8 (15.1-17.0)
Neutrophils	%	275	63.3	64.2	9.5	29.3	83.0	NG	42.4 (37.4-46.0)	78.8 (77.5-81.3)
Neutrophils	10^3/μL	273	6.1	5.8	1.9	2.8	12.3	NG	3.2 (2.9-3.3)	11.1 (10.5-12.3)
Lymphocytes	%	274	24.8	22.9	8.4	8.6	54.2	NG	11.6 (10.5-12.5)	44.4 (43.6-49.3)
Lymphocytes	10^3/μL	273	2.3	2.2	0.9	0.7	5.9	NG	1.0 (0.8-1.1)	4.7 (4.5-5.9)
Monocytes	%	274	5.4	4.9	2.1	2.0	16.1	NG	2.7 (2.4-2.9)	11.9 (10.2-15.9)
Monocytes	10^3/μL	274	0.5	0.5	0.2	0.1	1.8	NG	0.2	1.2 (1.0-1.6)
Eosinophils	%	273	4.9	4.4	3.4	0.1	18.1	NG	0.3 (0.2-0.5)	13.6 (13.1-18.1)
Eosinophils	10^3/μL	273	0.47	0.40	0.36	0.01	2.06	NG	0.03 (0.02-0.05)	1.45 (1.40-2.06)
Basophils	%	275	0.5	0.4	0.4	0	2.8	NG	0.1	1.5 (1.2-1.9)
Basophils	10^3/μL	275	0.04	0.04	0.03	0	0.29	NG	0.01	0.14 (0.11-0.16)
LUC	%	275	1.0	0.8	1.0	0	6.9	NG	0 (0-0.1)	4.0 (3.3-4.9)
LUC	10^3/μL	275	0.10	0.07	0.10	0	1.04	NG	0	0.37 (0.27-0.42)
Platelets	10^3/μL	273	354	330	115	149	844	NG	193 (170-206)	661 (630-844)
MPV	fL	274	11.7	11.1	2.3	8.2	22.5	NG	8.9 (8.5-9.0)	17.5 (17.0-21.8)

CIs, confidence intervals; G, Gaussian; LUC, large unclassified cells; Max, maximum; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; Min, minimum; MPV, mean platelet volume; NG, non-Gaussian; RDW, red cell distribution width; SD, standard deviation.

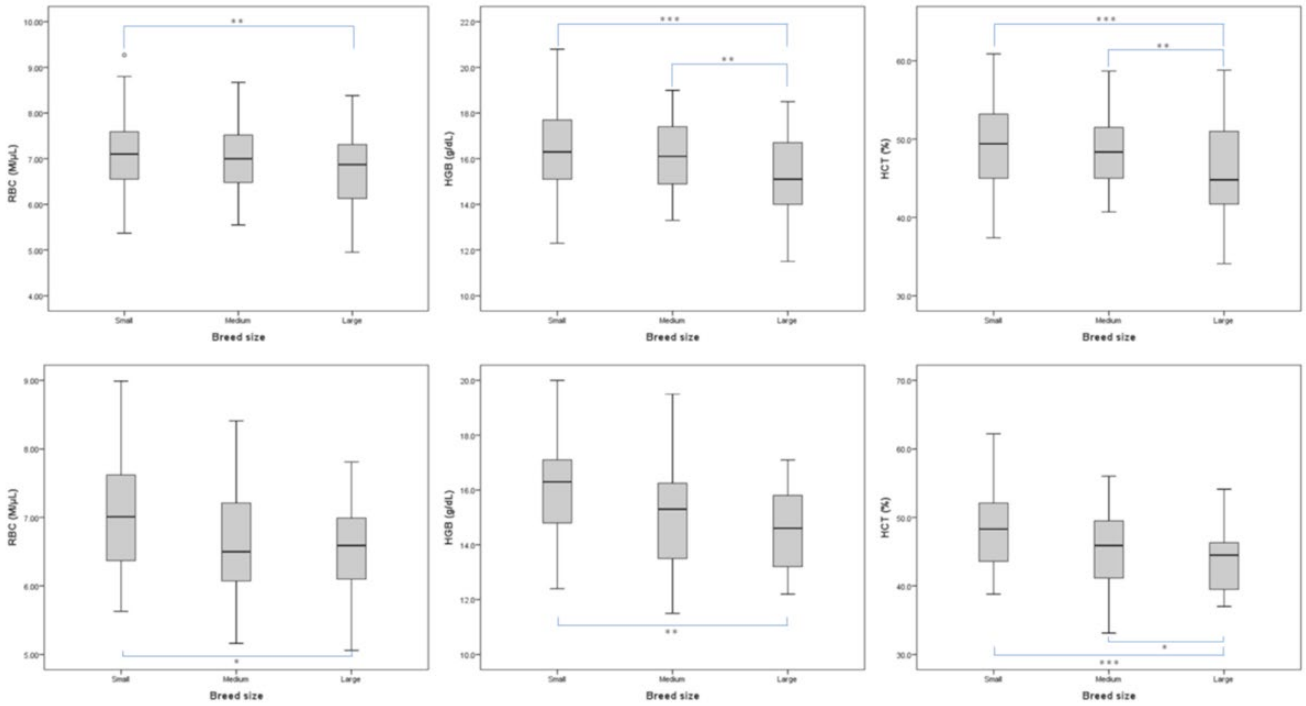

Figure 1. Boxplots of the red blood cell (RBC), hemoglobin (HGB), and hematocrit (HCT) values of the different breed size-specific subgroups. In the upper row, boxplots of RBC, HGB, and HCT values of small-, medium-, and large-sized dogs breeds with indoor lifestyle are depicted. In the lower row, boxplots of RBC, HGB, and HCT values of small-, medium-, and large-sized dogs breeds with outdoor lifestyle are presented. The lines represent the main body of data, whereas the dot represents an observed outlying point. The boxes represent the interquartile ranges; they are bisected by a line, which stands for the mean value. *$P < 0.10$, **$P < 0.05$, ***$P < 0.01$.

tical criteria previously proposed were applied (Lahti et al., 2002; Lahti et al., 2004). However, for the final decision, non-statistical criteria were employed, laying emphasis on the clinical relevance of the reported differences between the sex-specific RIs.

RESULTS

Nine dogs were excluded from the study based on profoundly abnormal CBC results (moderate to severe leukopenia [n=1], moderate to severe leukocytosis [n=1], severe monocytosis [n=1], severe eosinophilia [n=2] and severe thrombocytopenia [n=3]). Few outliers (n=1-2) were identified for some analytes using Horn's method. The results of the statistical analysis and calculated CBC RIs are presented in Table 2.

Sex-dependent partitioning of RIs was statistically indicated for lymphocytes count (based on distance statistical criteria) and monocytes count (based on proportion statistical criteria). Statistically significant differences were noted between small-, medium- and large-sized dog breeds in the following analytes: RBC count (ANOVA, p=0.010), HGB concentration (ANOVA, p<0.001), HCT (ANOVA, p<0.001), MCV (ANOVA, p=0.020), eosinophils count (Kruskal-Walis Test, p=0.020) and PLT count (ANOVA, p<0.001). However, only the differences in RBC count, HGB concentration, and HCT were proven statistically significant between the different breed-specific subgroups with the same lifestyle (Figure 1). Specifically, among the dogs with indoor lifestyle, statistically significant differences were observed between small-, medium-, and large-sized dog breeds in RBC count (ANOVA, p=0.020), HGB concentration (ANOVA, p=0.004), and HCT (ANOVA, p=0.002). Among the dogs with outdoor lifestyle, statistically significant differences were documented

between the three breed size subgroups in HGB concentration (ANOVA, p=0.021) and HCT (ANOVA, p=0.007), while marginally insignificant difference was noted in RBC count (ANOVA, p=0.064).

DISCUSSION

In the present study, CBC RIs in dogs were calculated using Advia 120 by extracting the reference individuals from the medical records of a veterinary teaching hospital. Although the *a posteriori* determination of RIs is not considered ideal, it is acceptable by the ASVCP QALS Committee (Friedrichs et al., 2017). The main advantage of this method, compared with the *a priori* method, is the capacity to include a large reference population, whereas the primary disadvantage is the limited control of some preanalytic and analytic factors. In an attempt to overcome the aforementioned limitation, strict inclusion and exclusion criteria were set. Moreover, in the context of the present study, it was investigated whether sex-dependent partitioning of CBC RIs is necessitated or not, while the effect of breed size on CBC RIs was also assessed.

In general, our CBC RIs are in agreement with those previously reported by Moritz et al. (2004). From a clinical point of view, the most significant differences are noted in RIs for RBC count, HGB concentration, HCT and WBC, neutrophils and lymphocytes counts. Specifically, our RIs for RBC count, HGB concentration and HCT are generally shifted downwards. The upper reference limit of WBC count and the lower reference limit of lymphocytes count are also shifted downwards in the present study. In terms of neutrophils count, our RIs are wider than those of Moritz et al. (2004). The observed differences between our RIs and those reported in the literature (Moritz et al., 2004) may be attributed, first and foremost, to preanalytic factors (notably the size, the demographic characteristics and the selection method of the reference population) and secondarily to analytic factors (e.g. statistical analysis).

The need for partitioning of RIs is increasingly discussed in veterinary medicine (Reynolds et al., 2010; Lavoué et al., 2013; Lawrence et al., 2013; Paltrinieri et al., 2014; Hegstad-Davies et al., 2015; ; Oikonomidis et al., 2018)). The rationale behind partitioning is the decrease of the variability of the reference population, which eventually leads to the narrowing of RIs. The reference population should comprise a minimum of 40 animals in order to be considered for partitioning of RIs (Friedrichs et al., 2017). Since the aforementioned criterion was met in our study, we investigated whether sex-dependent partitioning of RIs is necessitated. Based on previously proposed statistical criteria for Gaussian and non-Gaussian data distributions (Lahti et al., 2002; Lahti et al., 2004), partitioning was warranted only for lymphocytes and monocytes counts. From a clinicopathologic point of view though, sex-dependent partitioning of RIs is questioned and seems to not be required for any of the aforementioned analytes, in accordance with the relevant literature (Bourgès-Abella et al., 2011). In that study, sex-specific RIs were required for HCT and PLT count (Bourgès-Abella et al., 2011), this is not confirmed by our results. However, on the grounds of the well-known effect that breed has on hematologic analytes (Lawrence et al., 2013), we suggest that sex should be investigated as a partitioning factor in dogs of the same breed. The need for breed-dependent partitioning of RIs was not investigated in the present study, due to the insufficient number of individual canine breeds.

Several differences were observed between small-, medium- and large-sized breeds; a rather expected finding, based on the existing literature (Lawrence et al., 2013). However, when the breed size-specific subgroups were compared in the context of the same lifestyle, the differences were proven statistically significant only for RBC count, HGB concentration, and HCT, with the larger breed dogs having generally lower values.

Some limitations are recognized in the present study. Our RIs were determined *a posteriori*, by extracting the reference individuals from a database of medical records. Despite the fact that the inclusion criteria were strict, they were not set before sampling, as ideally should have been. Additionally, although the preanalytic and analytic procedures are well standardized in our laboratory, we cannot exclude a minor effect of preanalytic and analytic factors on the calculated CBC RIs. Finally, a limitation concerning the RIs for PLT count is recog-

nized, since data of the microscopic evaluation of platelets in the blood film were available only for dogs that had platelets count <200,000/μL. In this context, dogs with platelets count ≥200,000/μL and platelet clumps were inevitably included in our reference population, while dogs with platelets count <200,000/μL and evidence of platelet clumps in the blood film were indiscriminately excluded from our study. Thus, our platelets count RIs should be considered somewhat biased.

CONCLUSIONS

Complete blood count RIs using Advia 120 were established based on a large reference canine population with pre-existing CBC results and in accordance with ASVCP QALS Committee guidelines for the determination of RIs in veterinary species. In addition, the necessity of sex-dependent partitioning of RIs was investigated, using both statistical and non-statistical criteria, while the effect of breed size of dogs on CBC RIs was also assessed. Our RIs can potentially be adopted by veterinary laboratories using Advia 120 after a proper validation. Sex-dependent partitioning of RIs seems to not be required, while the effect of breed size of the dogs should be taken into consideration when evaluating CBC results. Finally, the present study can also be used as a paradigm for the *a posteriori* determination of RIs based on the official ASVCP QALS Committee guidelines.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effects of diazepam, ketamine HCl and sevoflurane anesthesia on vital and recovery values of nine long legged buzzards (*Buteo rufinus*) upon wing amputation

CT İşler [1]*, ME Altuğ [1], Z Yurtal[1], MZY Deveci [1]

[1]*Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Surgery, 31040, Hatay, Turkey*

ABSTRACT. In this clinical study, effects of diazepam + ketamine HCl + sevoflurane anesthesia on vital functions and recovery duration and qualityofnine long legged buzzards were evaluated upon wing amputation. Operation was decided for long legged buzzards and heartand breathingrate, body temperature, and reflexes of long legged buzzards were evaluated before, during and after the anesthesia. Diazepam and ketamine HCl injection increased the heart rate whereas it was decreased by sevoflurane. Respiratory rate decreased upon sevoflurane application. Body temperature decreased during anesthesia. Recovery began in the 3rd minute after cessation of sevoflurane administration with return of eye reflexes and completedin the 35th minute. It was observed that although birdsrecovered from anesthesia, danger of hypothermia persisted for a long time.There was a significant difference between the respiratory and heart rates during the ketamine HCl and sevofluraneanesthesia from those in the preoperative period. However, there was no statistically significant difference between pre and post operative periods in terms of vital parameters. For the first time, effects of diazepam + ketamine HCl + sevoflurane anesthesia combination on vital parameters are evaluated in long legged buzzards in Turkey.

Keywords: long legged buzzard (*Buteorufinus*), wing amputation, diazepam, ketamine HCl, sevoflurane

Corresponding Author:
CT İşler
E-mail: cafer.isler@gmail.com

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INTRODUCTION

In the literature, the reliability of anesthesia application to birds has been discussed. The advantages of injectable anesthetics are the ease of use, being economical and they ensure fast induction. Their disadvantages are inadequate muscle relaxation, necrosis at the injection site, maintenance dose risks and low recovery success. Diazepam, midazolam, xylazine or medetomidine in combination with ketamine is preferred for premedication (Durrani et al., 2009;Girling, 2009;Lierz and Korbel, 2012; Forbes, 2014; Lennox, 2015).

The dose of diazepam has been reported as 0,1-0,5 mg/kg (Girling, 2009). Ketamine ensures approximately 30 minutes surgical anesthesia when it is given intramuscularly (IM) at 4-10mg/kg (Forbes, 2014). Due to complications such as hyperthermia, tachycardia, and superficial respiration, it is not recommended to use alone ketamine HClin some birds e.g pigeons (Durrani et al.,2009).Isoflurane and sevofluranearepreferred for inhalation anesthesia(Mckeown and Hennigh, 2014).

In this study, it is aimed to evaluate effects of diazepam+ ketamine HCl + sevoflurane anesthesia on vital functions and recovery period before, during and after anesthesia in long legged buzzards upon wing amputation.

MATERIAL AND METHODS

Animals

In this study; raptors were injured with gun-pow and transported by rescue team for the treatment of Mustafa Kemal University, Faculty of Veterinary Medicine, and Department of Surgery in Hatay/ Turkey Of all 23 wild birdswhich were provided to the clinics in one year, 14 of them were determined as long legged buzzards. Upon clinical and radiological examinations of these animals, they were classified according to treatment processes. Nine of the long legged buzzards animals had infections as well as necrotic, open and comminuted fractures.According to the results of examinations, wing amputation was decided in 9 of the long legged buzzards

Clinicial examination

General health statuses of animals were assessed. Animals which had bad health statuses were orally administered antibiotics of Baytril %10 (1ml/1lt),A andB vitamins.Five cc of lactated Ringer's and 5% dextrose solutions were givento animals subcutaneously. During this process, antiseptic wound dressings and bandages were applied to the wings of the animals. Animals which were sufficiently healthy for surgical interventions were operated. Continuous ECG monitoring (Petas, KMA 800) was usedto follow heart and, respiration rates, body temperature before, during and after the operation. The number of normal heart, respiratory rate and body temperature are given in table 1.

Anesthetic procudures

Anesthesia induction was performed by using 0.5 mg/kg IM diazepam and 40 mg/kg IM ketamineHCl (alpha, Egevet) 10 minutes after diazepam application (Girling, 2009) at the muscles semimembranosous and semitendinosous. Patient was up on the anesthesia. During ketamine anesthesia is done preoperative procedure such as patients' feather collected, asepsia

Table 1: Change in Vital Parameters of Long Legged Buzzards (n=9)

Period	The heart rate (mean±SD)	Respiratory rate (mean±SD)	Body temperature (mean±SD)
Before the application	157±32^{a}	44±24	39,4±5,1^{*,d}
Diazepam	240±24^{b,d}	60±18^{a,c,d}	39,0±4,7^{a,b,c}
Ketamine HCL	286±46^{a,c,e}	56±13^{b}	38,2±3,8
Sevoflurane	118±15^{a,b,c}	30±9^{a,b,c}	36,0±4,1^{a,c,d}
20 minutes after waking up	134±22^{d,e}	38±13^{d}	36,4±2,1*^{,b}

^{*,a,b,c,d,e}When cells in the same column were evaluated between each other, there were significant differences between same letters ($p<0.05$).

and antisepsia. After then, upon induction, tracheal intubation (endotracheal tube, internal diameter 2.5) was applied 10 minute later and 100%O_2 and 1-4% concentrations of sevoflurane anesthesia were applied by a non-rebreathing system with spontaneous respiration. Operative interventions were finalized during the 80-90 minutes anesthesia process. During interventions, and vital parameters (body temperature, heart and respiratory rates) were continuously followed up (Table 1). Statuses of reflexes were controlled before and after the anesthesia (Table 2).

Table 2: Status of Long Legged Buzzards Before and After the Anesthesia (+: present, -: absent, min: minutes) (n=9)

Reflex/Anesthesia	Diazepam	KetamineHCl	Sevoflurane	Awakening
Eye Reflex	+	+	- (5±1,1 min)	+ (3±0,8 min)
Swallowing	+	+	- (6±1,7 min)	+ (5±1,1 min)
Foot Movement	+	-(5±2,1 min)	-	+ (9±1,8 min)
Wing beat	+	-(6±1,9 min)	-	+ (13±2,3 min)
Head Movement	+	-(7±2,1 min)	-	+ (18±5,1 min)
Full Recovery				+ (35±5,4 min)

Statistical methods

Statistical analyses were performed by using SPSS 17.0 program. Independent Samples T-Test was used to compare two independent groups and One-way ANOVA and Post-hoc Tukey tests were used to compare more than two groups. Values were represented as mean±standard deviation (mean±SD) and the statistical significance was accepted when p value was lower than 0.05 ($p<0.05$).

RESULTS

It was determined that diazepam led to decrease in reflexes but it did not eliminate any of the reflexes in long legged buzzards. It was observed that diazepam increased the hearth and respiratory rates whereas it did not change the body temperature. Ketamine HCl stopped coordination and convulsive reflexes. It increased the heart rate and prominently decreased the body temperature. It was determined that ketamine HCl did not influence significantly breathing, swallowing and eye reflexes. Even though animals had swallowing reflex, intubation was applied and there was no complication in*Buteo rufinus*.It was also shown that sevoflurane eliminated all reflexes from the 6th minute of its application.It decreased the hearth rate, and prominently reduced the respiratory rate and body temperature.

When the 3% sevoflurane inhalation anesthesia was applied, even though all conditions of the anesthesia were ensured, there were pain and leg motions in three animals during plucking of feathers. When 4% of sevoflurane was given, these reflexes were not observed and it was decided that the ideal starting concentration of sevoflurane was 4%. In the25th minute upon anesthesia, all patients were stabile when they were administered the 2% concentration sevoflurane anesthesiaand when 1% concentration anesthesia was given to patients in the 40th minute (during the suture applicationprocess).Upon suture application process, sevoflurane inhalation was stopped and observed the animals recovered (Table 2).

In case of recovery parameters, eyelid motions started in the 3rd minute. Swallowing reflex, head movements to sides and pedal reflexes were observed in the 8th minute. The animal flapped its wings in the 18th minute and the full recovery was observed in the 35th minute. It took long time to have the ideal body temperature and it was observed that the danger of hypothermia continued for 2 hours after the recovery. There was no significant difference between pre and post operative periods in terms of vital parameters. On the other hand, there was a significant difference between the body temperature and preoperative sevoflurane anesthesia ($p<0,05$). Statistical differences were shown in Table 2.

When postoperative periods were assessed between each other, two animals died presumably due to hypothermia in 0to 6 hours and two animals died because of the stress and bad general condition on the 4th and 5th days. Full recovery and healing were observed in remaining animals and sutures were removed on the 10th day. Long legged buzzards which could not be left in their natural habitat due to amputated wings were delivered to Forestry and Water ministry officials. Then, healthy animals were given to Gaziantep Zoo.

DISCUSSION

The aim of the premedication is to decrease the salivation and mucus amount, but on the other side it may lead to the risk of airway obstruction by increasing the mucus viscosity (Paul-Murphy and Fialkowski, 2001). Ketamine + Diazepam combination is preferredin case of painfulprocedures in patients. The use of ketamine alone in birds is not recommended (Mahmud et al., 2014). Diazepam dose is 0.1-0.5mg/kg (IM) (Girling, 2009). Diazepam can lead to ataxia (Abou-Madi, 2001). In this study, preanesthetic induction was performed by giving 0.5mg/kg IM diazepam. It was determined that 0.5mg/kg diazepam diddecrease in reflexes but it did not eliminate, there was no ataxia and airway obstruction, the animal only become tranquilized, and did not allow the intubation. Diazepam slightly increased the heart and respiratory rates and it did not lead to prominent alteration in the body temperature (table 1.,2.).

Intubation can be hard due to laryngospasm (Şenel, 2008). Insufficient premedication and anesthesia led to complications of bronchospasm during the intubation in a study (Ünsaldı,2015). In wild poultries, 10mg/kg ketamine HCl ensures a short time anesthesia(Kibar and Bumin, 2006, Aslan et al., 2009, Forbes, 2014). In our study, 40mg/kg IM ketamine HCl was used. Although the swallowing reflex was present, intubation was slowly performed without complications. This dose of ketamine, although high enough, was accepted appropriate for sufficient anesthesia, and intubation without pain and complication. In poultries, when 20-50mg/kg ketamine alone is used, it can lead to both insufficient anesthesia and excitation and hypothermia can also be observed. There can also be low recovery success (Girling 2009). Dose of 60 mg/kg Ketamine HCl leads mild sedation and anesthesia. However, it should not be used alone also for its possible complications such as hypothermia, tachycardia and shallow breathing (Durrani 2009). In our study, higher doses of ketamine HCl were not required since the anesthesia was continued by using sevoflurane. 40mg/kg IM ketamine HCl was eliminated the wing and pedal reflexes, but eye and swallowing reflexes were still observed. Acceleration of the heart rate from awake state was statistical significant (table 1.,2.).

In both injectable and inhalational anesthesia applications, hypothermia is one of the important risks (Lierz and Korbel, 2012). Warming the body during the delivery of inhalants, and taking precautions for heating the body minimize the hypothermia until the end of the recovery process (Chan et al., 2013). In this study, it was observed that hypothermia occurred and the body temperature of the animal reduced to 36.4^{0}C, hypothermia led to deaths in the postoperative period and hypothermia risk continued even in case the full recovery was observed. Furthermore, it was determined that external heating precautions had vital roles during the 5 to 6 hours of the postoperative period.

It has been reported that use ofsevofluranehas been recently increasing despite its high costs; sevoflurane have faster inductionand reanimationand these drugs can be preferred in the inhalation anesthesia (Chan et al., 2013, Mckeown and Hennigh, 2014). Sevoflurane inhalation is stated that its induction dose is 5-6% and its maintaining dose is 3-4% (Girling 2009). In this study, the clinical importance and effects of sevoflurane in rapptorsare clearly shown.It was decided that the ideal starting concentration was 4% and the maintaining dose was 2%. No reflexes and pain sensations were observed when these concentrations were applied to animals.Two of our animals died due to hypothermia in the postoperative period. Even though the full recovery was seen, hypothermia continued for two hours. During the reanimation period, the standing position of the animal took 35±5,4 minutes, the eyelid motions were observed in the 3rd minute after the anesthesia was stopped. The full recovery was completed in totally 35±5,4 minutes.

For the first time in Turkey, effects of anesthetic combination Diazepam + ketamine HCl+ Sevoflurane on vital parameters of long legged buzzards were evaluated in this study. It was determined that 0,5mg/kg IM, 40mg/kg IM Ketamine HCl, %100 O_2 and %1-4 Sevoflurane anesthetic combination was acceptable for free living buzzards.

CONFLICT OF INTEREST STATEMENT

All the authors declares that there is no conflict of interest for the presented case repost.

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Research article
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Relationship of the bovine *IGF1*, *TG*, *DGAT1* and *MYF5* genes to meat colour, tenderness and cooking loss

S. Ardicli[1], H. Samli[1], D. Dincel[1], B. Ekiz[2], H. Yalcintan[2], B. Vatansever[3], F. Balci[1]

[1]Laboratory of Genetics, Department of Genetics, Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey
[2] Department of Animal Breeding and Husbandry, Faculty of Veterinary Medicine, Istanbul University, Istanbul, Turkey
[3]Department of Biology, Institute of Science, Uludag University, Bursa, Turkey

ABSTRACT. Bovine insulin-like growth factor 1 (*IGF1*), thyroglobulin (*TG*), diacylglycerol-O-acyltransferase 1 (*DGAT1*) and myogenic factor 5 (*MYF5*) genes play an important role in the physiology of lipid and muscle metabolism and are therefore considered as candidate genes for meat production traits in farm animals. The objectives of this study were to investigate single nucleotide polymorphisms (SNPs) in *IGF1*, *TG*, *DGAT1* and *MYF5* genes and to evaluate whether these polymorphisms affected meat colour, tenderness and cooking loss in Holstein cattle. Initially, the SNPs were detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. Meat samples (N= 50) derived from *M. longissimus thoracis et lumborum* (LTL) were used in the current study. Significant differences in variations of meat colour parameters were observed at 24 hours post-mortem. *IGF1* was associated with colour parameters of a* and chroma values. In addition, effects of *TG* were statistically significant on L* and a* values, while, effects of *MYF5* were significant on a* value. There was no association of the tested SNPs with meat pH, tenderness and cooking loss. The results presented here may give the valuable information for improving meat colour in cattle.

***Keywords*:** Genetic markers, meat quality, single nucleotide polymorphisms, marker-assisted selection

Corresponding Author:
F. Balci, Department of Genetics, Faculty of Veterinary Medicine, Uludag University, 16059, Nilufer, Bursa, Turkey
E-mail: fbalci@uludag.edu.tr

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INTRODUCTION

The trend of beef production in many countries has gradually changed from meat yield to meat quality (Sañudo et al., 2004; Li et al., 2013). Carcass and meat quality traits, which are under the control of polygenic inheritance, are economically important traits and because meat quality is defined by the traits that consumer perceives as desirable (Warner et al., 2010). Among the characteristics considered, meat colour and tenderness influence the consumer's satisfaction about the quality of a piece of meat and determine the price accordingly (Grunert et al., 2004; Mullen et al., 2006; Li et al., 2013). Several factors have been identified when trying to evaluate meat colour and tenderness such as breed of animal, slaughter age, pre- and post slaughter conditions, aging process, meat pH, amount of connective tissue and packaging method (Priolo et al., 2001; Warner et al., 2010; Li et al., 2013; Frylinck et al., 2015). Apart from these environmental factors, many studies have investigated genetic effects on the mentioned traits and on the other hand, several genes associated with meat quality have been identified and single nucleotide polymorphisms (SNPs) of many candidate genes have been determined to be highly effective markers in beef production (Warner et al., 2010; Li et al., 2013).

Bovine insulin-like growth factor 1 (*IGF1*) gene is localized in chromosome 5 and consists of 6 exons (Bishop et al., 1991; Miller et al., 1992). *IGF1* has been shown to be a candidate gene for growth rate and meat production traits due to its role in regulation of cell proliferation and animal growth (Siadkowska et al., 2006). A thymine/cytosine transition (T/C), also recognizable as RFLP-*Sna*BI, at position 472 in the 5'-noncoding region of the *IGF1* gene (GenBank Acc. No: AF210383) has been reported to be candidate marker for growth performance and carcass traits (Li et al., 2004; Curi et al., 2005b; Siadkowska et al., 2006). Thyroglobulin (*TG*) gene is the molecular regulator for the thyroid hormones including T3 and T4 which are known to affect adipocyte differentiation and lipid metabolism (Shin and Chung, 2007; Pannier et al., 2010). *TG* gene has been mapped to the centromeric region of bovine chromosome 14 (Casas et al., 2005) and has been associated with lipid metabolism and meat quality traits in various cattle breeds (Barendse, 1999; Barendse et al., 2004; Gan et al., 2008). A cytosine/thymine (C/T) polymorphism at position 422 of the *TG* gene (GenBank Acc No: X05380) has been associated with marbling score (Shin and Chung, 2007) and back fat thickness (Moore et al., 2003; Casas et al., 2005). The diacylglycerol-O-acyltransferase 1 (*DGAT1*) gene (Gen Bank Acc. No: AY065621), maps to chromosome 14, encodes the microsomal enzyme DGAT1 which is a catalyzer in the triglyceride synthesis pathway (Li et al., 2013). A lysine/alanine amino acid substitution in exon 8, at amino acid position 232 (K232A) of *DGAT1* gene has been demonstrated to be associated with milk components (Banos et al., 2008; Hradecká et al., 2008; Cerit et al., 2014) and higher levels of intramuscular fat (IMF) content in *semitendinosus* muscle (Thaller et al., 2003; Pannier et al., 2010; Tait et al., 2014). The genes of the muscle regulatory factors (*MRF*) gene family including myogenin (*MYOG*), myogenic factor 5 (*MYF5*), myogenic differentiation 1 (*MYOD1*) and myogenic factor 6 (*MYF6*) also called *MRF4* or herculin. All four genes are composed of 3 exons and share homology (Bhuiyan et al., 2009). Among them, *MYF5*, mapped to bovine chromosome 5, was evaluated as a muscle-specific factor and its expression was associated with myoblast lineage (Kisacova et al., 2009). In previous studies, polymorphisms in *MYF5* gene have been reported to be associated with growth traits (Li et al., 2004; Chung and Kim, 2005; Zhang et al., 2007; Bhuiyan et al., 2009).

The candidate genes were selected for this study with the objectives to estimate the effects of polymorphisms at the *IGF1*, *TG*, *DGAT1* and *MYF5* genes which were previously hypothesised to be genetic factors influencing adipocyte and myoblast differentiation, lipid metabolism, IMF deposition in bovine muscle regulation that has noteworthy effects on meat quality traits (Barendse, 1999; Thaller et al., 2003; Siadkowska et al., 2006). In the literature there is a limited information about the effects of the mentioned genes on meat colour and tenderness. Hence the objective of the current study was to evaluate the association of polymorphisms at four candidate genes with meat colour, tenderness and cooking loss in Holstein bulls.

MATERIALS AND METHODS

Animals and sampling

All animals selected for this study belonged to the Pedigree Project of the Turkish Ministry of Food, Agriculture and Livestock, and Cattle Breeders Association. Ethical approval was received from Uludag University Local Ethical Committee of Animal Experiments (Grant No: 2010-03/05). A total of 50 meat samples derived from purebred Holstein bulls that were grown on a private farm in the South Marmara region with the same feeding conditions and slaughtered at 15-17 months of age were used in the present study. The mean pre-slaughter weight of the animals was 469.04±7.65 kg. Previous to slaughter, stunning method was applied with a captive bolt stunner. After slaughter, all of the carcasses were electrically stimulated for a duration of 30 s (60V), suspended through the Achilles tendon. The carcasses were kept in a room at 4°C overnight. 5 cm-thick meat samples were collected from the *M. longissimus thoracis et lumborum* (LTL) at the 12^{th}-13^{th} intercostal area at 24 h after slaughter. The samples were vacuum-packed and exposed to ageing period for 6-7 days at 2°C. In addition, 4 mL blood samples were collected in K_3EDTA tubes (Vacutest Kima, SRL, Italy) from the vena jugularis of each of the bulls for genomic DNA extraction.

Meat quality evaluation

Meat colour parameters with the CIELAB colour space system including L* (lightness), a* (redness) and b* (yellowness) values were evaluated using Minolta CR 400 colorimeter (Minolta Camera Co., Osaka, Japan) with illuminant D65 as the light source. The device was set to make three measurements and take their average after the calibration corresponding to the standard white plate. Colour measurements were repeated 3 times on the meat samples from a fat-free area and the average of these measurements was evaluated as the final value. Chroma value (C) was calculated as $(a^{*2}+b^{*2})^{1/2}$ and hue angle (H) as arctan (b*/a*). Initial measurements of colour parameters were performed 24 h after slaughter. Additionally, ultimate pH at 24 h postmortem was measured using a digital pH meter (Testo 205, Lenzkirch, Germany). After 6-7 days ageing period, meat colour was measured immediately after cutting, after 1 h storage and after 24 h storage on cut surface of 2.5 cm thick samples from fat-free area (Ekiz et al., 2009).

In order to measure cooking loss (%), meat samples were firstly weighed (initial sample weight), repacked in vacuum, cooked in a waterbath at 75°C for 1 h and the samples were reweighed (cooked sample weight). Mean initial weight of the samples was 300.204 ± 9.14 g. Cooking loss (%) was evaluated from the percentage of weight loss of the cooked sample compared to initial sample weight by using the formula of (initial sample weight - cooked sample weight) / initial sample weight x 100 (Ekiz et al., 2009). After the measurement of cooking loss, cooked samples were used to determine shear force value.

Six rectangular cross section (parallel to the muscle fibres) subsamples (3 cm long and 1x1 cm surface area) removed from cooked samples were sheared across the widest dimension using an Instron Universal Testing Machine (Model 3343, Norwood, MA, USA) which was set to 150 mm/min crosshead speed and 50 kg force applied to the meat. An average of the values for six sub-samples was accepted as the Warner-Bratzler shear force (WBSF) value for that sample (Ekiz et al., 2012).

DNA isolation and genotyping

DNA extraction was performed by a phenol-chloroform method as described by Green and Sambrook (2012). The concentration range (ng/µl) and purity (absorbance at 260–280 nm) of the DNA samples was measured with a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA). In the current study, genotyping of the SNPs in the *IGF1*, *TG*, *DGAT1* and *MYF5* genes was carried out by PCR-RFLP. Primers used, PCR conditions and corresponding restriction enzymes are shown in Table 1. The PCR amplification was performed in a total volume of 50 µL containing 33.5 µL dH_2O, 5 µL 10 x Buffer, 5 µL $MgSO_4$, 1 µL dNTPs (2.5 mM), 2.5 U *Taq* DNA polymerase (Biomatik, A1003-500 U, 5 U/µL), 1 µL (0.025 µM) of each primer and 3 µL of the DNA sample at a concentration of 100 ng/µL. PCR amplicons of the *IGF1*, *TG*, *DGAT1* and *MYF5* genes were subjected to restriction enzyme digestion with suitable restriction enzymes according to the

Table 1. Primers used, PCR conditions and corresponding restriction enzymes in the present study

Gene	PCR amplicon (bp)	Forward primer	Reverse primer	PCR conditions	Restriction enzyme	Reference
IGF1	249	5'ATTACAAAGCTGCCTGCCCC3'	5'ACCTTACCCGTATGAAAGGAATATACGT3'	94°C 5′ (94 °C 1′, 64°C 1′, 72°C 1′) 31 cycles, 72°C 5′	*Sna*BI	Siadkowska et al. (2006)
TG	545	5′GGGGATGACTACGAGTATGACTG3′	5′GTGAAAATCTTGTGGAGGCTGTA3′	94°C 5′ (94 °C 1′, 55°C 1′, 72°C 1′) 35 cycles, 72°C 7′	*Mfl*I	Shin and Chung (2007)
DGAT1	411	5'GCACCATCCTCTTCCTCAAG3'	5'GGAAGCGCTTTCGGATG3'	*94°C 4′, 10 cycles (94°C 60 s, 66°C 60s; −1°C per cycle, 72°C 60s), 25 cycles (94°C 60s, 56 °C 120 s, 72°C 60s), 72°C 15′	*Cfr*I	Lacorte et al. (2006)
MYF5	445	5'ACAGCGTCTACTGTCCTGATG3'	5'CGTGGTATATACTAAGGACAC3'	94°C 4′ (94 °C 30s, 58°C 60s, 72°C 60s) 38 cycles, 72°C 4′	*Taq*I	Kisacova et al. (2009)

IGF1: insulin-like growth factor 1, *TG*: thyroglobulin, *DGAT1*: diacylglycerol-O-acyltransferase 1, *MYF5*– myogenic factor 5 * Touch-down PCR protocol was performed.

suppliers instructions. Briefly, enzyme-buffer mix was prepared by mixing 2 μL of restriction enzyme with 8 μL of the respective buffer. Reaction mix was prepared by mixing 10 μL PCR product with 2 μL of enzyme buffer mix. Volume was made up to 20 μL with autoclaved MilliQ water. Afterwards, these reaction mixtures were incubated at 37°C for 16 h. The samples were put into the 3% agarose gel (Sigma Aldrich, Steinheim, Germany) with 1 x TBE buffer for electrophoretic separation. Ethidium bromide (1 μg.mL^{-1}) was used as an intercalated reagent. Electrophoresis ran at voltage max. 90 V per 1 h. Visualization of DNA fragments was made in using of gel imaging system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). As a reference to estimate the size of fragments we applied DNA ladder (100–1000 bp, Biomatik).

Statistical analysis

The genotype and allele frequencies of each SNP were calculated by the standard procedure (Falconer and Mackay, 1996). The Hardy–Weinberg equilibrium (HWE) was tested for all alleles by using the POPGENE software v1.32 (Yeh et al., 2000). The population genetic indexes including gene heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) were estimated by using the following formulas as described by Nei and Roychoudhury (1974) and Botstein et al. (1980):

$$He = 1 - \sum_{i=1}^{n} P_i^2$$

$$Ne = 1/\sum_{i=1}^{n} P_i^2$$

$$PIC = 1 - (\sum_{i=1}^{n} P_i^2) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Where:

P_i was the frequency of the i^{th} allele,

n was the number of alleles.

Statistical analyses were carried out using SPSS v23.0 (IBM, Armonk, NY, USA). Levene's test was used to test for homogeneity of the variances. Meat colour parameters were considered as repeated measures and analyzed by using the Repeated Measures of General Linear Model (RM-GLM) procedures. Orthogonal polynomial contrasts were used to estimate the linear, quadratic and cubic effects of meat colour variation and time as well as their interactions. The mix models in the present study, as shown below, were selected by evaluating the adjusted R^2 to compare the explanatory power of models with different numbers of predictors.

$$Y_{ijklmno} = \mu + IGHF1_i + TG_j + DGAT1_k + MYF5_l + LW_m + S_n + \sum_{o=1}^{4} b_o X_{o_{ijklmno}} + e_{ijklmno}$$

Where:

$Y_{ijklmno}$ = the studied traits; μ = the overall mean;

$IGF1_i$ = the fixed effect of the *IGF1* genotype; TG_j = the fixed effect of the *TG* genotype; $DGAT1_k$ = the fixed effect of the *DGAT1* genotype; $MYF5_l$ = the fixed effect of the *MYF5* genotype; LW_m = the fixed effect of pre-slaughter live weight (m = <430, 431-490, 491<); S_n = the fixed effect of season at the slaughter (n = winter, spring, summer); b = regression coefficient; X_o = meat colour parameters measured at different times (o = 1: meat colour parameters at 24h post-mortem, 2: 0h after ageing, 3: 1h after ageing, 4: 24h after ageing). In addition, the model for the independent variables (meat pH at 24h post-mortem, shear force values and cooking loss) is as follows:

$$Y_{ijklmno} = \mu + IGHF1_i + TG_j + DGAT1_k + MYF5_l + LW_m + S_n + e_{ijklmno}$$

When significant associations were identified, the mean values for each effect were contrasted using Tukey's test.

RESULTS

We have amplified the 249 bp fragment in the 5'-non-coding region of the *IGF1* gene. Digestion of the PCR product with the *Sna*BI nuclease resulted in two bands (223 bp and 26 bp) for homozygote TT and three bands (249 bp, 223 bp and 26 bp) for the heterozygote genotype. The DNA amplified from CC animals remained undigested with the corresponding restriction enzyme (Fig 1). The amplified fragment of the C422T polymorphism (545 bp) in *TG* gene showed three genotypes including CC, CT and TT by digestion with the restriction enzyme *Mfl*I and the C allele was cleaved into three bands of 72 bp, 178 bp and 259 bp, while T allele showed two bands of 72 bp and 473 bp (Fig 2). However, TT genotype was not present in the current study. The cleavage of a 411 bp PCR product by *Cfr*I yielded two fragments of 203 bp and 208 bp and was diagnostic for the AA genotype in the *DGAT1* assay. These two bands (203 bp and 208 bp) were observed as a single band (203 bp + 208 bp) in gel electrophoresis pattern. Heterozygote genotype was cleaved into three bands of 411 bp, 208 bp and 203 bp. Besides the DNA amplified from KK animals remained undigested with the *Cfr*I nuclease (Fig 3). Typing *MYF5* allelic variation by PCR-RFLP

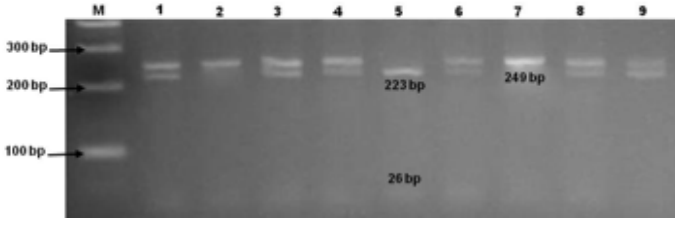

Figure 1. The electrophoresis pattern of C472T polymorphism within the bovine *IGF1* gene (M: Marker; Lanes 2 and 7: CC; Lanes 1,3,4,6,8 and 9: CT; Lane 5: TT).

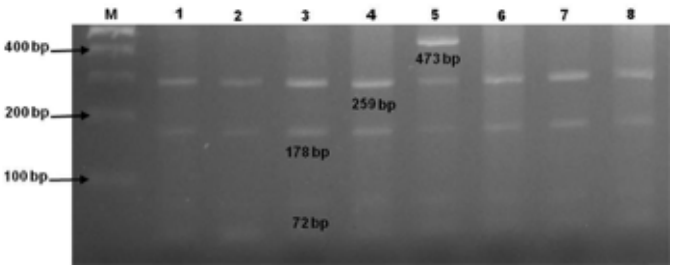

Figure 2. The electrophoresis pattern of C422T polymorphism within the bovine *TG* gene (M: Marker; Lane 5: CT; Lanes 1-4 and lanes 6-8: CC; Note: TT genotype was not present).

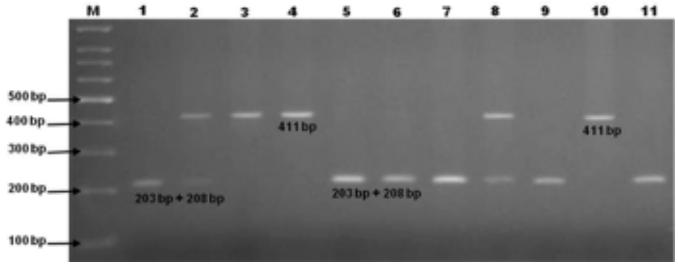

Figure 3 The electrophoresis pattern of K232A polymorphism within the bovine *DGAT1* gene (M: Marker; Lanes1, 5-7, 9,11: AA; Lanes 2 and 8: KA; Lanes 3, 4 and 10: KK).

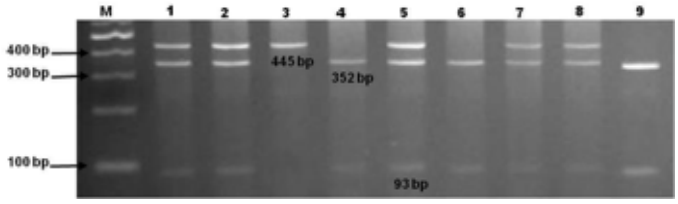

Figure 4 The electrophoresis pattern of g.1911A>G polymorphism within the bovine MYF5 gene (M: Marker; Lanes 4,6 and 9: *Genotype* '22'; Lanes 1,2,5,7 and 8: Genotype '12'; Lane 3: Genotype '11').

Table 2. Allele and genotype frequencies of polymorphisms in *IGF1*, *TG*, *DGAT1* and *MYF5* genes, population genetic indices (He, Ne, PIC) and compatibility with the Hardy-Weinberg equilibrium.

SNP	*IGF1*			*TG*			*DGAT1*			*MYF5*		
Genotypes	CC	CT	TT	CC	CT	TT	AA	KA	KK	11	12	22
N	4	40	6	45	5	0	7	36	7	3	26	21
%	8	80	12	90	10	0	14	72	14	6	52	42
MAF	0.48			0.05			0.50			0.32		
He	0.4992			0.0950			0.5000			0.4352		
Ne	1.9968			1.1049			2.0000			1.7705		
PIC	0.3745			0.0904			0.3750			0.3405		
χ^2(HWE)*	18.15*			26.36*			9.68*			1.89		

χ^2(HWE): Hardy-Weinberg equilibrium χ^2 value, * P<0.001: not consistent with HWE, N: number of experimental bulls, MAF: minor allele frequency, He: heterozygozity, Ne: effective allele number, PIC: polymorphism information content

bp when digested with *Taq*I restriction enzyme, while for allele 1, the 445 bp band was diagnostic. The presence of both alleles (heterozygote genotype: 12) was characterized by fragment sizes 445 bp, 352 bp and 93 bp (Fig 4).

The genotypic and allelic frequencies, population genetic indices including He, Ne and PIC and compatibility with the Hardy-Weinberg equilibrium (HWE) are shown in Table 2. Two alleles and three genotypes in each SNP were found in the current study, except for *TG* because TT genotype was not present. Results indicated that the population were determined not to be compatible for *IGF1*, *TG* and *DGAT1* genotypes in the Hardy-Weinberg equilibrium (P<0.001). The minor allele frequencies ranged from 0.05 to 0.50 were observed and all markers in the present study were polymorphic except for *TG*, according to the classification reported by Menezes et al. (2006). In addition to this, He values ranged from 0.0950 to 0.5000, PIC values ranged from 0.0904 to 0.3750 and Ne values ranged from 1.1049 to 2.0000 were observed in the chi-square statistics.

In Table 3, significant probability values obtained in the present study are given for linear, quadratic and cubic effects. Results indicated that, time effect on meat colour parameters was statistically significant except for linear effect on L* value, quadratic and cubic effects on a* value and cubic effect on chroma value. Time x *IGF1* interaction revealed linear effect on L*, a* and chroma value (P<0.05).

Table 3. Significant probability values obtained in the ANOVA

Effects	Meat colour parameters				
	L*	a*	b*	C	H
Time					
Linear	⋯	0.000	0.028	0.000	0.000
Quadratic	0.005	⋯	0.000	0.000	0.000
Cubic	0.020	⋯	0.000	⋯	0.000
Time x *IGF1*					
Linear	0.038	0.003	⋯	0.044	⋯
Quadratic	⋯	0.000	⋯	0.002	⋯
Cubic	⋯	⋯	⋯	⋯	⋯
Time x *TG*					
Linear	0.050	0.023	⋯	⋯	⋯
Quadratic	0.032	0.014	⋯	0.050	⋯
Cubic	⋯	⋯	⋯	⋯	⋯
Time x *DGAT1*					
Linear	⋯	⋯	⋯	⋯	⋯
Quadratic	⋯	⋯	⋯	⋯	⋯
Cubic	⋯	⋯	⋯	⋯	⋯
Time x *MYF5*					
Linear	⋯	0.027	⋯	⋯	⋯
Quadratic	⋯	⋯	⋯	⋯	⋯
Cubic	⋯	⋯	⋯	⋯	⋯
Time x Season					
Linear	⋯	⋯	⋯	⋯	⋯
Quadratic	⋯	⋯	⋯	⋯	0.049
Cubic	⋯	⋯	⋯	⋯	⋯
Time x Live weight					
Linear	⋯	⋯	⋯	⋯	⋯
Quadratic	⋯	⋯	⋯	⋯	⋯
Cubic	⋯	⋯	⋯	⋯	⋯

L*: lightness, a*: redness, b*: yellowness, C:chroma, H: hue angle.

Table 4. Least squares means (±standard errors) for *IGF1, TG*, *DGAT1* and *MYF5* genotype effects on meat colour parameters.

Parameters	Genotype																					
	IGF1						TG						DGAT1						MYF5			
	CC (N=4)		CT (N=40)		TT (N=6)		CC (N=45)		CT (N=5)		AA (N=7)		KA (N=36)		KK (N=7)		11 (N=3)		12 (N=26)		22 (N=21)	
$(L^*)^S$	33.80	2.10	31.53	1.37	34.21	1.98	35.93	1.14^a	30.43	1.97^b	32.50	1.85	33.66	1.42	33.38	1.97	34.18	2.50	33.83	1.17	31.53	1.28
$(L^*)^{0h}$	25.94	2.07	30.83	1.35	28.91	1.94	28.62	1.13	28.50	1.93	28.21	1.82	28.53	1.40	28.93	1.93	27.07	2.46	29.54	1.15	29.07	1.26
$(L^*)^{1h}$	26.47	2.38	31.97	1.55	29.88	2.24	29.43	1.30	29.45	2.23	29.03	2.09	29.24	1.61	30.06	2.22	28.16	2.83	30.10	1.33	30.06	1.45
$(L^*)^{24h}$	28.39	2.27	32.88	1.48	30.31	2.14	30.85	1.24	30.20	2.12	30.03	1.99	30.80	1.54	30.75	2.12	28.99	2.70	31.60	1.27	30.98	1.38
$(a^*)^S$	15.90	1.15^a	11.074	0.75^b	11.47	1.08^b	11.60	0.63^b	14.03	1.08^a	13.35	1.01	12.91	0.78	12.19	1.08	12.25	1.37^{ab}	12.22	0.64^b	13.96	0.70^a
$(a^*)^{0h}$	14.59	0.85	15.42	0.55	15.47	0.81	15.20	0.47	15.12	0.80	14.74	0.75	15.56	0.58	15.19	0.80	15.50	1.02	15.12	0.48	14.86	0.52
$(a^*)^{1h}$	17.44	1.26	18.18	0.82	18.60	1.19	18.41	0.69	17.73	1.18	17.98	1.11	18.49	0.85	17.75	1.18	18.63	1.50	17.97	0.70	17.61	0.76
$(a^*)^{24h}$	22.33	1.55	20.55	1.01	21.28	1.46	21.53	0.84	21.24	1.45	21.67	1.36	21.33	1.05	21.16	1.45	23.24	1.85	20.87	0.87	20.05	0.95
$(b^*)^S$	8.90	2.06	9.22	1.35	9.83	1.94	9.07	1.12	9.57	1.93	8.73	1.82	10.36	1.40	8.87	1.93	10.23	2.46	9.13	1.15	8.60	1.26
$(b^*)^{0h}$	-3.82	0.76	-2.24	0.49	-2.88	0.72	-2.98	0.41	-2.98	0.71	-3.05	0.67	-2.88	0.52	-3.00	0.71	-3.53	0.91	-2.56	0.43	-2.85	0.46
$(b^*)^{1h}$	-0.76	1.67	2.41	1.09	1.83	1.57	1.32	0.91	1.00	1.56	1.12	1.47	1.33	1.13	1.03	1.56	0.51	1.99	1.59	0.93	1.37	1.02
$(b^*)^{24h}$	2.91	1.42	4.53	0.93	3.51	1.34	3.87	0.78	3.43	1.33	3.22	1.25	4.01	0.97	3.72	1.33	3.18	1.70	4.19	0.79	3.58	0.87
$(C)^S$	18.75	1.37^a	14.72	0.89^b	15.28	1.29^{ab}	15.04	0.75	17.46	1.28	16.43	1.20	16.97	0.93	15.36	1.28	16.16	1.63	15.69	0.77	16.88	0.84
$(C)^{0h}$	15.09	0.79	15.66	0.51	15.81	0.74	15.55	0.43	15.49	0.74	15.16	0.69	15.85	0.53	15.55	0.74	15.91	0.94	15.43	0.44	15.22	0.48
$(C)^{1h}$	17.50	1.39	18.51	0.91	18.83	1.31	18.64	0.76	17.91	1.31	18.27	1.23	18.65	0.95	17.92	1.31	18.75	1.66	18.24	0.78	17.85	0.85
$(C)^{24h}$	22.54	1.75	21.15	1.14	21.65	1.64	21.96	0.95	21.60	1.64	21.99	1.53	21.76	1.18	21.60	1.63	23.49	2.08	21.38	0.98	20.48	1.07
$(H)^S$	0.52	0.14	0.69	0.08	0.71	0.13	0.66	0.07	0.62	0.13	0.59	0.12	0.68	0.09	0.65	0.13	0.71	0.16	0.65	0.07	0.56	0.08
$(H)^{0h}$	-0.26	0.05	-0.15	0.04	-0.19	0.05	-0.19	0.03	-0.21	0.05	-0.21	0.05	-0.19	0.04	-0.19	0.05	-0.23	0.07	-0.17	0.03	-0.19	0.03
$(H)^{1h}$	-0.05	0.09	0.11	0.06	0.08	0.08	0.06	0.05	0.04	0.08	0.04	0.07	0.06	0.06	0.05	0.08	0.01	0.11	0.07	0.05	0.06	0.05
$(H)^{24h}$	0.11	0.06	0.20	0.04	0.15	0.056	0.17	0.03	0.14	0.05	0.12	0.05	0.17	0.04	0.16	0.05	0.11	0.07	0.19	0.03	0.16	0.04

L*: lightness, a*: redness, b*: yellowness, C: chroma, H: hue angle

S: represents colour parameters measured at 24h post-slaughter, 0h, 1h, 24h: represents colour parameters measured at 0h, 1h and 24h after ageing period

[a,b] Different superscripts within a raw indicate significant difference

significantly affected the linear and quadratic terms of fitted meat colour development curves. Time x *MYF5* interaction revealed a significant effect on the linear term of the a* value (P<0.05). Besides, phenotypic interactions including time x season and time x live weight were evaluated in the current study. However, only quadratic term of the time x season interaction was observed (P<0.05).

Least squares means (±standard errors) for *IGF1*, *TG*, *DGAT1* and *MYF5* genotype effects on meat colour parameters are shown in Table 4. The results indicated that *IGF1* was associated with colour parameters of a* and chroma value at 24 h post-slaughter (P<0.05). Meat derived from CC animals had higher values of mentioned colour parameters compared to alternative genotypes. In the present study the effects of *TG* was statistically significant on L* and a* values (P<0.05). In this respect, on the one hand meat from CC animals seemed to be brighter in colour; on the other hand meat from heterozygous animals was redder. In addition, a significant association was observed between the *MYF5* marker and a* value at 24h post-mortem. The '22' genotype seemed to be associated with redder meat compared to alternative variants of the polymorphism (P<0.05). There was no association between any of the tested SNPs with meat colour parameters after ageing period, nor was there any association with variation in pH, tenderness and cooking loss, as shown in Table 5 (P>0.05).

DISCUSSION

In this work, the result of Hardy-Weinberg disequilibrium (P<0.001) in *IGF1*, *TG* and *DGAT1*genotypes

Table 5. Least squares means (±standard errors) for *IGF1*, *TG*, *DGAT1* and *MYF5* genotype effects on pH at 24h post-mortem, shear force and cooking loss.colour parameters.

Parameters	Genotype																					
	IGF1						TG				DGAT1						MYF5					
	CC (N=4)		CT (N=40)		TT (N=6)		CC (N=45)		CT (N=5)		AA (N=7)		KA (N=36)		KK (N=7)		11 (N=3)		12 (N=26)		22 (N=21)	
pH (24h)	5.94	0.14	5.88	0.09	5.76	0.14	5.74	0.07	5.98	0.14	5.82	0.13	5.83	0.11	5.93	0.14	5.91	0.18	5.90	0.08	5.78	0.09
WBSF (kgf/cm2)	3.75	1.31	6.16	0.85	4.22	1.23	4.92	0.71	4.5	01.22	4.15	1.15	5.26	0.88	4.71	1.22	6.71	1.56	3.72	0.73	3.69	0.79
CL (%)	21.01	3.36	24.92	2.20	22.0	03.16	23.00	1.83	22.29	3.15	21.84	2.96	25.60	2.28	20.49	3.15	24.39	4.01	21.78	1.88	21.77	2.05

WBSF: Warner-Braztler shear force, CL: cooking loss

may have been due to limited sample sizes from the population and artificial selection of parents for the milk production traits that should be considered for Holstein breed. According to the classification of PIC values (PIC<0.25= low informative polymorphism; 0.25<PIC <0.50= intermediate/mildly informative polymorphism; PIC>0.50= high informative polymorphism), population had intermediate levels of genetic diversity (Botstein et al., 1980). However, *TG* may not be included to the mentioned classification because of the T allele with low frequency (0.05) resulting in low genetic variabilities of He, Ne and PIC.

Meat colour is an extremely influential trait for commercial markets. Although colour is only slightly correlated with the eating characteristics of meat, it is very important for the consumer's choice (Priolo et al., 2001; Modika et al., 2015). Recently, many studies have investigated genetic effects on meat colour, however, testing of the SNPs in candidate genes may provide novel aspects to genetic background of meat quality. Based hereupon, we aimed to assess novel associations between *IGF1*, *TG*, *DGAT1* and *MYF5* genes and meat colour at pre- and post ageing process. Among them, the polymorphism in *IGF1* was significantly associated with colour parameters of a* and chroma values at 24 h post-mortem (P<0.05). The CC genotype seemed to be favourable in the present study. However, there was no association between the *IGF1* and any of the colour parameters after 6-7 days of ageing period. Curi et al. (2005a) observed significant associations of *IGF1* genotypes with body weight and subcutaneous back fat, and the effects of these genotypes on *M. longissimus thoracis et lumborum* area tended to be significant in different genetic groups of beef cattle.

Moreover, Li et al. (2004) reported that, CC genotype of *IGF1* was associated with higher live body weight at weaning in commercial lines of *Bos taurus*. As shown by Siadkowska et al. (2006) the CC genotype was found favourable also for feed consumption and conversion. In the literature, there is insufficient information about the association between the *IGF1* C472T marker and variation in meat colour to draw conclusions. Previous studies indicated that, effective quantitive trait loci (QTL) for meat production traits (Casas et al., 2000; MacNeil and Grosz, 2002) were assigned to bovine chromosome 5 (BTA5), where the *IGF1* gene is located. Our results suggest that focusing on this genomic region may be useful in improving not only growth traits but also meat colour.

Thyroid hormones play an important role in regulating adipocyte growth and differentiation, fat depots and homeostasis of the entire lipid metabolism. *TG* is the molecular precursor for thyroid hormones (Casas et al., 2005). Hence, *TG* is considered as a functional candidate gene as well as a positional candidate and is widely used in marker assisted selection programs to improve meat quality (Shin and Chung, 2007). In the literature, *TG* C422T polymorphism was considered as an effective marker for marbling scores (Barendse et al., 2001; Grisart et al., 2002; Burrell et al., 2004; Shin and Chung, 2007), *longissimus* muscle area (Casas et al., 2005), intramuscular fat content in *M. longissimus thoracis et lumborum* (Thaller et al., 2003) and back fat thick-

ness (Casas et al., 2005) in various cattle populations. We report herein the results of a study in which we evaluated how appropriately *TG* C422T polymorphism influenced meat colour parameters (linear and quadratic terms). Meat samples derived from animals with genotype CC had significantly higher L* values, but lower a* values at 24h post-mortem compared to those with heterozygous genotype ($P<0.05$). Visual appraisal of meat colour may also be affected by marbling and a large amount of intramuscular fat can increase lightness (Fiems et al., 2000). One possible explanation about the existence of the association between *TG* and meat colour parameters observed in the current study may be through intramuscular lipid deposition. The relatively small number of meat samples involved in the present study does not allow to draw final conclusions about the effects of *TG* C422T polymorphism on meat colour. However, it would be particularly interesting to evaluate the novel associations at the corresponding locus in future studies.

MYF5, which is included in the *MYOD* gene family, was considered as a candidate gene for meat production traits due to its potential roles in proliferation through muscle fiber development (Bhuiyan et al., 2009). Results of the gene-specific SNP marker association analysis for the *MYF5* reported by Li et al. (2004) and Bhuiyan et al. (2009) indicated that, g.1911A>G SNP genotypes of *MYF5* gene had significant effect on average daily gain in Canadian beef cattle and Korean cattle (Hanwoo) respectively. Moreover, similar results were observed in the studies performed by Chung and Kim (2005) and Zhang et al. (2007). To the best of our knowledge, there is no information about the association of *MYF5* g.1911A>G polymorphism with meat colour parameters. Our results revealed that the genotype '22' was significantly associated with redder meat (higher values of a*) and seemed to be favourable ($P<0.05$). Nevertheless, no significant difference was obtained for the quadratic and cubic nonlinear terms. *MYF5* was mapped to BTA5 including considerable overlap of QTLs regulating not only growth traits but also meat quality (Casas et al., 2000). Moreover, muscle fibre characteristics may influence meat colour characteristics, although a few reports exist in cattle (Vestergaard et al., 2000). In this respect, the results of the current study may provide an alternative aspect for future studies.

DGAT1 gene, mapped to BTA14, is functional and positional candidate gene for traits related to fat synthesis in both dairy and beef cattle (Curi et al., 2011). However, there was no association between the *DGAT1* K232A polymorphism and any of the phenotypic traits evaluated in the current study. In addition, there was no association of the tested SNPs with meat pH at 24h post-mortem, WBSF values and cooking loss ($P>0.05$). Meat quality traits such as tenderness and cooking loss are regulated by many genes and affected by interactions among them, and thus, a candidate gene associated with a trait in one population may have a different effect, or show no effect at all, in another population due to negative effects of other genes and epistatic interactions of the candidate gene with other genes in the population (Ge et al., 2003). Hence, inconsistent results about the associations between the same genetic markers and the mentioned traits can be evaluated as a common circumstance. On the other hand, indirect selection of the mentioned loci from the selection for milk production should be considered for Holstein breed.

CONCLUDING REMARKS

This study focused on the associations of markers in the *IGF1*, *TG*, *DGAT1* and *MYF5* genes with meat colour, tenderness and cooking loss. Novel associations between the *IGF1*, *TG* and *MYF5* genotypes and meat colour parameters traits were observed. The present results could therefore be indicative for future studies on meat quality, especially colour evaluation, in livestock.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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Research article
Ερευνητικό άρθρο

Comparative examination of the serological response to bluetongue virus in diseased ruminants by competitive and double recognition enzime-linked immunosorbent assays

A. Gavrilović[1*], P. Gavrilović[2], S. Radojičić[3], D. Krnjaić[4]

[1] Veterinary Clinic "Pančevo", Pančevo, Serbia
[2] Veterinary Specialised Institute "Pančevo", Department of Serology, Pančevo, Serbia
[3] Faculty of Veterinary Medicine, Belgrade University, Department of Infectious Animals Diseases and Diseases of Bees, Belgrade, Serbia
[4] Faculty of Veterinary Medicine, Belgrade University, Department of Microbiology and Immunology, 11000 Belgrade, Serbia

ABSTRACT. Bluetongue (BT) is a viral non-contagious disease of ruminants which is transmitted by insects of the genus *Culicoides*. In recent years, BT has been a serious threat to livestock and to the economies of European countries. In Serbia the disease appeared for the first time in 2001, and after a 12 year period of freedom, it broke out again in 2014. Considering the actuality of this infectious disease, especially the need for prompt and rapid diagnostics, the aim of this paper was to determine the possibility of detecting the serological response in sheep and cattle with manifested clinical signs of the disease using two different methods: double recognition enzyme-linked immunosorbent assay (sELISA) and competitive enzyme-linked immunosorbent assay (cELISA). A total of 105 blood serum samples of cattle and sheep, which had exhibited clinical signs of BT during 2014, were taken for examination from a serum bank. Out of 74 blood serum samples of sheep and 31 blood serum samples of cattle, 52 samples of sheep and 18 samples of cattle tested positive using sELISA, while 50 samples of sheep and 18 samples of cattle gave positive reactions with cELISA. The results confirm the high sensitivity of sELISA which detected 4% more seropositive sheep in comparison with cELISA. Using Cohen's *kappa* statistical analysis, almost perfect agreement was determined between the results (k>0,81) obtained by cELISA and sELISA.

Keywords: bluetongue, cELISA, sELISA, ruminants, Serbia

Corresponding Author:
E-mail: aleksandra.gav@yahoo.com,
T: +381641640723,
Veterinary Clinic "Pančevo",
Stevana Šupljikca 151/24, 26000 Pančevo, Serbia

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INTRODUCTION

Bluetongue (BT) is a viral non-contagious disease of ruminants which is transmitted by *Culicoides* biting midges. The agent belongs to the genus *Orbivirus*, family *Reoviridae*. All ruminants are susceptible to the infection, but clinical signs are most often manifested in sheep and white-tailed deer (*Odocoileus virginianus*) (Johnson et al., 2006; Sprelova and Zendulkova, 2011). The disease is manifested as an acute, chronic or subclinical condition. After an incubation period of four to eight days, clinical signs in the form of fever, apathy, tachypnea, and hyperaemia of the lips and nostrils, with excessive salivation and serous nasal discharge, appear in infected sheep. The clinical manifestation of the disease is influenced by the strain of the virus (Sprelova and Zendulkova, 2011). So far, 27 bluetongue virus (BTV) serotypes have been identified world-wide (van Rijn et al., 2016).

The economic losses may be direct such as death, abortions, weight loss or reduced milk yield and meat production inefficiency, and indirect as a result of export restrictions for live animals, semen and animal products. The annual world-wide losses due to BT have been estimated at 3 billion US$ (Tabachnick, 1996). In recent years BT has constituted a serious threat to the livestock and agricultural economies of European countries. In Serbia the disease appeared for the first time in 2001 (Đuričić et al., 2004), and after a 12 year period of freedom, it broke out again in 2014, when the virus was spreading rapidly throughout the countries of the Balkan Peninsula (Ostojić et al., 2014).

Serological tests for the detection of specific antibodies to the agent are very important diagnostic methods. The current OIE manual (OIE, 2014) describes the complement fixation test (CFT), agar gel imunodiffusion (AGID), competitive ELISA (cELISA) and indirect ELISA (iELISA). The bluetongue competitive or blocking ELISA was developed to detect specific antibodies against BTV without detecting cross-reacting antibodies to other orbiviruses. The specificity is the result of using serogroup-reactive monoclonal antibodies which bind to the amino-terminal region of the major core protein VP7 (Lunt et al., 1988; Afshar et al., 1989).

Considering the actuality of this infectious disease, especially the need for prompt and rapid diagnostics, as well as the lack of data in the current OIE Manual about the use of sELISA, the aim of the investigations was to compare the possibility of detecting the serological response in sheep and cattle with manifested clinical signs of BT, using two different enzyme-linked immunosorbent assays: the double recognition enzyme-linked immunosorbent assay (sELISA) and the competitive enzyme-linked immunosorbent assay (cELISA).

MATERIAL AND METHODS

Animals

The material for examination consisted of blood serum samples of sheep and cattle originating from South Banat, Serbia, the plains area that is bounded by the river Danube in the south. The numbers of sheep, goats and cattle is estimated at 45,000, 5,000 and 28,000, respectively. The investigation included 74 blood serum samples of sheep from 10 communities belonging to four municipalities and 31 blood serum samples of cattle from six communities within three municipalities. The samples originated from 19 sheep farms and 13 cattle farms.

Sampling

Samples of sheep and cattle, which had exhibited clinical signs of bluetongue during the 2014 epizootic were taken for the investigations from the serum bank of the Veterinary Specialised Institute "Pančevo". During that epizootic, the blood of ruminants with manifested clinical signs of BT was sampled for examinations in accordance with the government protocol which implied that, when the signs of the disease appeared in a community for the first time, blood samples of the diseased animals were examined for the presence of viral RNA by RT-PCR (reverse transcription polymerase chain reactions) and also serologically for the presence of antibodies against the agent by ELISA. In the latter cases suspected ruminants were tested only serologically for the presence of specific antibodies. After the examinations, the samples were saved in the serum bank.

Serological examination

The presence of antibodies against BTV was exam-

ined using the sELISA produced by *Ingenasa*, Spain and the cELISA produced by *VMRD*, USA. Both tests had been previously verified in the laboratory, based on repeatability, by testing positive and negative internal control samples in six replicates, and reproducibility, by examining the same replicates of the positive and negative controls under the same conditions at a seven-day interval. The coefficients of variation (CVs) for both tests were <10%. Since the samples originated from animals with manifested clinical signs and from communities in which the first suspicious cases were diagnosed by the detection of viral RNA by RT-PCR, they were interpreted as positive if they gave a positive reaction in either of the ELISA methods.

Double recognition enzyme-linked immunosorbent assay

According to the instructions of the manufacturer, the sELISA kit has been designed to detect antibodies against BTV in sheep, goats and cattle. The sensitivity and specificity of the assay are 100% and 99.8%, respectively. Microtiter plates are coated with VP7 protein of BTV. After adding a sample to the well, if it contains BTV specific antibodies, they will bind to the antigen. When VP7 protein conjugated with peroxidase is added, they will catch the labeled VP7. In such a way antibodies are caught between two antigens (double recognition). Presence or absence of labeled VP7 will be detected by the addition of a substrate which, in the presence of the peroxidase, will develop a colorimetric reaction.

Competitive enzyme-linked immunosorbent assay

The cELISA has been designed to detect BTV antibodies in ruminant sera. The sensitivity and specificity of the assay are 100% and 99%, respectively. If present in samples, antibodies inhibit the binding of horseradish peroxidase-labeled bluetongue virus-specific monoclonal antibody to BT viral antigen coated on the plastic wells. Binding of the horseradish peroxidase-labeled monoclonal antibody conjugate is detected by the addition of a substrate and quantified by subsequent colour product development.

Statistical analysis

The agreement between the two ELISA tests was evaluated using Cohen's *kappa* statistical analysis. The calculation of the k (*kappa*) value is based on the difference between how much the agreement is actually present ("observed" agreement) compared to how much the agreement would be expected to be present by chance alone ("expected" agreement). The common interpretations of kappa are as follows: < 0 Less than a chance agreement; 0.01–0.20 Slight agreement; 0.21–0.40 Fair agreement; 0.41–0.60 Moderate agreement; 0.61–0.80 Substantial agreement; >0.80 Almost perfect agreement.

RESULTS

Out of 74 blood serum samples of sheep, 50 samples tested positive by cELISA, while 52 samples gave a positive reaction in sELISA. Out of 31 blood serum samples of cattle 18 samples tested positive in both ELISA tests (Table 1). It was determined, by applying the *kappa* statistical analysis, that there was almost perfect agreement (k>0,81) between the cELISA and sELISA for both classes of sample. The *kappa* value for blood serum samples of sheep is 0.93, whereas for blood serum samples of cattle the *kappa* value is 1.00

Table 1. Results of the examination for the presence of antibodies against bluetongue virus in diseased sheep and cattle using cELISA and sELISA

Number of samples	cELISA		sELISA	
	Number of seropositive samples	Percent of seropositive samples	Number of seropositive samples	Percent of seropositive samples
Sheep				
74	50	67.57%	52	70.27%
Cattle				
31	18	58.10%	18	58.10%

(Table 2).

Seropositive sheep were discovered in 15 out of 19 tested farms while seropositive cattle were discovered in 11 out of 13 tested farms. All of the clinically suspected animals were confirmed serologically in 10 sheep farms and in 7 cattle farms.

DISCUSSION

While the cELISA is described as a specific method for the serological diagnosis of bluetongue, the possibility of using sELISA is not cited in the current edition of the OIE Manual (OIE, 2014). This was one of the reasons for comparing cELISA and sELISA tests in our investigations. The *kappa* statistical analysis excludes the possibility of the high percentage agreement between the tests obtained in the present investigation being present by chance.

Several studies confirmed a high sensitivity of sELISA in relation to the cELISA tests of different manufacturers (Oura et al., 2009; Eschbaumer et al., 2011; Niedbalski, 2011). Comparing the values relevant for the assessment of the reaction, obtained for two samples that gave different reactions in applied ELISA methods (Table 3), it can be seen that they are close to the cut off values in both tests indicating their different sensitivity. In addition, it was taken into account for the interpretation of the results that the samples originated from animals that had manifested signs of BT and were from settlements in which the agent had been proven to be present by the RT-PCR method.

Afshar et al. (1987) determined, using different serological methods, that cELISA was superior to iELISA in the detection of anti-BTV antibodies in the sera and whole blood samples from both cattle and sheep early after infection with BTV. Similar to our results obtained in naturally infected ruminants, Oura et al. (2009) reported that sELISA tests of different manufacturers were more sensitive in detecting

Table 2. Examination of the agreement between the results obtained by cELISA and sELISA using *kappa* statistical analysis

	Sheep, total of 74 samples			**Cattle, total of 31 samples**		
	sELISA			**sELISA**		
cELISA	positive	negative	total	positive	negative	total
Positive	a = 50	b = 0	$m_1 = 50$	a = 18	b = 0	$m_1 = 18$
Negative	c = 2	d = 22	$m_0 = 24$	c = 0	d = 13	$m_0 = 13$
Total	$n_1 = 52$	$n_0 = 22$	n = 74	$n_1 = 18$	$n_0 = 13$	n = 31
***Kappa* statistical analysis**	$pe = [(n_1/n)x(m_1/n)] + [(n_0/n)x(m_0/n)]$ pe = 0.572 po = (a + d)/n = 0.97 k = (po-pe)/(1-pe) = 0.93			$pe = [(n_1/n)x(m_1/n)] + [(n_0/n)x(m_0/n)]$ pe = 0.33 po = (a + d)/n = 1 k = (po-pe)/(1-pe) = 1		

po = the observed agreement; pe = the expected agreement; k = *kappa* value

Table 3. Comparison of the results obtained by cELISA and sELISA for the samples that gave different reactions

No	**cELISA**				**sELISA**			
	OD value	**S/N %**	**Cut off** (S/N%)	**Result**	**OD value**	**PP%**	**Cut off** (PP%)	**Result**
1	0.39	58.22	positive	-	0.49	25.80	positive	+
2	0.41	57.69	< 50%	-	0.37	21.30	>15%	+

OD value = optical density; S/N% = the OD value of a sample in relation to the OD value of the negative control; PP% = the OD value of a sample in relation to the OD value of the positive control

antibodies in vaccinated sheep than cELISA methods. Niedbalski et al. (2011) investigated the performances of commercial ELISAs in cattle vaccinated or infected with BTV serotype 8. The authors found that the relative sensitivity for cELISA, *VMRD* and sELISA, *Ingenasa* in vaccinated cattle amounted 69.5 and 98.3%, respectively, while the relative sensitivity for infected cattle with BTV serotype 8 was 98.6% for *VMRD* and 100% for *Ingenasa*. Similarly, in naturally infected ruminants with serotype 4 in our investigations, the difference in relative sensitivity between tests was small and amounted to 4% for sheep and 0% for cattle. The present investigations coupled with the results of Niedbalski et al. (2011) lead to conclusion that neither the different BTV serotypes (the serotypes 4 and 8) nor the species of ruminants naturally infected influence on the relative sensitivity of sELISA (*Ingenasa*).

The relatively small percentage of serologically confirmed clinical cases in the present investigations can be explained by the possibility that some herds of sheep were infected with contagious ecthyma virus, which had previously been diagnosed in the area of investigation. That is the most likely reason for negative reactions for all of the tested samples in four sheep farms. For some negative results, the reason could be found in the fact that the veterinarians, beside the samples of the animals that exhibited clinical signs typical of BT, also took a number of samples from animals which exhibited nonspecific signs that were not caused by BTV.

Unlike the epizootic of 2001, during which clinical signs had not been recorded in cattle (Debeljak et al., 2003), the results of this study show that a large percentage of infected cattle exhibited clinical signs during the epizootic of 2014. Comparing the population numbers with the number of serologically confirmed clinical cases of the disease in cattle and sheep in the area of investigation, no differences in susceptibility between the ruminant species examined could be seen. The fact that these two epizootics were caused by different serotypes of the agent, the former by BTV serotype 9 and the latter by BTV serotype 4, could provide an explanation for differences in the susceptibility of cattle to bluetongue.

CONCLUDING REMARKS

Using Cohen's *kappa* statistical analysis, almost perfect agreement ($k>0.81$) was determined between the results obtained by cELISA and sELISA in detecting the serological response in ruminants with manifested clinical signs of BTV. The high percentage agreement between the results obtained by the two different ELISA methods shows that either of them can reliably be used for the detection of antibodies against BTV in blood sera of naturally infected sheep and cattle.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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Research article
Ερευνητικό άρθρο

L-lysine determination in animal feed using microbiological microtiter plate assay

S. Jakšić[1], I. Jajić[2], S. Krstović[2*], Ž. Mihaljev[1]

[1] *Scientific Veterinary Institute, Rumenački put 20, 21000 Novi Sad, Serbia*

[2] *University of Novi Sad, Faculty of Agriculture, Trg D. Obradovića 8, 21000 Novi Sad, Serbia*

ABSTRACT. Chromatographic methods are most commonly used for the analysis of amino acids; however, there is growing need for faster, simpler and more price-effective assays. In this paper, the applicability of a rapid microbiological assay for quantification of the total content of L-lysine in feed samples was evaluated. The assay relies on the dependency of bacterial growth of *Pediococcus acidilactici* on the presence of L-lysine. Microbiological microtiter plate assay method for the quantitative determination of total (added and natural) L-lysine in feed samples has been verified, and parameters such as accuracy, precision, limit of detection, and limit of determination were evaluated. Results of total L-lysine determination in different feed samples have been compared with results of validated HPLC method. The microbiological microtiter plate assay method can be employed as a qualitative and quantification method for total L-lysine determination with detection and determination limit of 0.040 % and 0.085 %, respectively. However, further research on the influence of sample matrix on the determination of low lysine levels is required.

Keywords: L-lysine, determination, feed, microbiological assay, HPLC

Corresponding Author:
Saša Krstović,
University of Novi Sad, Faculty of Agriculture, Trg D. Obradovića 8,
21000 Novi Sad, Serbia;
e-mail: sasa.krstovic@stocarstvo.edu.rs;

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INTRODUCTION

Amino acids play very important role in animal nutrition. In modern animal nutrition, pure crystalline amino acids are replaced with trade products that contain amino acid mixtures. With the development of premixes and formulations, methods for amino acid determination have to change to be enough specific and selective for complex matrixes (Fontaine and Eudaimon, 2000). Lysine, as essential amino acid is not only a building block for proteins, but also a substrate for non-peptide molecules in animal bodies. Lysine can also affect the metabolism of other nutrients such as Ca and cholesterol (Liao et al., 2015). Deficiency of dietary lysine will impair animal immunity and elevate animal susceptibility to infectious diseases. Lysine in nutrition of monogastric meat animals can significantly increase body muscle protein accretion (Liao et al., 2015).

A range of analytic methods for amino acid analysis are used: spectrophotometry (Hasani et al., 2007), a whole cell green fluorescent sensor (Chalova et al., 2008), capillary electrophoresis (Latorre et al., 2001, Latorre et al., 2002), potentiometric sensor array (Garcia-Villar et al., 2001), and cyclic voltammetry (Saurin et al., 1999). Still, various chromatographic methods are mostly applied. The initial step involves protein hydrolysis, which can be acidic (Cottingham and Smalidge, 1988, Fontaine and Eudaimon, 2000, Bartolomeo and Maisano, 2006, Khan and Faiz, 2008, Jajić et al., 2013, Culea et al., 2015) alkaline (Fountoulakis and Lahm, 1998, Culea et al., 2015), or enzymatic (Fountoulakis and Lahm, 1998, Culea et al., 2015), followed by different chromatographic separations like ion exchange (Fontaine and Eudaimon, 2000, Khan and Faiz, 2008), reverse phase liquid chromatography (Cottingham and Smalidge, 1988, Bartolomeo and Maisano, 2006, Jajić et al., 2013), and gas chromatography (Culea et al., 2015). In addition to these methods, diverse derivatization reactions of amino acids such as pre- or post-column with nynhidrine (Fontaine and Eudaimon, 2000, Khan and Faiz, 2008), ortho-phthalaldehyde (OPA) (Fontaine and Eudaimon, 2000, Bartolomeo and Maisano, 2006), 9-fluorenylmethyl chloroformate (FMOC) (Cottingham and Smalidge, 1988, Jajić et al., 2013), and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Fiechter and Mayer, 2014) are applied. Recently, methods using liquid chromatography–tandem mass spectrometry (LC–MS/MS) have been reported for amino acid analysis in physiological samples (Le et al., 2014). Many studies have been done to validate and compare different chromatographic methods, which were improved with development of instruments for chromatographic separations and spectrophotometric detections (Couch and Thomas, 1976, Cottingham and Smalidge, 1988, Fontaine and Eudaimon, 2000, Bartolomeo and Maisano, 2006).

Although chromatographic methods were most commonly used for separation and determination of a mixture of amino acids, there is a need for development of faster and cheaper methods. To that end, new enzymatic methods were designed for the selective assay of L-lysine in biological samples utilizing oxidase reaction and decarboxylation reaction by the L-lysine/specific decarboxylase/oxidase from *Burkholderia* sp. AIU 395 (Sugawara et al., 2015). Besides enzymatic techniques, novel microbiology methods for L-lysine determination were developed. Analytical microbiology technique relies on the fact that test microorganism and the medium used as reagents give the sensitivity and the specificity due to the metabolic process involved. Microbiological methods have a long history of development. First of all, selection of a suitable microbial culture, on which amino acids have impact, is of crucial importance, but avoidance of side effects and adverse reactions are equally significant. The development of methods for microbiological assay is comparative to the development of any analytical chemical procedure, test organism and the medium are used as reagents and the reaction should bring sensitivity, precision and selectivity of the analyte measurement (Loy and Wright, 1959). Microbiological methods involving different microorganisms and application of tube methods are described (Horn et al., 1946, Horn et al., 1947, Loy and Wright, 1959). The similar principle can be used in the form of microtiter plate assay, but instead of tube and titration, microtiter wells and spectrophotometric determination are used in form of commercial test kit (ifp, VitaFast® L-Lysin).

In this paper, we investigated possibility of use commercial set kit (VitaFast® L-Lysin) for total L-lysine determination in different feed samples. Also, we compared results obtained by using this

microbiological method with those obtained with HPLC method. To the best of our knowledge this is the only available test kit based on the microbiological method, and there are no studies about its comparison with the standard method for L-lysine determination in feed samples.

MATERIALS AND METHODS

Samples

For this study, different feed samples were used. Microbiological microtiter plate assay (MMPA) was applied for the determination of total L-lysine in maize, wheat, soy grits, wheat, and soybean meal, as well as in complete mixtures for laying hens, ducks and in dog and cat food.

First group of samples are presented in Table 1. Maize, soybean, and soybean meal were analyzed for natural L-lysine content. Then, a mixture of maize, soybean, and soybean meal samples was made in the ratio of 70:15:15 (w/w/w). The mixture was divided into four parts, and one part, marked "zero", was used as such, while the other three parts were supplemented with L-lysine in different concentrations. Thus, the mixture "one" contained 0.1%, "two" 0.2%, and "five" 0.5% of the added L-lysine.

The samples used for inter-laboratory studies, were also analyzed. Since robust average values for these samples were available based on analysis by a large number of laboratories, they were considered reference materials (RMs). The samples included: a total mixture for laying hens, dog food, wheat, two total mixtures for ducks, cat food, and soybean grits. Samples were purchased from National Reference Laboratory of Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic).

Microbiological microtiter plate assay

L-lysine microtiter plate assay is microbiological method for the quantitative determination of total L-lysine content (added and natural) in animal feed (VitaFast®, Art. No. P1012, R-Biopharm, Germany). Sample was treated by hot acid extraction with 2 M HCl in an autoclave (Colussi S.r.l. L 40E, Italy) 1 h at 121 ºC. After extraction of L-lysine from the sample, filtration and dilution of extract was done in sterile working conditions and by using sterile consumables.

The L-lysine assay was performed according to manufacturer's instruction. The preparation of samples for MMPA analysis was performed according to guidelines provided by the manufacturer of test kit for determination of total L-lysine (natural and added). After extraction of L-lysine from the sample and before assay implementation step, samples with expected L-lysine content higher than 0.40% were diluted 10 times in sterile conditions. Calibration curve for total L-lysine determination with MMPA was constructed in the working range of 0.020–0.400 g/100 g, by 4-parameter evaluation. Calibration curve was described by the following equation (Herman et al., 2008):

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^{b}}$$

where x = mass concentration, y = turbidity, a = 0.073 (the minimum value that can be obtained, *i.e.*, value at 0 dose;); b = 1.127 (Hill's slope of the curve *i.e.*, b is related to the steepness of the curve at point c); c = 96501 (the point of inflection *i.e.* the point on the S shaped curve halfway between a and d), and d = 757,408 (the maximum value that can be obtained *i.e.*, value at infinite dose), with coefficient of correlation r = 0.9944.

The medium and diluted extracts are pipetted into the wells of a microtiter plate coated with *Pediococcus acidilactici* bacteria. The growth of *P. acidilactici* is dependent on the supply of L-lysine. Following the addition of L-lysine either as a standard or a compound of the sample, the bacteria grow until the L-lysine is consumed. The incubation is done in the dark at 37 °C for 44–48 h. The intensity of metabolism of growth of *P. acidilactici* in relation to the extracted L-lysine is measured as turbidity and compared with a standard curve (VitaFast®, ifp, Germany). The measurement is done using a microtiter plate reader at 620 nm (Multiscan MCC/340, Labsystem, Finland). Special software, the Rida®Soft Win (Art. No. Z9999, R-Biopharm, Germany), was used for the evaluation of the results.

HPLC method

The sample preparation for HPLC determination and the chromatographic conditions were in accord-

ance with Jajić et al. (2013). The samples were finely ground to pass through a 0.5 mm sieve. A sample weight, equivalent to 10 mg nitrogen content, was hydrolyzed using 6 M HCl (Lach-Ner, Neratovice, Czech Republic) containing 0.1 % (p.a. grade, Sigma-Aldrich, St. Louis, MO) of phenol for 6 h at 150 °C in vacuum. After the hydrolysis, the samples were cooled to room temperature and evaporated to dryness at 70 °C under a stream of nitrogen. The residues were quantitatively transferred into 50 cm3 volumetric flasks using 0.1 M HCl. The solutions were filtered through quantitative filter paper into glass tubes. The hydrolyzed samples and standard amino acid mixture solutions were automatically derivatized with OPA and FMOC (Agilent Technologies, Waldbronn, Germany) by programming the autosampler. The analysis was performed on an Agilent 1260 Infinity Liquid Chromatography system equipped with a μ-Degasser (G1379B), 1260 binary pump (G1312B), 1260 standard autosampler (G1329B), 1260 thermostated column compartment (G1316A), 1260 diode array and multiple wavelength detector (G1315C). After derivatization, 0.5 μl of each sample was injected into a Zorbax Eclipse-AAA column (150 × 4.6 mm, i.d., particle size 5 μm), at 40 °C, and detected on a DAD detector at 338 nm and 262 nm. The mobile phase A consisted of 5.678 g of Na_2HPO_4 per 1 L water, adjusted to the pH 7.8 with a 6 M HCl solution. The mobile phase B was acetonitrile–methanol–water (45:45:10, v/v). The separation was performed at a flow rate of 2 ml/min employing a solvent gradient. The calibration curve was constructed using five standard solutions containing 10, 25, 100, 250, and 1000 μM of each amino acid (Agilent Technologies, Waldbronn, Germany). The data of peak area vs. amino acid concentration were treated by linear least squares regression analysis.

Statistical Analysis

Statistically significant difference between the content obtained by the MMPA and the HPLC method, as well as the expected total L-lysine content in samples supplemented with L-lysine in different concentrations was analyzed using *t* test. The differences between the total L-lysine content obtained by the MMPA and the HPLC method in RMs, as well as difference between MMPA and reference value were analyzed using paired *t* test as well as linear regression analysis (PAST, Version 2.12, Oslo, Norway).

RESULTS AND DISCUSSION

The results of determination of total L-lysine in the first group of samples prepared by mixing of feeds and supplementation of L-lysine are presented in Table 1.

As it can be seen, in samples 1, 2, 4, and 7 there is no significant difference between MMPA and HPLC methods, as well as between the tested MMPA method and reference value at the 0.05 level for samples 1–4, and 7. For samples 3 and 5, there is no significant difference between tested and HPLC method at the 0.01 level. The *t* test revealed significant difference between the two methods, as well as between MMPA determined and declared content for total L-lysine only for maize sample No. 6 ($p<0.001$). Because of the low expected concentration of L-lysine in maize, which would be outside the range of the calibration curve after extract dilution, the extract was not diluted during preparation. Thus, potential interference of sample matrix with assay interpretation should be taken into consideration. Determination of total L-lysine in this sample has been repeated six times. The obtained high value of relative standard deviation (RSD) being 49% as compared to required 5% (Reason, 2003), points to the inaccuracy and imprecision in the determination of total L-lysine in this sample.

To the purpose of determining of intermediate precision, the same sample was extracted and assay was implemented in three different days (Table 2). The RSD values were 2.2% and 3.9% for samples with total L-lysine contents of some 1% and 3%, respectively. Since it was below 10%, which is maximum recommended value by both assay producer and international requirements (Reason, 2003), it can be considered acceptable. RSD is also comparable with values for coefficient of variation given in EC No152/2009 (2.1–2.8%) (European Commission, 2009), as well as the precision of the HPLC method (3.24%) (Jajić et al., 2013). Method specificity and selectivity is achieved by selecting suitable bacteria (*Pediococcus acidilactici*) which growth is conditioned by the presence of lysine (ifp, VitaFast® L-Lysin).

Table 1. Results of total L-lysine determination in different samples using two analytical methods

Sample No.	Sample type	Total L-lysine content, %		
		MMPA*	HPLC†	Reference value‡
1	Mixture „zero”	1.0	0.992	1.045
	p§		0.258	0.128
2	Mixture „one”	1.1	1.110	1.145
	p§		0.295	0.374
3	Mixture „two”	1.2	1.341	1.245
	p§		0.030	0.295
4	Mixture „five”	1.7	1.616	1.545
	p§		0.158	0.073
5	Soybean	2.8	2.462	1.81–2.59 (2.21)
	P§		0.046	0.027
6	Maize	0.03	0.304	0.14–0.37 (0.24)
	p§		<0.001	<0.001
7	Soybean meal	2.9	2.894	2.32–3.05 (2.73)
	p§		0.935	0.122

* samples 1, and 7, $n = 3$; samples 2–5, $n = 2$; sample 6, $n = 6$; † $n = 5$; ‡ For sample No. 1 reference value is obtained on the basis of HPLC analysis of each component (samples No 5–7), for samples No. 2–4, reference value was calculated from added L-lysine, and for samples No. 5–7 as reference L-lysine value is considered literature average value in parenthesis (Redshaw et al., 2010); § *t* test value (*p* values) comparison of data obtained using MMPA and HPLC, *i.e.*, reference value.

Table 2. Intermediate precision of total L-lysine determination by microbiological microtiter plate assay

	Total L-lysine content, %	
	Sample 1	Sample 7
Day 1	0.99	2.8
Day 2	1.0	2.8
Day 3	1.0	3.0
Average (%)	1.0	2.9
SD (%)	0.02	0.11
RSD (%)	2.2	3.9

MMPA has been applied also for the determination of total L-lysine in reference samples originating from international proficiency testing programs (Table 3). It is obvious that L-lysine content obtained in sample No. 10 is significantly lower as compared with the value obtained by HPLC method as well as the reference values. In this sample, the expected total L-lysine level was within the range of calibration curve, thus, sample extract was not additionally diluted. This also supported the likely matrix interference with assay results, so it can be concluded that the MMPA method is not suitable for every matrix. The analysis of paired *t* test revealed significant differences neither between the results for total L-lysine content in RMs obtained using MMPA and HPLC methods ($p = 0.396$), nor between contents determined by MMPA and reference values ($p = 0.274$), at the level 0.05.

The accuracy of the method was analyzed based on the results of determinations of L-lysine in all samples except in samples No. 6 and No. 10 (Table 4). As it can be seen, the obtained recovery (109.5%) was somewhat higher than indicated by assay producer (90–105%) but it was comparable to recovery recommended by international requirements (90–110%) (Reason, 2003) as well as these obtained using HPLC method (109.4%) (Jajić et al., 2013). However, investigation of correlation between total L-lysine content in all samples determined using two methods revealed high linear correlation coefficient ($r = 0.9942$; $p < 0.001$). Also, high correlation coefficient was recorded for total L-lysine content obtained by MMPA method and reference value ($r = 0.9905$; $p < 0.001$).

Table 3. Results of total L-lysine determination in different RMs using two analytical methods

Sample No.	Sample type	Total L-Lysine content, %		
		MMPA	HPLC	Reference value*
8	Total mixture for laying hens	0.80	0.882	0.745
9	Dog food	1.0	1.057	0.895
10	Wheat	0.05	0.307	0.311
11	Total mixture for ducks 1	0.9	0.918	0.904
12	Total mixture for ducks 2	1.0	1.157	0.904
13	Cat food	1.1	1.360	1.012
14	Soybean grits	3.9	3.614	3.088

* National Reference Laboratory of Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic)

Table 4. Recovery of total L-lysine by MMPA

Sample No.	Recovery, %	Bias, %
1	96.7	3.35
2	98.7	1.31
3	98.8	1.20
4	108.1	8.09
5	126.7	26.70
7	106.2	6.23
8	107.6	7.65
9	114.0	13.97
11	105.1	5.09
12	112.6	12.65
13	113.9	13.94
14	125.6	25.65
Average	109.5±9.7	

Limit of detection of MMPA method was calculated from average value ($n = 3$) of turbidity of standard solution mass concentration 0.020% and threefold standard deviation for turbidity being 0.040%. Based on the sum of average turbidity values for this standard solution and tenfold value of standard deviation for turbidity, the limit of quantification (LOQ) was calculated, being 0.085% (Thompson et al., 2002). However, analysis of real samples without additional dilution does not allow determination of lysine content, which is due to matrix interference. Recalculation of LOQ based on additional dilution of the sample reveals the LOQ 0.85%. Contrary to MMPA, the HPLC method yielded lower values for LOD and LOQ. Determination of LOD and LOQ of HPLC method for lysine quantification was also performed according to standard solution analysis (Jajić et al., 2013). LOQ of the HPLC method for lysine determination based on the standard deviation of the response and the slope of the linearity plot, calculated as 10α/b, where α is the standard deviation of the *y*-intercept and *b* is the slope of the calibration curve being 0.106% (Jajić et al., 2013).

According to test manufacturer, indicated standard range of the method is 0.02–0.40% (ifp, VitaFast® L-Lysin) however, the results revealed that this method can be used for total L-lysine quantitative determinations in samples of feed and total mixtures for animal feed with content of total L-lysine ≥ 0.085% after 10-fold dilution, meaning that the content should be ≥ 0.85%. Another shortcoming of this method is test kit format, because kit for 96 determinations must be used in three times. It means that 32 wells should be used at once. Further, in contrast to HPLC method which can be developed for the determination of several amino acids at a time, MMPA is designed for determination of single amino acid.

CONCLUSIONS

The applied MMPA method proved to be simple and fast for the determination of total L-lysine in animal feed samples. According to presented results, investigated MMPA gave results comparable to HPLC method. Even though some earlier reports suggested potential application of microbiological techniques for determination of amino acids, current scientific literature still does not provide any distinctly described method. This article offers for the first

time the results of the investigation and validation of commercial test relying upon this principle.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Monitoring research of somatic cells count in goat milk in the eastern region of Ukraine

T.I. Fotina[1], H.A. Fotina[1], V. I. Ladyka[1], L.M. Ladyka[1], N.M. Zazharska[2*]

[1]*Sumy National Agrarian University*

[2]*Dnipro State Agrarian and Economic University*

ABSTRACT. The aim of research was to analyze the contents of somatic cells (SC) in goat milk in the East of Ukraine, level of SC translocation in the process of milking, speed of SC evacuation in their secret, to conduct monitoring and ranging on the level of SC goat milk of herds in the Eastern region during 2015 seasonally. Somatic cell count was studied on a large number of animals in different zones of the East of Ukraine (1800 milk samples). Somatic cell count in goat milk of the Eastern region of Ukraine appears up to 800×10³ cells/ml at 65-71% of studied milk samples by the method of laser-running cytometry with the account of only those cells having DNA in the nucleus. While analyze of SC subpopulations in goat milk it is shown that at increase to 2 million cells/ml and more the concentration of neutrophils and lymphocytes does not change and the number of macrophages and eosinophils ($p \leq 0,05$) rises from 1,5 to 4 times accordingly. It is marked that SC contents in goat milk in the morning and evening yield of milk is different. In evening yield of milk, the somatic cell count is up to 30 % higher than in the morning one. A clear pattern of distribution of somatic cells in milk portions during milking was not detected. Average sample of yield is required to determine the somatic cell count in the milk of goats. Six of the eight goats over six months of lactation were given milk with a fairly constant somatic cell count. Low and fairly constant somatic cells content (15 to 63 × 103 cells / ml) is observed in the milk of primiparous goats.

Keywords: goat milk, somatic cell count, cell subpopulations, lactation, yield

Corresponding Author:
Nadiia Zazharska, Department of Parasitology,
Veterinary and Sanitary Expertise; Faculty of Veterinary Medicine,
Dnipro State Agrarian and Economic University,
Yefremov Str., 25, Dnipro, 49027, Ukraine
E-mail: zazharskayan@gmail.com

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INTRODUCTION

Nowadays a big question in the science and dairy industry remains the establishment of referent levels of somatic cells (SC) in goat milk, and estimation of top concentration limit which can lie in pricing and establishing of milk quality grade. It is known that somatic cell count in milk is the indicator of udder health and subclinical mastitis. Determination of somatic cells is a very important factor in the estimation of milk quality which is used besides other parameters such as chemical composition of milk (concentration of fat and protein) for determination of milk cost (Dankow et al., 2003; Shapovalov et al., 2015). In addition, somatic cell count can be an excellent index in the monitoring programs of mastitis. The increase of somatic cells is connected with the reduction of milk yield and changes in its composition.

An inflammatory process in the mammary gland of goats as a result of pathogenic microorganisms influence, toxins or tissue damage leads to the changes of milk secretion, its amount, composition and properties, increase of albumen contents in a whey, decline of milk components secretion (casein, fat, lactose), reduction of thermal stability of milk, increase of time for coagulation of milk, and its safety (Katic et al.,1994; Heeschen, 1995, 2000, Bernacka, 2006). On the basis of the above-mentioned it is possible to make a conclusion that the level increase of somatic cells in goat milk influences on its appropriateness for processing (Auldist and Hubble, 1998).

Goat milk on the average has a higher level of somatic cells (can be several millions in 1 ml) comparing with cow milk (Dankow et al., 2003). When correlative dependence between the level of SC and health of udder is clearly defined for cows the question of mastitis diagnostics by the level of SC in goat milk is debatable. So, in the United States (USA) the possible index of somatic cell count for cow milk is 750 $\times 10^3$ cells/ml, for goats and sheep - 1 million/ml. In the European Union (EU), (Directive 92/46 EEC, 1992) - the possible level of SC in cow milk is set at 400 $\times 10^3$ cells/ml, but also legislatively, the level of SC is not defined for goat and sheep milk. At present the procedure of milk quality control is regulated by international standards (By regulation of Commission (EU) № 1662/2006 - is regulation with alteration made in Regulation (EU) № 853 /2004 about hygienical rules for the food products of animal origin) in which a possible level limit of SC in goat milk also is not set. Reference levels of SC are shown only for a raw goat milk at a temperature of 30 °C, $\leq 1500\times10^3$ cells/ml, if it is intended for production of thermally processed products and $\leq 500\times10^3$ cells/ml if it is intended for making products without thermal processing. Determination of SC level is suggested to express as the average geometrical meaning received during two months. However, the last research of the Norwegian scientists at IGA Conference "Quality of Goat Milk" (2013) in Tromse showed that concentration of SC in milk of healthy goats must be at the level of 500×10^3 cells/ml (Solverod, 2013). The level of SC in milk set by the method of direct count in some herds of goats in Norway was within the limits of 549×10^3 cells/ml, and at the end of lactation 415×10^3 cells/ml, on other farms a maximal level of SC was: 943×10^3 cells/ml in 2009, 839×10^3 cells/ml in 2010, 842×10^3 cells/ml in 2011, 824×10^3 cells/ml in 2012 (method of direct count) (Solverod, 2013). Somatic cell count in goat milk of herds in Brazil in 2010 (state San Paulo) depending on the stage of lactation was: 159, 508 and 277×10^3 cells/ml (Madureira et al., 2010, Gomes et al., 2006).

Divergences in received values are connected first with methods of SC level determination and usage of direct microscopic methods of count or methods with painting DNA SC and estimation of their amount by the method of laser running cytometry from one side and also with the features of goat milk secretion. As it is generally known in the process of secretion for goats and sheep cytoplasmic, apocrine particles are evolved in milk, these structures have similar diameter and morphology of leucocytes (of the basic SC population) though they contain more albumen and RNA but not DNA (Andrade et al., 2001, Dulin et al., 1982). At microscopic count, these particles are mistakenly considered as SC of goat milk. Perhaps contradictory data are also explained by the fact that the row of works was done in the period when the method of laser-running cytometry at painting DNA with etidium bromide and with the following multiple detection was not used yet. It is also necessary to mark that technical requirements outlined in the project of standard of the Eurasian council of metrology standardization and certification, Minsk 2013 "MILK

is WHOLE DRINKABLE GOAT" are the following - contents of SC in goat milk must be no more than 750×10³ cells/ml and for the product intended for a preschool and school feed no more than 500×10³ cells/ml. In Ukraine, long-term research results of T. N. Ryzhkova concerning the study of goat milk quality (2000 – 2014) became the base of the national standard of Ukraine "Goat milk raw material" elaborated and put into operation since 01.01.2010. This standard shows that for the first grade it is 500, for the second 600, for the third 800×10³ cells/ml.

The aim of research was: to analyze the contents of SC in goat milk in the East of Ukraine (Sumy, Kharkov, Donetsk regions), to determine the level of SC translocation in the process of milking, to offer the optimal methods of sampling, to conduct monitoring and ranging on the level of SC goat milk of herds in the Eastern region seasonally.

MATERIALS AND METHODS

Experimental part of work included studies of lactating goats in the period of 2015. For experiment, clinically healthy goats were selected in accordance with the generally accepted methods of zootechnic research. The level of SC was studied on the greater number of animals (527 goats) in different zones of the East of Ukraine. 26 herds were involved in monitoring research. Herds of Saanen and local breed goats were from 8 to 30 animals. The goats were of 1 – 5 lactations, hand milking twice daily. In summer goats graze, while in the cold season they stay indoors. The goats have free access to feed and water. The data collection and sampling of milk was conducted in a few stages: the first two stages: since 13 till 20 of April and since 12 till 12 of June 2015 in the Sumy region, others during a year since 14 till 17 of every following month: February, April, July, October in the Sumy region: Sumy, Lebedyn, Krasnopillja, Nedryhailiv districts, Kharkov region, Donetsk region: Starobeshevsk district, Dnepropetrovsk region. Samples of milk from each experimental goat on a farm were filtered and cooled to the temperature of (6±2) ºC. Determination of SC (1800 milk samples) was conducted in the Trial center of stock-raising institute of National agrarian academy of science of Ukraine, which is accredited according to requirements of SSDU ISO/IEC 17025: 2006 (ISO/IEC 17025 : 2005, certificate of accreditation № 2T621 in the National accreditation agency of Ukraine). In the laboratory, the samples of milk were heated to 40±º0,9, homogenized and conducted measuring on Somacount 150 (USA) using principle of laser running cytometry of fluoro - optic electronic method in accordance with ISO 13366-2: 2007 (IDF 148-2: 2006). For contents determination of certain SC subpopulations in goat milk (eosinophils, neutrophils, monocytes, lymphocytes) was used the running cytometer with the method of immunophenotyping with specific monoclonal antibodies of CD 45 marked by fluorescent dye.

RESULTS

While ranging the results of goat milk quality monitoring in the Eastern region of Ukraine in 2015, almost the same distribution of somatic cells in milk of goats by seasons is marked (table. 1).

Somatic cell count in the goat milk was up to 800×10³ cells/ml at 65-71% of all studied animals by

Table 1: Ranging of somatic cell count in goat milk by seasons in 2015 in the Eastern region of Ukraine

Level of ranging of somatic cell count	Spring		Summer		Autumn		Winter	
	n	% from sampling	n	% from sampling	n	% from sampling	n	% from sampling
A: < 100×10³	75	14	66	17	88	20	77	17
B: 100×10³- 400×10³	121	23	60	16	86	19	75	17
C: 400×10³ - 600×10³	89	17	58	15	87	20	74	17
D: 600×10³ - 800×10³	77	15	64	17	54	12	69	16
E: 800×10³ - 10⁶	76	14	62	16	71	16	67	15
I: ≥ 2×10⁶	89	17	75	19	60	13	80	18

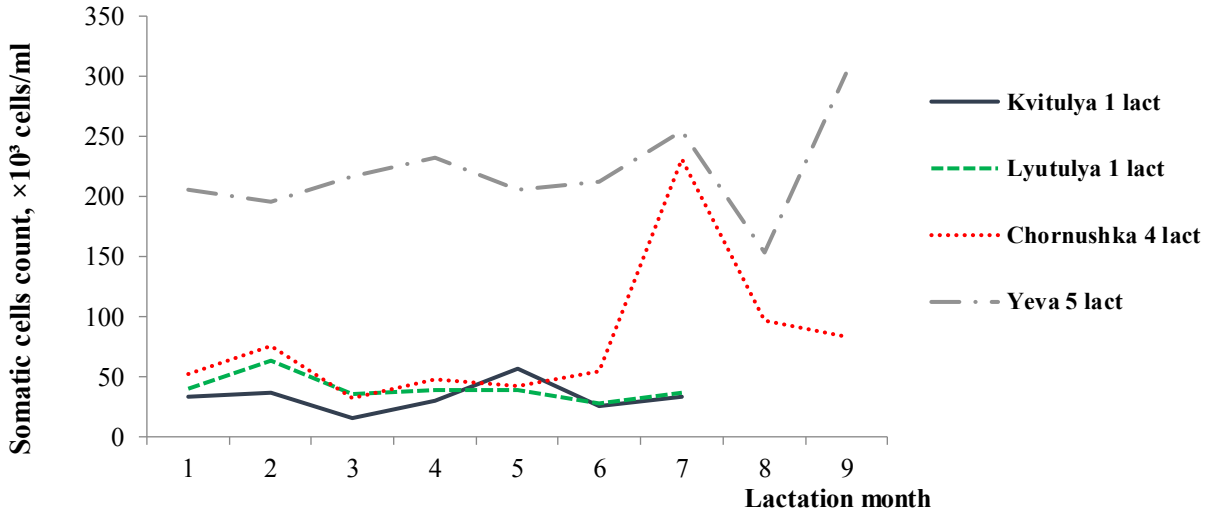

Fig 1: Somatic cell count in goat milk depending of lactation month

seasons, that is considerably lower than in researches of other authors. SC contents more than 2 million/ml was from 13 to 19% during a year, thus a minimum percent was in autumn time. This research can become pre-condition in the analysis of goat milk rating in the Eastern region of Ukraine.

At contents determination of certain SC subpopulations in goat milk: epithelial cells and eosinophil's, neutrophils, macrophages, lymphocytes, it was shown that level of epithelial "shelling cells" was in the range of 2,1-3,7% and did not have reliable differences in milk with the different level of summary SC contents. The count of other cells was taken later on for 100% and their mass stake was determined with the use of running cytometry method (table 2).

It is shown that lymphocytes level in goat milk remains permanent enough and does not have reliable distinctions even in case of SC increase in goat milk more than 1 million/ml. However, considerable level increase of eosinophil's (to 4 times) and macrophages (1, 5 times) has been marked. The level of neutrophils at general increase of SC goes down not for certain,

Table 2: Ranging of SC contents, contents of SC subpopulations in goat milk in 2015 conducted in the result of milk quality monitoring in the Eastern region of Ukraine

Level of ranging	n	Mass portion of sampling, %			
		Neutrophils	Macrophages	Lymphocytes	Eosinophils
A: < 100×10^3	30	87,80 ± 4,8	8,1 ± 3,1	2,10 ± 0,2	2,0 ± 0,7
B: 100×10^3- 400×10^3	12	84,50 ± 6,2	11,1 ± 5,6	2,00 ± 0,1	2,4 ± 0,5
C: 400×10^3 - 600×10^3	14	82,90 ± 5,3	11,8 ± 3,4	2,40 ± 0,4	2,9 ± 0,1
D: 600×10^3 - 800×10^3	14	81,10 ± 2,5	12,0 ± 2,1	2,40 ± 0,7	4,5 ± 0,1
E: 800×10^3 - 10^6	15	77,40 ± 9,7	14,2 ± 3,1	2,40 ± 0,7	6,0 ± 0,1
I: ≥ 2×10^6	17	73,40 ± 5,4	15,8 ± 2,7	2,70 ± 0,5	8,1 ± 0,2

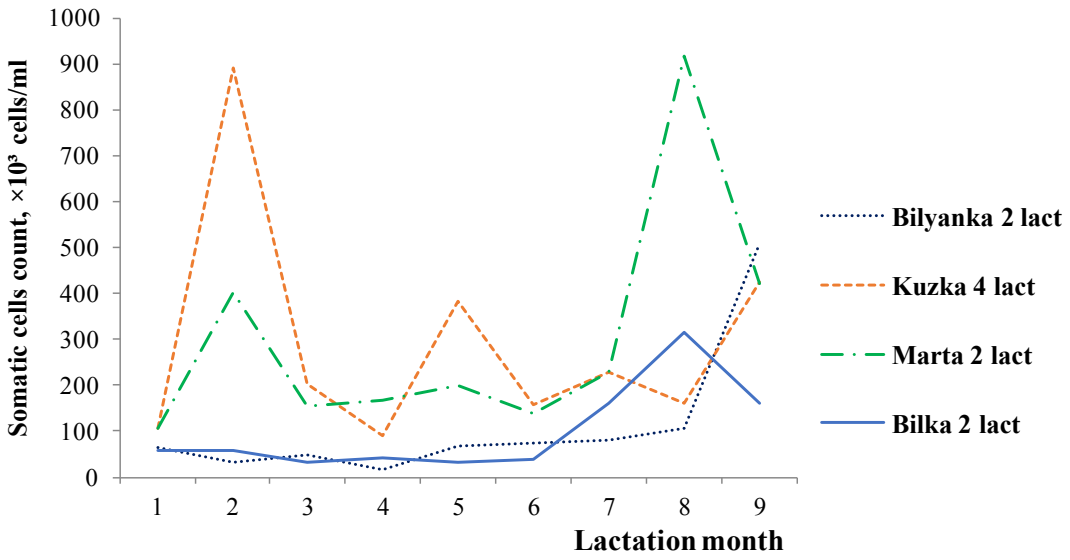

Fig 2: Somatic cell count in milk from other goats depending of lactation month

perhaps due to the increase of other subpopulations of lymphocytes.

It has been discovered that the range of SC contents in goat milk was from 11 thousand to 2 million when using method of laser-running cytometer (painting DNA of every SC) (table. 3). A difference

Table 3: Somatic cell count in goat milk in the morning and evening yield of milk at farm in Sumy region

Names of experiment animals	Spring (March) 2015				Summer (June) 2015			
	14.04 evening, ×10³ cells/ml	15.04 morning, ×10³ cells/ml	Δ evening / morning, ×10³ cells/ml	Δ evening / morning, %	13.06 evening, ×10³ cells/ml	14.06 morning, ×10³ cells/ml	Δ evening / morning, ×10³ cells/ml	Δ evening/ morning, %
Belka	442	198	244	55,2	703	282	421	59,9
Kvitka	1824	1053	771	42,3	716	297	419	58,5
Rosa	255	141	114	44,7	721	731	-10	-1,4
Chernushka	2070	1458	612	29,6	1416	1488	-72	-5,1
Dina	571	268	303	53,1	504	444	60	11,9
Anfisa	619	465	154	24,9	494	614	-120	-24,3
Ljuta	63	37	26	41,3	567	633	-66	-11,6
Aza	53	34	19	35,8	598	621	-23	-3,8
Marta	190	64	126	66,3	71	90	-19	-26,8
Berezka	170	71	99	58,2	62	80	-18	-29,0
Roza	175	116	59	33,7	52	47	5	9,6
Marta	177	115	62	35,0	45	27	18	40,0
Belka	526	471	55	10,5	839	744	95	11,3
Kvitka	362	376	-14	-3,9	1131	536	595	52,6
Romashka	11	12	-1	-9,1	1441	2177	-736	-51,1
Mean value	500,5	325,3	175,3	34,5	624,0	587,4	36,6	6,0

between the morning and evening yield of milk in individual samples was from 1 to 770 thousand. A middle difference of SC contents in goat milk between the morning and evening yield of milk was from 35 to 150 thousand cells.

Somatic cell count during lactation of eight goats is presented in two charts (fig.1-2). Lactation curves of animals milks which are characterized by a more or less constant and low somatic cell count are placed at Figure 1. Kvitulya, Lyutulya – primiparous goats, their first lactation lasted only 7 months. But milk differed of very low and fairly constant somatic cell content (from 15 to 63×10³ cells/ml). Figures 1, 2 shows that six of the eight goats were given milk with a fairly constant level of somatic cells over six months of lactation. On the contrary, milks of goat Kuzka (fourth lactation) and Marta (second lactation) are characterized of frequent changes of somatic cell count (fig. 2).

Somatic cell count at the last month of lactation in three goats increase, in the other three goats, on the contrary, – decrease compared to eighth month.

An important achievement is that goat milk of the first month of lactation (5-8 days after kid birth) contains a very low somatic cell count – from 33 to 107 ×10³ cells/ml. The exception is goat Eva milk which contains 206×10³ cells/ml. But milk from this animal is researched for several years, and her level of somatic cells is always 250-300×10³ cells/ml in the main months of lactation, which is her physiological norm.

DISCUSSION

According to Ukraine requirements it is permitted to 500 **×10**³ cells / ml for the highest quality goat milk. In Europe, the best goat milk is considered with somatic cells count ≤ 1 million / ml. Somatic cell count in the goat milk was up to 1 million cells/ml at 81-87% of all studied animals according to our results. We have to review Ukrainian requirements for somatic cells count of goat milk.

It is defined that in spring period of experiment on the herd of goats at farm in Sumy region general contents of SC in the evening milk was at the level of 500×10³ cells/ml and in the morning milk - 325×10³ cells/ml i.e. 34 % more (table. 3).

Table 4: Somatic cell count, ×10³ cells/ml in goat milk in different milking portions

	Milk portions			Ratio accumulation of SC in %		
	1*	2**	3***	Δ 2/1	Δ 2/3	Δ 3/1
Sabina	27	27	449	0,00	93,99	93,99
Ljuta	77	85	225	9,41	62,22	65,78
Regina	106	98	103	-8,16	4,85	-2,91
Marta	205	300	335	31,67	10,45	38,81
Dina	450	550	620	18,18	11,29	27,42
Kvitka	700	1252	1658	44,09	24,49	57,78
Kuzka	37	40	47	7,50	14,89	21,28
Marta	1033	642	1388	-60,90	53,75	25,58
Chornushka	39	43	45	9,30	4,44	13,33
Bilka	43	41	43	-4,88	4,65	0,00
Kvitulya	90	111	74	18,92	-50,00	-21,62
Lyutulya	45	47	46	4,26	-2,17	2,17
Bilyanka	47	50	50	6,00	0,00	6,00
Eva evening 19.12.13	346	391	600	11,51	34,83	42,33
Eva morning 20.12.13	334	374	448	10,70	83,48	25,45
Eva evening 28.01.14	431	448	351	3,79	-27,64	-22,79
Eva morning 29.01.14	650	700	675	7,14	-3,70	3,70

Notice: * cisternal, ** transit - alveolar, *** alveolar

Analogical researches were got by F. Cedden et al. in 2008 in Turkey, however in a summer period such regularity was practically at the level of tendency (on the average 6% higher) in count to 1000/ml, but at count to the total evening and morning yield of milk SC contamination was higher in the evening milk.

Such difference may be explained by influence of different factors: the factor of photoperiodism, greater motion activity in the interval of light day and correspondingly the speed of metabolism, the interval of time from milking to milking - the bigger interval the more yield of milk and value of milk components concentrations. Thus, it is shown that this index is very variable even for one animal when milking in different time of the day. Others study led for this same conclusion (Zazharska N. et al., 2015). 170 milk samples from eight goats were studied during more than year and a half. In each animal there was an increase of somatic cell count as the evening and morning. The dependence of the number of somatic cells occasionally yield time was not found. The index can change twice and more during the day. The somatic cell count in goats increases with age, late lactation and influenced by other factors, even without infectious agents (Maurer J. et al., 2013). Noted the big somatic cell count (> 2000 thousand / ml) at low bacterial contamination (19,6 × 10^3 CFU / mL) of goat milk (Zazharska N., 2016).

On the next stage, the level of SC at milk excretion was studied (table. 4). It is known that at milking the first streams differentiate on chemical composition: less high-fat than in the last streams. It is explained by complicated mechanisms and concerted reflex reactions of myoepiteliy and smooth musculature of udder, walls and sphincter of nipple. It is found that somatic cells count in different portions of milk within a milking change in healthy goats. It is shown that amount of SC in 65% samples in cisternal milk is considerably lower than in alveolar one (from 2 to 94%). In four samples, largest number of somatic cells is defined in the middle of milking and in two cases – observed in cisternal milk. Dependence SC content in the alveolar milk from primary level in cisternal one is also not defined. Perhaps stimulation of milk giving simultaneously causes the intensive excretion of SC by alveolar epithelium that leads to their rapid and considerable increase in the following portions of milk yield. It is also set by numerous studies that alveolar portion is relatively steady while cisternal part of milk yield varies considerably. Their ratio changes depending on individual distinctions of animals and stages of lactation, age of goats, interval and stereotype of milking, capacity of udder, cisternal pressure and other. Moreover, even in milk samples of one animal somatic cells are distributed differently. For example, at the eighth month of lactation of goat Eva the largest somatic cells count is noticed in the end of milking, and next month before dry period – in the middle of milking. That also points to the great volatility of this parameter. So, sample of goat milk cannot be taken at the beginning, middle or end of milking for determination of somatic cell count. It is necessary to select an average sample.

CONCLUSIONS

Somatic cell count in goat milk of the Eastern region of Ukraine appears up to 800×10^3 cells/ml at 65-71% of studied milk samples by the method of laser-running cytometry with the account of only those cells having DNA in the nucleus.

At ranging of SC contents, contents of SC subpopulations in goat milk it is shown that at increase to 2 million cells/ml and more the concentration of neutrophils and lymphocytes does not change for certain and the number of macrophages and eosinophils certainly ($p \leq 0,05$) rises from 1,5 to 4 times accordingly.

It is marked that SC contents in goat milk in the morning and evening yield of milk is different. In evening yield of milk, the level of SC is up to 30 % higher than in the morning one. It causes the necessity of milk sampling during two adjoining days proportionally to daily yield of milk for determination of SC concentration in milk.

A clear pattern of distribution of somatic cells in milk portions during milking was not detected. Average sample of yield is required to determine the somatic cell count in the milk of goats.

Six of the eight goats over six months of lactation were given milk with a fairly constant level of somatic cells. In the first month of lactation milk of seven goats contains a very low somatic cells count from 33 to 107 × 10^3 cells / ml. Low and fairly con-

stant somatic cells content (15 to 63 × 10^3 cells / ml) is observed in the milk of goats-firstborn, though their first lactation lasted only 7 months.

CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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Research article
Ερευνητικό άρθρο

Effect of microencapsulated butyric acid supplementation on growth performance, ileal digestibility of protein, duodenal morphology and immunity in broilers

M. Imran[1], S. Ahmed[1], Y.A. Ditta[1], S. Mehmood[2], M. I. Khan[1], S. S. Gillani[3], Z. Rasool[4], M. L. Sohail[5], A. Mushtaq[6], S. Umar[6]

[1] *Department of Animal Nutrition, University of Veterinary and Animal Sciences Lahore, Pakistan*

[2] *Department of Poultry Production, University of Veterinary and Animal Sciences, Lahore, Pakistan*

[3] *Poultry Research Institute (PRI) Rawalpindi, Pakistan*

[4] *Animal nutrition and health emeakemineuropan.v, Toekomstlaan 42, Herentals, Belgium*

[5] *University College of Veterinary and Animal Sciences, the Islamic University of Bahawalpur, Bahawalpur, Pakistan.*

[6] *Department of pathobiology, pmas arid agriculture university Rawalpindi, Pakistan*

ABSTRACT. This experiment was conducted to evaluate the effect of microencapsulated butyric (MEB) acid on growth performance, apparent ileal digestibility of protein (AID), duodenal morphology and immunity in broilers reared to 35-days. In total, 336 one-day-old Hubbard classic broiler chicks were randomly assigned to 4 dietary treatments (Control, 0.25, 0.35 and 0.45g/kg of MEB). Each treatment was replicated 3 times with 28 birds in each replicate. Feed intake, body weight gain and feed conversion ratio (FCR), parameters of growth performance and intestinal morphology, AID of protein and immunity parameters were evaluated. At the end of the experiment (35-d), 3 birds / replicate were randomly selected and slaughtered to collect blood, duodenal samples, and ileal digesta. The result indicated improved body weight gain ($P<0.05$), feed conversion ratio ($P<0.05$) and AID ($P<0.05$) whereas, treatments remained unresponsive with respect to feed intake ($P>0.05$), duodenal villous height ($P>0.05$) and antibody titer against Newcastle disease (ND) ($P>0.05$). There is an indication that MEB improves the digestion and consequently bird's performance.

Keywords: Butyric acid, duodenal morphology, immunity, broilers

Corresponding Author:
Dr. Sajid Umar,
Department of Pathobiology,
PMAS Arid Agriculture University Rawalpindi (46000), Pakistan.
Email: sajncvi@gmail.com

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INTRODUCTION

Better intestinal health and high digestibility of nutrients in broilers are supremely important in order to achieve higher body weight and better feed conversion ratio (FCR) (Roberts et al., 2015). Maintenance of gut development and health is very important to support development and health of the bird (Choct et al., 2009).

Organic acids (OA) and their salts are generally considered as harmless and have been approved by most technologically advanced countries to be used as a feed additive for animals. The acidifiers, including sodium butyrate (SB) is known for decreasing the gut mucosal pH, thus creating an acidic environment for the growth of normal commensals. OA are one of the potent and effective feed additives that can be used in animal nutrition to achieve higher body weight (BW) and better feed conversion ratio (FCR) (Abdel-Fattah et al., 2008). Among the OA supplements, especially BA is a suitable candidate to improve gut health resulting in more nutrients being absorbed throughout the gastrointestinal tract (GIT). Butyric acid ($CH_3CH_2CH_2$-COOH), which is a short chain fatty aicd (SCFA) having 4 carbons. Butyric Acid improves bioavailability which helps enterocytes to absorb more nutrients for the development of birds. The BA is a readily available energy source for intestinal villi and stimulates their differentiation and multiplication (Dalmasso et al., 2008) and consequently increased feed efficiency (Adil et al., 2011). It induces the production of host defense peptides when it enters in the blood stream (Guilloteau et al., 2009). These peptides stimulate the repair and development of the lower intestinal tract by improving cell proliferation (Bartholome et al., 2004). Butyrate produced by fermentation of carbohydrates is rapidly absorbed and locally affect the large intestine. Endogenous butyrate imparts no direct useful effects in small intestines (Niewold, 2014), however, the exogenous uncoated BA is readily absorbed and metabolized by crop and proventriculus of birds before reaching the small intestine (Borne et al., 2015; Kaczmarek et al., 2016).

It has been observed that the microencapsulated (coated with fatty acid matrix) type of organic acid was more effective than an antibiotic growth promoter (Enramycin) in rising growth performance in broilers. The microencapsulated butyrate delivered portion of the butyrate to be free further distal in the intestinal tract because of slow release during digestion and causes mucosal modulation in the gut. Its use led to a tendency towards better growth performance, lower colonization and fecal shedding of *Salmonella* compared to the non-protected feed supplements (Chamba et al., 2014). Consequently, the protection of BA with microencapsulation, such as MicroPEARL® technology (Kemin, Herentals, Belgium), improves its efficacy. The MicroPEARL technology helps to prevent the rapid absorption of BA in the GIT, and thus its utilization, thereby increasing the surface area exposed to the molecule (Smith et al., 2012).

To-date, limited reports have been published to evaluate the effects of SB on performance of broilers, ileal digestibility of protein, duodenal morphology and immunity in broilers. A comprehensive study was, therefore, needed to assess such effects in commercial chickens. Keeping in view the above-mentioned properties of BA, in this experiment, MicroPEARL encapsulation using hydrogenated palm oil calcium butyrate (MEB) (ButiPEARL, Kemin) was used in broilers to study its effect on overall performance of broilers, ileal digestibility of protein, duodenal morphology and immunity in broilers.

MATERIALS AND METHODS

The study was carried out in accordance with the guidelines of Animal Care and ethics Committee, University of Veterinary and Animal Sciences, Lahore, Pakistan. The trial was conducted at Research and Development Farm Sharif Feed Mills (Pvt) Ltd, Okara, Pakistan for the duration of 35 days. In total, 336 one-day-old broiler chicks were procured from a local commercial hatchery and randomly assigned to 4 dietary treatments as MEB-I (control), MEB-II (0.25g/kg), MEB-III (0.35g/kg) and MEB-IV (0.45g/kg) of MEB in control diet. The MEB (ButiPEARL™, Kemin) contained 50% calcium butyrate. Each treatment was replicated thrice with 28 birds each. Experimental birds had been raised in 12-floor pens on a concrete floor with rice husk as a bedding material. All standard management practices were followed throughout the trial. Birds

were vaccinated according to the prescribed schedule. Birds were observed twice daily for any clinical sign.

Table 1 shows the formulation and nutrient composition of the control diets which was formulated to meet or exceed the nutrient requirements of broiler chickens (NRC 1994). All the diets were fed in crumbs form in two feeding phase starter (1 to 21 days) and grower (22 to 35 days) and all diets were iso-caloric & iso-nitrogenous in both phases. In all diets Celite® at 2% were added on 32-day of the experiment as an inert marker for the estimation of AID of protein.

Table 1. Composition of the diets (% as-fed basis).

Ingredients	Starter	Grower
Maize	42.3	52.5
Rice Tips	15	5
Rice Polish	6	2.95
Soybean	19.75	18.15
Rape Seed Meal	_	2
Canola Meal	11.15	12
Fish Meal	1.5	1.5
Animal protein concentrate	1.5	2
L-Lysine HCl	0.62	0.55
DL-Methionine	0.17	0.15
L-Threonine	0.09	0.09
DCP	0.73	0.55
Calcium Carbonate	0.57	0.57
Sodium Bicarbonate	0.13	0.1
Sodium Chloride	0.42	0.25
**Vitamin mineral premix	0.07	0.09
Tallow	_	1.60
Phyzyme XPTPT	0.01	0.01
Analyzed Nutrients (%)		
ME kcal/kg	3070	3127
CP (%)	21.67	20.73
dLys (%)	1.20	1.10
dM+C (%)	0.90	0.85
CF (%)	4.32	4.97
Calcium (%)	0.95	0.87
Avail. P (%)	0.56	0.44

**Premix composition (per kg of diet): retinol12000 IE, cholecalciferol 2400 IE, dl-a-tocopherol 0.05g, thiamine 2.0 mg, riboflavin 7.5 mg, pyridoxine 3.5 mg, cyanocobalamin 20 mcg, niacin 35 mg, D-pantothenic acid 12 mg, choline chloride 460 mg, folic acid 1.0 mg, biotin 0.2 mg, iron 80 mg, copper 12 mg, manganese 85 mg, zinc 60 mg, cobalt 0.40 mg, iodine 0.8 mg,selenium0.1 mg, anti-oxidant mixture125 mg.
dLys= digestable Lysine, ME=metabolizable energy, CP=crude protein, dM+C= digestible Methionine + Cysteine , CF= crude fiber

The birds had *ad libitum* access to water and feed. Body weight and feed intake (FI) were measured weekly with the pen as the experimental unit. Before weighing, mean body weight gain, FI, and FCR ratio were used to determine the growth performance.

At the end of the experiment, n=3 birds/replicate were selected randomly for the collection of ileal digesta and blood samples. To harvest serum, blood samples were allowed to stand at room temperature for 1 hour and then centrifuged (Beckman J25I; Beckman Instruments, Inc. USA) at 1500 × g at 4°C for 20 min. The serum was divided into aliquots and stored at -20°C for analysis of antibody titer against Newcastle disease (ND). The stored serum samples were used to determine the antibody titer against NDV through Haemagglutination and Haemagglutination-inhibition (HI) tests. Digesta samples within a pen were pooled and stored at -20°C until used for acid insoluble ash (AIA) and CP analysis (Lemme et al., 2004). Feed and digesta samples were analyzed for crude protein and AIA contents using a standard method (AOAC International, 2000).

The data collected for AIA and CP contents of the feed and digesta samples were used to calculate the ileal digestibility of proteins using following equation (Ravindran et al., 2006).

Apparent Protein Digestibility (%) = [(NT/AIA) d − (NT/AIA) I] / [(NT/AIA) d] x 100

Where, (NT/AIA) d = ratio of nutrient and AIA in diet, and (NT/AIA) I = ratio of nutrient and AIA in ileal digesta.

On slaughtering, thymus, spleen and bursa were collected and weighed. The weight of these organs g/100g of live body weight were calculated (Taherpour and Ghasemi, 2014).

Histomorphological Evaluation

Gut morphology is an important indicator of gut health. Histomorphological evaluation included

determination of the villus height (VH), crypt depth (CD) and villus height to crypt depth ratio (VH:CD). Duodenal samples from slaughtered birds were collected as described by (Qaisrani et al., 2015). Briefly, a duodenal sample, 2 cm in length was collected from the middle of the duodenum, washed with normal saline (0.9% NaCl) solution and instantly stored in 10% formalin solution until further processing. The preserved duodenal tissue samples were processed according to conventional haematoxylin and eosin method described by (Chen et al., 2016). The tissue slides were examined for villus height and crypt depth using a compound microscope (Olympus CX31, Olympus USA) equipped with a digital imaging system (Olympus DP20, Olympus USA).

Statistical Data analysis

The collected data were analyzed through completely randomized design (CRD) under one way analysis of variance (ANOVA) (Steel et al., 1997). Data were mentioned as means ±SEM (standard error of the mean) and analyzed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Means were separated through Duncan's Multiple Range test using SAS 9.1. Differences were taken as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Table 2 shows the effect of MEB supplementation on FI, BWG and FCR. Feed intake ($P > 0.05$) was unaffected by supplementation of different levels of MEB. However, it was numerically lower in birds fed 0.35g/kg MEB. The supplementation of encapsulated butyric acid positively influenced the BWG ($P < 0.05$) and FCR ($P < 0.05$) but the results were more pronounced with 0.45g/kg of MEB. The present findings are in coherence with Levy et al. (2015) and Kaczmarek et al. (2016) who reported that graded levels of MEB supplementation in broiler diet improved broilers performance without affecting FI. The results of this experiment are also in line with the reports of other researchers (Chamba et al., 2014; Eshak et al., 2016), who reported that addition of BA in broilers diets improved BWG. The improvement in BWG and FCR may be due to microencapsulation of butyric acid with palm oil allowed for the target release of butyrate at the ileum level, improvement in duodenal morphology and especially improved protein digestibility.

In contrary to current findings Mahdavi and Torki, (2009) and Aghazadeh and TahaYazdi, (2012) reported that different levels of unprotected butyric acid did not influence the BWG and FCR. This might be due to the fact that BA was unprotected, which was absorbed in gizzard and proventriculus and did not reach the target site. Smith et al., (2012) revealed that encapsulating butyrate delays BA absorption, allowing it to reach the small intestine.

Small intestine is the site for absorption in which the available nutrients are taken up through epithe-

Table 2..- Effect of microencapsulated butyric acid on feed intake, body weight gain and feed conversion ratio at 35-d (Mean ± SEM).

Groups	Feed intake (g)	Body weight gain (g)	Feed conversion ratio (g/g)
MEB-I	3190.84±55.63	1823.50^{b}±34.24	1.75^{b}±0.01
MEB-II	3179.97±69.93	1937.32^{a}±23.71	1.64^{a}±0.02
MEB-III	3177.22±44.83	1940.02^{a}±14.45	1.63^{a}±0.01
MEB –IV	3153.31±48.57	1967.5^{a}±30.56	1.60^{a}±0.02
P value	0.9685	0.0222	0.0056

$^{a-b}$Means with different superscripts in a column are significantly different (P<0.05)

Table 3.. Effect of microencapsulated butyric acid on duodenal villus height (VH), crypt depth (CD), villus height to crypt depths ratio(µm) and apparent ileal digestibility (AID) of protein at 35-d (Mean ± SEM).

Groups	Villus height(µm)	Crypt depth(µm)	Villus height to crypt depth ratio (µm)	Apparent ileal digestibility of protein (AID %)
MEB-I	1043.71±22.29	103.33±8.81	10.21±0.71	70.87^{b}±1.41
MEB-II	1223.79±53.70	145.33±3.92	8.42±0.34	73.30^{b}±0.35
MEB-III	1323.33±98.20	147.33±23.91	9.26±1.01	$74.66^{a,b}$±0.66
MEB-IV	1373.33±89.87	130.00±5.13	10.54±0.35	76.72^{a}±0.76
P value	0.0501	0.1448	0.1813	0.0098

$^{a-b}$Means with different superscripts in a column are significantly different (P<0.05)

lial cells and drained into the general circulation. Architectural modulation of the small intestine is assumed to have a relationship with production performance of animals (Table 3). Butyrate acts as a rich source of energy for the enterocytes (Ahsan et al., 2016), and it may possibly increase the cell mitosis in the crypts. The SB may protect the mucosal epithelium from injury and alleviate the enteropathic stress (Ashraf et al., 2013) by increasing thyroid hormone in the circulation. We found improved histomorphometrics in MEB offered groups in duodenum and jejunum. These findings proposed that the incoming ingesta containing MEB at ileum had earlier been presented to utmost absorption in the former gut lumen and displayed better effect there. Duodenum is the major site of digestion in broilers. Butyrate supplementation, however, did not significantly influence villus height (P > 0.05), crypt depth (P >0.05) and villus to crypt depth ratio (P >0.05) of duodenum (Table 3). Our findings are in line with Levy et al. (2015), who did not find any significant effect on duodenal morphology with the addition of MEB. Likewise, Smulikowska et al. (2009) reported non-significant effect of coated BA supplementation on jejunal morphologyon.

In contrast to our findings, Kaczmarek et al. (2016) found that microencapsulation made a difference and the supplementation of MEB had a significant effect on VH. Similarly, morphometric results, Panda et al. (2009) who reported that BA, regardless of concentrations in feed, increased VH. This can be attributed to the BA that is a readily available energy source for intestinal villi and stimulates their differentiation and multiplication (Dalmasso et al., 2008). These contrary results of duodenal morphology might be due to day on which samples were taken or dose difference.

AID of protein (P<0.05) was higher with a higher level of MEB supplementation (Table 3). The results of AID are in line with Kaczmarek et al. (2016) who reported that encapsulated calcium butyrate supplementation improved ileal digestibility of amino acid in broilers. Likewise, Jahanian and Golshadi, (2015) found that butyric acid glycerides (BAG) improved ileal protein digestibility in laying hens. Our results are also in agreement with Dehghani-Tafti and Jahanian, (2016). The improvement in AID might be due to the fact that butyric acid supplementation increased pancreatic fluid, amylase, and dose dependent secretion of trypsin (Ohbo et al., 1996; Sileikiene et al., 2005). Proteolysis of proteins by pepsin produced peptides which activated the release of hormones including cholecystokinin and gastrin (Adil et al., 2011).

It had been reported that BA and it glycerides improved immunity in broilers. In this experiment no significant difference was found on the weight of immune organs and antibody titer of NDV at 35th day of age (Table 4). Mahdavi and Torki, (2009) found that inclusion of BAG in broilers diet did not

Table 4. Effect of microencapsulated butyric acid on relative organs weight* and antibody titer against Newcastle Disease at 35-d (Mean ± SEM)

Groups	Spleen*	Thymus*	Bursa of Fabricius*	ND titer
MEB-I	0.096±0.010	0.108±0.008	0.095±0.006	6.11±0.26
MEB-II	0.093±0.006	0.133±0.010	0.128±0.015	6.23±0.35
MEB-III	0.098±0.006	0.112±0.005	0.118±0.009	7.00±0.23
MEB-IV	0.104±0.005	0.112±0.010	0.113±0.015	6.33±0.28
P value	0.7884	0.2030	0.3100	0.1142

*Relative organs weight = organ weight/body weight×100.

have significant effect on spleen, thymus and bursa of Fabricius weight at an age of 35 days. Contrary to our findings, Jahanian (2011) reported that the supplementation of 0.2% BAG improved ND antibody titer at the 12th day post vaccination. Eshak et al. (2016) also reported contrary findings to our findings, which might be due to the longer interval among sampling and vaccination days.

CONCLUSION

It can be concluded from the present study that microencapsulated butyric acid supplementation at the levels of 0.25g/kg to 0.45g/kg in broilers diet improve body weight gain and feed conversion ratio and protein digestibility. It did not influence duodenal morphology in the broilers.

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FINANCIAL DISCLOSURE STATEMENT

This study was not funded by any funding agency and was conducted at the expense of the research group to combat the rising problem in the poultry industry of the country.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

In ovo hepatocarcinogenicity of N-nitrosodimethylamine and N-nitrosodimethylamine in White Leghorn chickens

A. Kril[1], A. Georgieva[1], B. Nikolov[2], R. Pepovich[2], K. Hristov[2], G. Stoimenov[2*], E. Nikolova[3], R. Petrova[3], J. Ananiev[4], Vassil Manov[2]

[1] *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*

[2] *University of Forestry, Faculty of Veterinary Medicine, Sofia, Bulgaria*

[3] *National Diagnostic Veterinary Research Institute, Sofia, Bulgaria*

[4] *Trakia University, Faculty of Medicine, Stara Zagora, Bulgaria*

ABSTRACT. Avian embryos have been gaining an increasing scientific interest as a valuable model system for the experimental cancer research that could contribute to a significant reduction of the number of laboratory animals. In the present study, the liver lesions induced by N-nitrosodimethylamine and N-nitrosodiethylamine in 15I line, White Leghorn embryos were identified and studied by routine histopathological methods. Foci of altered hepatocytes with basophilic and eosinophilic phenotype, well known as preneoplastic alterations were identified in the avian embryonal livers after *in ovo* exposure to both N-nitroso compounds. These studies were further extended by histopathological, haematological and biochemical examinations on the effects of N-nitrosodimethylamine in chickens hatched from carcinogen-inoculated eggs. In addition to the preneoplastic lesions observed in the avian livers, proliferations of oval and hepatocellular carcinoma cells, with clearly expressed signs of malignancy were found. The *in ovo* application of the chemical carcinogen was found to affect both hematological and blood biochemistry parameters measured in experimental birds. The established conditions such as thrombocytopenia and increased levels of liver enzymes, as an essential part of the paraneoplastic syndrome, were associated with the process of hepatocarcinogenesis. The results of this study confirm the preneoplastic nature of the focal lesions in embryonal avian liver and their progression to liver neoplastic alterations after a single *in ovo* application of known hepatocarcinogens. Moreover, the results indicate that 15I line, White Leghorn embryos are a new, valuable *in ovo* model for studies on hepatocarcinogenicity of chemical compounds and underline the importance of research on the development of different avian models of carcinogenicity.

Keywords: *in ovo* models, avian embryos, nitrosamines, hepatocarcinogenesis

Corresponding Author:
Department of Infectious Pathology and Food Hygiene, Faculty of Veterinary Medicine, University of Forestry, Kliment Ohridski Street 10, Sofia 1797, Bulgaria.
E-mail: georgi.stoimenov.vm@gmail.com

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INTRODUCTION

Foci of altered hepatocytes (FAH) represent the most prevalent form of hepatic preneoplasia observed in animals for a long time and more recently identified in human chronic liver diseases associated with, or predisposing to, hepatocellular carcinomas Bannasch et al., 2003; Bannasch et al., 1997; Su et al., 2003). The lesions are composed of morphologically and functionally altered populations of cells that have no obvious neoplastic nature, but indicate an increased risk for the development of benign or malignant liver neoplasms (Bannasch et al., 1996; Bannasch et al., 2003). Sasaki and Yoshida (1935) were the first who described sequential series of cellular alterations, including the appearance of foci of cellular change, preceding the occurrence of rodent liver tumors in experimental studies on chemically-induced carcinogenesis in rats. Since then, the FAH have been the focus of numerous investigations on the early cellular events of hepatocarcinogenesis. The pathobiology of FAH and their relation to hepatic neoplasia have been studied most extensively in rats exposed to chemical carcinogens. Experimental data indicate, that the predominating sequence of cellular events in the hepatocarcinogenesis induced by DNA-reactive carcinogens begins with the appearance of clear cell and acidophilic foci, storing glycogen in excess, followed by their progression to mixed cell foci, composed of acidophilic and basophilic hepatocytes, and then to basophilic, glycogen-poor foci. The later are considered as the most advanced preneoplastic lesion, directly preceding the appearance of hepatocellular carcinomas (Bannasch et al., 1989). In addition to chemicals, other established hepatocarcinogenic agents, such as certain hormones, hepadnaviruses, transgenic oncogenes, *Helicobacter hepaticus* and radiation, have also been shown to induce FAH in appropriate animal models (Bannasch et al., 1996; Bannasch et al., 2003). Moreover, development of FAH has been found in all animal species studied, including primates (Bannasch et al., 1997). Morphological, biochemical, and molecular biological analysis revealed striking similarities in specific alterations of the cellular phenotype of preneoplastic FAH in experimental and human hepatocarcinogenesis, irrespective of the carcinogenic agents by which they was induced. The detection of phenotypically similar FAH in various animal models and in humans prone to develop or bearing hepatocellular carcinomas favors the extrapolation of data obtained in animals to humans (Georgieva et al., 2012). Consequently, preneoplastic FAH have been widely used as endponds in carcinogenicity testing, as well as in studies on the molecular mechanisms of early neoplasia (Bannasch et al., 2003; Iatropoulos et al., 2001; Ito et al., 1989; Pitot et al., 1935; Tsuda et al., 2010; Weisburger et al., 1999).

Long-term rodent bioassays have been the regulatory standard for carcinogenicity assessment of industrial and agro-chemicals, food additives, pharmaceuticals and environmental pollutants for over 50 years (Marone et al., 2014). The duration of the *in vivo* carcinogenicity tests in rodents is usually two years. The neoplastic alterations induced by the test chemical in the laboratory animals are the endpoints measured by this experimental approach (Knight et al., 2008; Williams et al., 2008). The obvious negative side of the standard bioassay in rodents is that it is extremely time-consuming and costly, and requires the sacrifice of large numbers of animals. For these reasons, the development of alternative predictive models remains a research priority. In order to shorten the experimental period of the *in vivo* carcinogenicity assays and to minimize the pain and suffering of the laboratory animals a large number of medium-term tests, with an average duration ranging from few weeks to few months, have been developed. These experiments are terminated before the appearance of solid tumors and metastases, and the induced preneoplastic lesions are used as endpoints (Hasegawa et al., 2009; Tsuda et al., 2010). In addition, numerous short-term *in vitro* mutagenicity and genotoxicity tests have been established in an attempt to reduce and/or replace the animals needed for carcinogenicity assessment (Anadón et al., 2014; Benigni et al., 2013). Investigations aimed at the development of alternative models and methods have been gaining an increasing importance since the adoption in 2010 and the implementation in 2013 of the new Directive 2010/63/EC of the European Parliament and the EU Council on the protection of animals used for scientific purposes.

During the last decades, avian embryos have attracted the interest of the scientific community as new and reliable alternative model systems (*in ovo* models) for studies on different pathological processes,

including viral and chemical carcinogenesis. It has been shown that *in ovo* experiments can provide valuable information about the carcinogenic potential of chemical compounds and may fill the gap between the *in vivo* and *in vitro* experiments, combining some advantages of both approaches (9). *In ovo* carcinogenicity assay (IOCA) has been described in detail by Enzmann and Brunnemann, (Enzmann et al., 1997) and the results revealed the appearance of eosinophilic (glycogen-rich, glycogenotic, glycogen-storing foci, basophilic foci of altered hepatocytes and mixed cell foci in the avian embryonal liver. These focal alterations are morphologically identical to those found in rodents and are considered as preneoplastic lesions able to progress to hepatocellular carcinomas, without further exposure to carcinogens. The *in ovo* experiments are more rapid, less expensive and safer for the personnel than the *in vivo* experiments in rodents. In the *in ovo* carcinogenicity studies, turkey and quail embryos were most frequently used as experimental models (Enzmann et al., 1998; Enzmann et al., 1997; Enzmann et al., 1996, Enzmann et al., 1992, Enzmann et al., 2013). The development of preneoplastic FAH was also found in the liver of chicken embryos after treatment with organic (Georgieva et al., 2012) and inorganic carcinogenic chemicals (Kril et al., 2011). Interestingly, identical hepatic lesions were identified in chicken embryos experimentally infected with oncogenic avian retroviruses (Georgieva et al., 2013).

In the present study, the preneoplastic liver lesions in 15I line, White Leghorn embryos, exposed *in ovo* to N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) were examined by histopathological methods. The progression of the neoplastic process, initiated *in ovo,* was studied in chickens, hatched from NDMA-treated eggs. In addition, some haematological and blood biochemical parameters in the experimental birds were followed eighteen weeks post hatching.

MATERIALS AND METHODS

Eggs. Fertilized eggs from 15I line, White Leghorn hens were obtained from diseases-free flock, bred in the animal housing facilities of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, BAS.

Carcinogens and treatment of embryonated eggs. The tested carcinogens N-nitrosodimethylamine (CAS № 62-75-9; Sigma-Aldrich) and N-nitrosodiethylamine (CAS № 55-18-5; Sigma-Aldrich) were diluted with sterile glass double distilled water and administered as a single dose of 0.3 mg/per egg, with an injection volume of 0.1 mL. Control eggs were injected with an equal volume of the vehicle. The eggs were treated during the first hours of incubation. Briefly, after sterilization of the pointed site with 70% ethanol the test substances were inoculated into the egg albumen and the incubation continued in an automatic rotating incubator at 37.8±0.5°C and 70±10% relative humidity. At the end of the incubation, the eggs were transferred to hatcher at 37°C ± 0.2°C and 80-85% humidity.

Avian embryos. A total of 93 avian embryos were examined at the 18th embryonic day- 36 treated with NDMA, 32 treated with NDEA and 25 vehicle-treated controls.

Experimental birds. Twelve birds hatched from NDMA-inoculated and control eggs were followed up to 18 weeks post hatching. The treatment and control group consisted of six birds each. Standard fodder mixtures and water were available *ad libitum*. This study was conducted in accordance with the European and National guidelines and regulations for animal welfare.

Histopathology. All experimental birds were exsanguinated 18 weeks post hatching. Tissue samples from control and treated embryos and birds were immediately fixed in 10% buffered formalin for subsequent histopathological examination. The tissues were routinely dehydrated, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Histopathological lesions were observed and documented with microscope Leica DM 5000 B, equipped with a digital camera and original software.

Hematology and blood biochemistry. Venous blood was taken from the wing vein of the treated and control birds at the 13th and 18th week post hatching. Haematological parameters (WBC, 10^9/L; LYM, 10^9/L; GRA,

10^9/L; Hgb, g/L; RBC, 10^{12}/L; Hct,%; Thr, 10^9/L) were measured in whole blood by veterinary automatic hematological analyzer Hema Screen 18 LIHD 170, (Hospitex diagnostics – Italy). Biochemical parameters (total protein, g/L; albumin, g/L; ALT, U/L; AST, U/L; GGT, U/L and Glucose, mM) were measured in the blood serum by a semi-automatic biochemical analyzer Screen Master LIHD 113, (Hospitex diagnostics – Italy) and reagent kits for biochemical analyses (Human – Germany).

RESULTS

The alterations of the embryo weight, the absolute and relative liver weight, induced by NDEA and NDMA, were examined as important indicators for the toxic and carcinogenic potential of the tested compounds. The *in ovo* treatment with NDMA and NDEA, induced a statistically significant reduction of the embryo weight ($p \leq 0.001$) and significantly increased the absolute and the relative liver weight, compared to controls (Table 1).

The reduction of the embryo weight was more pronounced in NDEA-treated group and an increase of the absolute liver weight was more prominent in NDMA-treated group. The relative liver weight values for both carcinogen-treated groups were similar.

The gross pathology revealed the presence of well-demarcated reddish-green areas in the livers of carcinogen-treated White Leghorn embryos (Fig. 1a). In the livers of chickens *in ovo*-treated with NDMA grayish-white nodular proliferations (2-4 mm) were found. In addition, bile imbibition and petechial hemorrhages were also present (Fig. 1b).

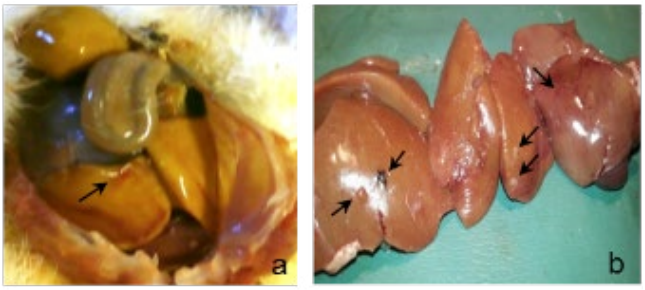


Fig. 1. Gross pathology of lesions in embryonic liver, induced by N-nitrosodiethylamine (a) and in the livers of 18 weeks old White Leghorn chickens, treated *in ovo* with N-nitrosodimethylamine (b)

The histopathological examination of liver sections from NDMA and NDEA-treated embryos revealed the presence of morphologically distinct foci of altered hepatocytes (Fig. 2). Basophilic FAH were found in embryonal livers after exposure to NDEA. The cells of the altered foci were smaller than the surrounding unaffected hepatocytes and showed an intense cytoplasmic basophilia (Fig. 2 A). Clear and acidophilic foci were the hepatic lesions most frequently found in NDMA-treated embryos (Fig. 2 B). In addition, small, mixed cell foci (composed of basophilic and acidophilic hepatocytes) and small groups of basophilic hepatocytes were detected in sections from embryonic livers of this experimental group. After treatment with both hepatocarcinogens, megalocytes and obstruction of bile ductules by bile plugs were also found (Fig. 2 C, D). Neither of the described lesions were detected in samples from vehicle-treated controls

Histopathology of liver samples from 18 weeks old chickens, *in ovo* treated with NDMA, showed neoplas-

Table 1: Effect of N-nitrosodimethylamine and N-nitrosodiethylamine-treatment on the body weight, absolute and relative liver weights of 15I line, White Leghorn embryos

Treatment groups	Dose (mg/egg)	Number of embryos	Embryo weight (g)	Liver weight (g)	Relative liver weight (%)
Control	0	25	20.31±0.41	0.31±0.01	1.51±0.06
NDEA	0.3	32	13.93±0.37***	0.35±0.01**	2.53±0.12***
NDMA	0.3	36	17.64±0.46***	0.42±0.01***	2.38±0.10***

Values are means ± SD; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ compared to control

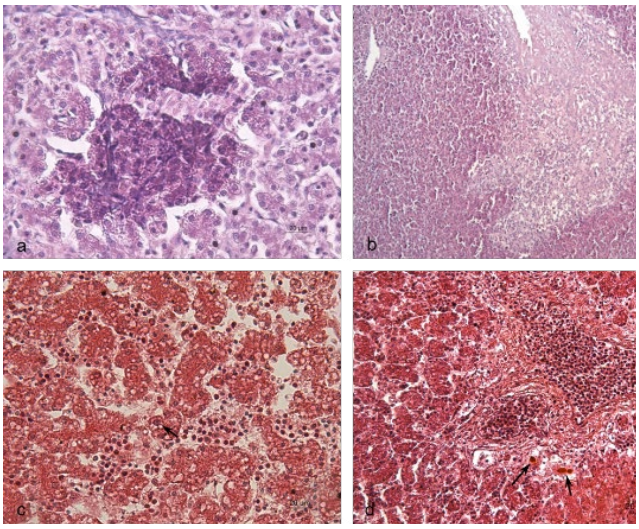


Fig. 2. Histopathology of liver lesions in White Leghorn embryos after *in ovo* exposure to N-nitrosodimethylamine and N-nitrosodimethylamine. A. Basophilic focus of altered hepatocytes; B. Eosinophilic focus of altered hepatocytes; C. Liver megalocytes; D. Bile plugs; NDEA treatment (A, C); NDMA treatment (B, D); H&E; bar=20 μm.

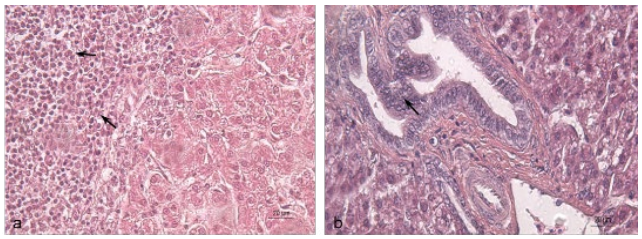
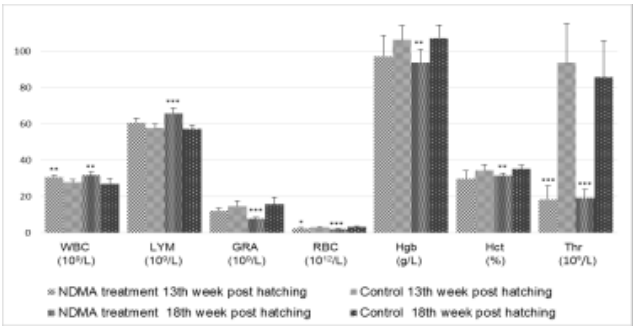
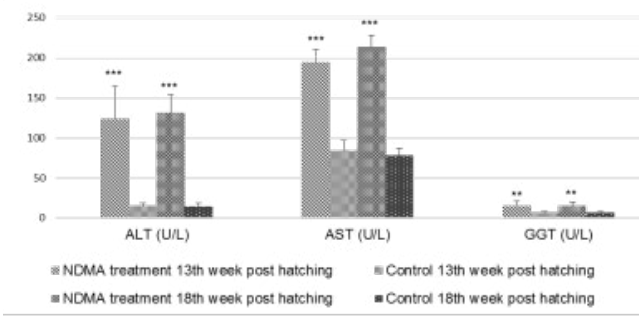


Fig. 3. Hyperplastic and neoplastic lesions in the liver of chickens, *in ovo* treated with N-nitrosodimethylamine. A. Proliferation of oval and hepatocyte-like carcinoma cells; B. Hyperplasia of cholangiocytes; H&E; bar=20 μm.



Values are means ± SD; *p≤0.05; **p≤0.01; ***p≤0.001 compared to control

Fig. 4. Hematological parameters of 15I line, White Leghorn chickens, treated in ovo with N-nitrosodimethylamine



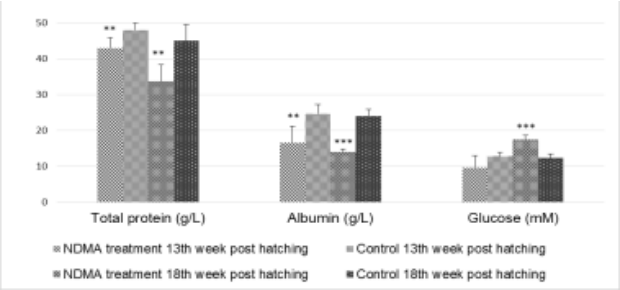
Values are means ± SD; *p≤0.05; **p≤0.01; ***p≤0.001 compared to control

Fig. 5. Blood serum activities of alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transferase in 15I line, White Leghorn chickens treated *in ovo* with N-nitrosodimethylamine

tic alterations, represented by the proliferations of oval cells and hepatocyte-like cells with clearly expressed signs of malignancy (Fig. 3 A). Moreover, hyperplasia of cholangiocytes with pseudopapillary intraluminal projections was frequently found (Fig. 3 B). In addition, preneoplastic changes such as basophilic, eosinophilic and mixed foci of altered hepatocytes were found in the livers of experimental birds. No such lesions were detected in the liver samples from the control birds.

The investigation of the blood samples from experimental birds 13 weeks after hatching revealed significant elevation (p≤0.01) of the number of the white blood cells, compared to control. An increase of lymphocyte count was also noted, without statistical significance. All other tested hematological parameters were lower than the those measured in the control group of birds. However, only the decrease of the values of red blood cells and thrombocytes reached statistical significance (Fig. 4). Eighteen weeks post hatching, the WBC and the LYM values were significantly elevated ($p<0.01$ and $p<0.001$, respectively), compared to controls. The other tested parameters were significantly lower, as compared to the controls (Fig. 4).

The results from the biochemical studies showed a statistically significant (p≤0.001) increase in the levels of the major hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as



Values are means ± SD; *p≤0.05; **p≤0.01; ***p≤0.001 compared to control

Fig. 6. Blood serum concentrations of total protein, albumin and glucose in 15I line, White Leghorn chickens treated *in ovo* with N-nitrosodimethylamine

well as significantly (p≤0.01) increased activity of gamma-glutamyl transferase (GGT) (Fig. 5).

The total serum protein content was significantly reduced (p<0.01) at both test points. The albumin values measured at 13th and 18th week post hatching were also lower, compared to the controls (p<0.01 and p<0.001, respectively). At the 13th week after hatching the blood sugar values were lower than the control, but the differences were not significant. In contrast, significant elevation (p≤0.001) of blood glucose was observed 18 weeks post hatching (Fig. 6).

DISCUSSION

The results indicate that the treatment of chicken embryos with NDMA and NDEA induces a pronounced hepatotoxic effect, evidenced by the substantial and statistically significant alterations in the embryo weight and the absolute and relative liver weights as compared to the control. The results obtained revealed that chicken embryos show higher sensitivity to the toxic effects of NDEA, compared to those of the NDMA. A number of studies on the effects of NDEA on turkey and quail embryos have been carried out (Brunnemann et al., 2002; Enzmann et al., 1995; Enzmann et al., 1997; Enzmann et al., 2013; Williams et al., 2011). These *in ovo* experiments showed a significant increase of the absolute and relative liver weights of the carcinogen-treated avian embryos. The changes of the same parameters, established in the present study correspond well with the previously published data.

The identified basophilic and eosinophilic foci of altered hepatocytes in White Leghorn embryos treated with NDEA are quite similar to the preneoplastic lesions described in embryos of other avian species after treatment with the same carcinogen. The basophilic FAH reported in NDEA-treated turkey embryos, were often composed of intensely basophilic small hepatocytes or large hepatocytes with a diffuse basophilia of the cytoplasm (Enzmann et al., 1995). The results from our study showed exclusively the presence of basophilic foci of smaller altered hepatocytes. Histopathological examination of the liver of chickens, treated with N-nitrosodimethylamine during early stages of embryonic development showed the presence of preneoplastic lesions in all birds studied. These lesions were classified as clear/acidophilic, mixed and basophilic foci of altered hepatocytes. In addition, clearly expressed hyperplasia of cholangiocytes was regularly observed in the liver of experimental birds. Similar histopathological alterations were found previously in quail embryos*, in ovo* treated with hepatocarcinogens (Brunnemann et al., 2002; Enzmann et al., 1996). In addition to the preneoplastic and hyperplasic alterations affecting hepatocytes and cholangiocytes, respectively, the development of neoplastic processes evidenced by the appearance of proliferations composed of oval and hepatocyte-like carcinoma cells were identified in the experimental birds. Similar results were obtained in experiments with line LM, Leghorn chickens exposed to NDEA, at doses ranging from 20 to 100 mg, applied through a drinking water or injection, at the 20th day post hatching. After 24 weeks, macroscopically visible neoplastic lesions were identified and classified histopathologically as hepatocellular carcinomas (Kawaguchi et al., 1987).

The results from the biochemical studies showed substantial and statistically significant increase in the levels of the major hepatic enzymes ALT and AST, as well as significantly increased activity of GGT. Marked hypoproteinemia and hypoalbuminemia, were also registered. Hematological investigations revealed a moderate leukocytosis with lymphocytosis, accompanied by prominent neutropenia and thrombocytopenia. In addition, anemia demonstrated by reduced erythrocytes count, decreased hemoglobin and hematocrit values was found in the experimental group. The blood biochemistry results complement

the observed morphological changes in the liver of experimental birds, showing a significant deterioration of the hepatocytes function and confirm the registered hyperplasia of cholangiocytes. The established elevated values of the key liver enzymes, the hypoproteinaemia, hypoalbuminaemia, the relative anemia and marked thrombocytopenia are not only indicators for general changes in liver function, but they are also an important part of the paraneoplastic syndrome, that accompanies the process of hepatocarcinogenesis.

CONCLUSION

This study shows that *in ovo* exposure to N-nitrosodimethylamine and N-nitrosodiethylamine induces preneoplastic liver lesions in 15I line, White Leghorn chicken embryos, morphologically identical to preneoplastic alterations found after similar treatment in embryonic livers of other avian species. To the best of our knowledge, we are the first to describe the development of neoplastic liver lesions in 15I line, White Leghorn chickens, as a result of the single *in ovo* treatment with hepatocarcinogens. These findings strongly support the statement about the progression of different types of FAH to hepatocellular carcinoma.

We also conclude that 15I line, White Leghorn embryos are valuable model that allows reliable, rapid and inexpensive assessment of the carcinogenic potential of chemical compounds and are strictly in line with the animal protection regulations and ethical aspects of the scientific investigations.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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Research article
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Comparison of two methods of making reduced-fat ovine Halloumi type cheese

S. Kaminarides[1]

[1]*Laboratory of Dairy Science and Technology, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece*

Σύγκριση δύο μεθόδων παρασκευής τυριού Χαλούμι με μειωμένο λίπος από πρόβειο γάλα

Σ. Καμιναρίδης[1x]

[1]*Εργαστήριο Γαλακτοκομίας, Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου, Αθήνα*

ABSTRACT. Halloumi is a popular cheese in Cyprus where reduced-fat Halloumi cheese is manufactured solely from bovine milk. In this study, two methods of making reduced-fat Halloumi type cheeses from ovine milk containing 1.8% fat were compared. Ovine milk was used because it has a greater biological and nutritional value than bovine milk. The first method used traditional technology (T) as a control whereas the second method was a modified technology (M), as described in material and methods. The M cheese had a significantly ($P<0.05$) higher yield, moisture content, protein in dry matter, pH value and lower content of fat, lactose, ash, Ca and Mg, acidity, hardness, adhesiveness, elasticity, gumminess and chewiness than the T cheese. The assessment panel suggested that the M technology was superior and can be used to produce a reduced-fat ovine Halloumi type cheese.

Keywords: Reduced-fat cheese, Ovine Halloumi cheese, physicochemical characteristics, texture profile, sensory attributes.

Corresponding Author:
Stelios Kaminarides, Laboratory of Dairy Science and Technology Department of Food Science and Human Nutrition Agricultural University of Athens 75, Iera Odos - 118 55 Athens - Greece, E-mail: skamin@aua.gr

Αλληλογραφία:
Στέλιος Καμιναρίδης, Εργαστήριο Γαλακτοκομίας, Τμήμα Επιστήμης Τροφίμων και Διατροφής του Ανθρώπου, Γεωπονικό Πανεπιστήμιο Αθηνών, Ιερά Οδός 75 - 118 55 Αθήνα, ΤΗΛ.: 210-5294641, E-mail: skamin@aua.gr

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ΠΕΡΙΛΗΨΗ. Το Χαλλούμι είναι ένα δημοφιλές τυρί στην Κύπρο. Τα τελευταία χρόνια στην αγορά της Κύπρου κυκλοφορεί Χαλλούμι με μειωμένο λίπος που παράγεται αποκλειστικά από αγελαδινό γάλα. Στην παρούσα μελέτη, έγινε σύγκριση δύο μεθόδων παρασκευής τυριού τύπου Χαλλούμι από πρόβειο γάλα που μειώθηκε το λίπος του στο 1,8%. Χρησιμοποιήθηκε πρόβειο γάλα γιατί είναι μεγαλύτερης βιολογικής και θρεπτικής αξίας από το αγελαδινό. Στην πρώτη, χρησιμοποιήθηκε ως μάρτυρας η παραδοσιακή τεχνολογία (Τ), ενώ στη δεύτερη, η τροποποιημένη τεχνολογία (Μ), όπως περιγράφεται στα υλικά και μέθοδοι. Το τυρί Μ είχε υψηλότερη απόδοση, περιεκτικότητα σε υγρασία και πρωτεΐνη υπολογισμένη σε ξηρή ουσία, υψηλότερη τιμή pH και χαμηλότερη περιεκτικότητα σε λίπος, λακτόζη, τέφρα, Ca, Mg, οξύτητα, σκληρότητα, συνάφεια, ελαστικότητα, πολτοποιητικότητα και μασητικότητα από το τυρί Τ. Η ομάδα οργανοληπτικής αξιολόγησης πρότεινε ότι η τεχνολογία Μ είναι καλύτερη και μπορεί να χρησιμοποιηθεί για την παραγωγή πρόβειου τυριού τύπου Χαλλούμι με μειωμένο λίπος.

Λέξεις κλειδιά: Τυρί με μειωμένο λίπος, τυρί Χαλλούμι, φυσικοχημικά χαρακτηριστικά, ρεολογικά, χαρακτηριστικά, οργανοληπτικά χαρακτηριστικά.

INTRODUCTION

Obesity has become a very important danger for the population's health mainly in the United States and Europe, but also in developing countries. Obesity causes serious health problems, such as arteriosclerosis, cardiovascular damage, coronary heart disease, diabetes in adults, as well as being implicated in some forms of cancer in the breast and colon (Ritvanen *et al*., 2005). Nowadays, more and more consumers buy foods with reduced-fat and energy, as part of a diet to reduce their weight (Ritvanen *et al*., 2005).

The production of reduced-fat cheeses has increased considerably recently, reflecting the increasing demand of consumers for lower-fat products (Molinaa *et al*., 2000). The cheeses with reduced fat, however, frequently present various negative characteristics, such as a very hard texture, the yield reduce, the crystallization of calcium salts with lactic acid, a lack of good flavour. These can be attributed to the important role of fat in determining the characteristic texture and flavour of cheese. However, other parameters that are altered by the reduction of fat, e.g. moisture, pH, salts etc., may play a role. Thus, a series of modifications of cheese technology have been proposed for the improvement of low-fat or reduced-fat cheese quality (Ritvanen *et al*., 2005).

Halloumi cheese is a traditional and distinctive cheese of Cyprus. Traditionally, raw ovine and caprine milks, or a mixture of the two, were used for the manufacture of Halloumi cheese. However, increased production of cow's milk in Cyprus has promoted the manufacture of Halloumi cheese from cow's milk alone or mixed with that of sheep or goat milk. It is widely popular in Cyprus and other countries of the Eastern Mediterranean and, more recently, the product has gained international acceptance and recognition. It is a semi-hard rindless cheese. The Committee for Standards of the Cyprus Ministry of Commerce and Industry (1985) established the definition and standards for 'fresh' and 'mature' Halloumi cheese. Two categories of Halloumi cheese with reduced-fat from cow's milk have been circulated in the Cypriot market in the past few years by the brand names of Light and Slim (Papademas *et al*., 2000). Attempts to produce reduced fat versions of the cheese are poorly documented. Theophilou and Wilbey (2007) reported the production of a low-fat Halloumi cheese from bovine milk, where bovine whey protein concentrate (Simplesse®) incorporated into the milk as replacement of milk fat. It should be noted that cow's milk contains more than 20 allergenic proteins, of which β-lactoglobulin and casein are reported to cause the most allergenic effects (El-Agamy, 2007), while for sheep's milk there are no scientific reports that it causes allergies. Furthermore, whey proteins in sheep's and goat's milk are increasingly recognized for their bioactivity or health-promoting benefits, such as their immunomodulatory and anti-microbial activities (Hernandez-Ledesma *et al.,* 2011).

Ovine milk is richer in fat, proteins, ash, calcium, iron, manganese, phosphorus, zinc, medium-chain fatty acids, monounsaturated fatty acids, linolenic acid, all essential amino acids and most vitamins (except for carotene) than bovine milk and caprine milk (Park *et al.,* 2007). Indeed, ovine milk is of high biological and nutritional value with high digestibility and hypoallergenicity (Park *et al.,* 2007). Thus, from a nutritional, and health point of view, ovine milk and its dairy products excel cow's milk (Kaminarides and Anifantakis, 2004; Kaminarides *et al.,* 2007).

Today, there are not data about the technology and quality of the production reduced-fat Halloumi type cheese from ovine milk. So, the objectives of this work were to determine differences between reduced-fat Halloumi type cheeses from ovine milk made by the traditional technology and by modified technology with six changes, especially by the step of incorporation of denatured ovine serum proteins, which are more than those of bovine milk, into the casein matrix during the heat treatment of milk (75°C/10min). The cheeses produced by two technologies were compared

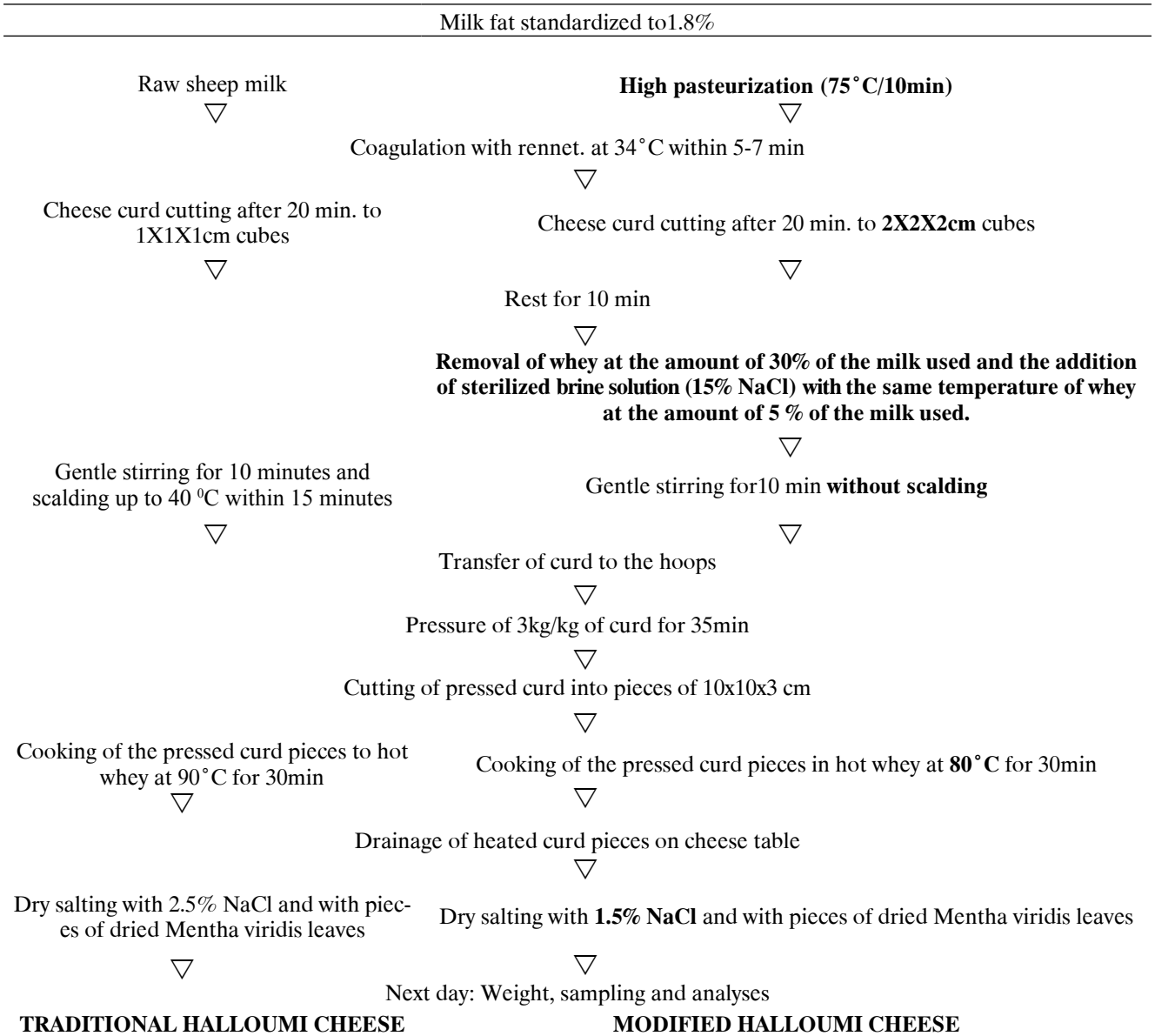

Figure 1: Flow chart for the prodution of reduced fat Halloumi type cheeses by traditional and modified technology. Bold letters indicate the modifications to the traditional technology.

for yield, recovery of milk solids, physicochemical microbiological, rheological and sensory properties.

MATERIALS AND METHODS

Milk

Fresh ovine milk, without antibiotics, was obtained from the farm of the Agricultural University of Athens and standardized to 1.8% fat by separation. The standardized milk was separated into two batches and used as cheese milk for the production of reduced-fat Halloumi type cheese by two different technologies in two different vats.

Cheese Making and Sampling

In a series of five parallel pilot plant experiments, reduced-fat Halloumi type cheese was manufactured over 5 successive weeks, using two different technologies. The first trial employed traditional Halloumi cheese making technology (T) but with the milk fat standardized at 1.8% and was used as a control. The second trial utilized modified technology (M) that differed from the traditional technology as shown in Figure 1. Based mainly on bibliographic data, these modifications are indicated in bold and aimed at increasing the yield, the moisture (Rodriquez, 1998; Lo and Bastian, 1998) and improving the texture of reduced-fat Halloumi type cheese. The weight of milk and cheese produced from each trial were measured. Samples of milk and cheese from each treatment and replication were analyzed 1 day after manufacture

Physicochemical Analyses of Milk Samples

The main constituents (fat, protein, lactose, ash and total solids) of the standardized ovine milk used for the production of reduced-fat Halloumi-type cheeses were determined by the Milkoscan apparatus (model 255 A/B, type 25700, Fosselectric, Denmark). Acidity was measured by the Dornic method and pH recorded using a pH meter (model 632, Metrohm, Herisau, Switzerland). All analyses were performed in triplicate and the results are presented as the average of fifteen analyses from five trials.

Enumeration of Microorganisms

Samples (50 g) of cheese curd were transferred under aseptic conditions to Petri dishes and analyzed on the day of sampling. A sample (5g) was suspended in 20 g L^{-1} trisodium citrate (45 ml) to give a 1:10 dilution. Further decimal dilutions were prepared in 1/4 strength Ringer's solution. The total microbial flora was enumerated by the pour-plate method of the American Public Health Association (APHA, 1967) using Plate Count Agar (Difco, Michigan, USA) with incubation at 30^0C for 2 days. Yeast and mould counts were enumerated using Yeast Extract Glucose Chloramphenicol Agar (IDF, 1991) with incubation at 25^0C for 4 days. Coliforms were enumerated according to IDF (1985) using Macconkey broth (Oxoid, Hampshire, England) and incubation at 37^0C for 2 days.

Physicochemical Analyses of Cheese Samples

Samples of reduced-fat Halloumi type cheese were analyzed for total N by the method of Kjeldahl (TN; IDF, 1964), total solids (TS; IDF, 1982), NaCl content (IDF 1972), fat by the method of Gerber-Van Gulic (Schneider, 1954) ash as specified in the AOAC (1975), pH was measured with a pH-meter (model 632, Metrohm, Herisau, Switzerland), acidity was measured by the method of Dornic, as specified in the AOAC (1975). Calcium, sodium, potassium and magnesium contents were determined according to procedure 119 of the IDF (2007) using a Varian spectrAA-200HT atomic absorption spectrophotometer (Varian, Victoria, Australia). Phosphorus content was determined according to spectrometric method 42: B of the IDF (1990). All analyses were performed in triplicate and the results are given as the average of 15 analyses from five trials.

Textural Evaluations

The textural properties of cheeses were measured with a Shimadzu testing instrument, model AGS-500 NG (Shimadzu Corporation, Kyoto, Japan), as described by Kaminarides and Stachtiaris (2000).

Sensory Evaluation

The Halloumi type cheese sensory characteristics were evaluated by a ten-member panel of the Dairy Laboratory of the Agricultural University of Athens, which was familiar with Halloumi cheese. Panel members evaluated each cheese for appearance, texture and flavour (odour and taste) using a 10-point scale, scoring 1 for worst and 10 for best quality. Results are expressed as a mean score for the whole panel for each cheese. Also, the panel of judges were requested to record their perception of elasticity and softness on a scale from 1 to 5. Elastici-

ty (the degree to which the cheese sample deforms) was assessed by pressing a piece of cheese between the forefinger and thumb [1= inelastic, 5= elastic]. Softness was estimated as the force required to penetrate the cheese sample with the molar teeth [1= very hard, 5= very soft].

Statistical analysis

Statistical analysis was performed using the general linear model (proc GLM) statistical program S.A.S. (SAS Institute, 2005), with independent variable the technology of cheese production. The level of significance was fixed at P = 0.05. The comparisons of means were made with the Duncan method. The results are presented as means ± standard error of mean.

RESULTS AND DISCUSSION

Milk Composition

The main constituents of the standardized ovine milk used for the production of reduced-fat Halloumi-type cheeses were as follows (mean values (±) the standard errors of the means): 1.80±0.01% fat, 5.29±0.18% total protein, 5.02±0.17% lactose, 0.91±0.03% ash and 13.10±0.35% dry matter.

Yield and Recovery of Milk Constituents in Reduced-fat Halloumi type Cheese

Cheese yield is one of the most economically important aspects of cheese manufacture. The cheese yield depends on the composition and quality of the milk used, the technology applied during cheese production and the conditions of cheese storage. A total reduction of cheese yield is inevitable during the production of cheese from milk with a low fat content, provided that the fat in the milk is one of the main components determining cheese yield (Romeih *et al*., 2002). The yield of the experimental cheeses T and M, 1 day after preparation, differed significantly ($P < 0.05$). From the results (Table 1), it appears that the av-

Table 1. Yield and physicochemical characteristics of reduced-fat Halloumi type cheeses produced from ovine milk by traditional (T) and modified (M) technology. (Means of 5 trials± standard error of mean).

Yield and physicochemical characteristics	Reduced-fat ovine Halloumi type cheeses	
	Traditional technology (T)	Modified technology (M)
Cheese yield (%)	12.86 ^{a} ± 0.57	15.95 ^{b}± 0.64
pH	5.9 ^{a} ± 0.09	6.3 ^{b} ± 0.06
Acidity (%)	0.38^{b} ± 0.05	0.19^{a} ± 0.03
Moisture (%)	48.70 ^{a} ± 1.14	54.51^{b} ±1.21
Dry matter (%)	51.30 ^{b} ± 1.14	44.79 ^{a} ±1.21
Fat (%)	12.58^{b} ± 0.27	10.66^{a} ± 0.32
Fat in dry matter (%)	24.46^{a} ± 0.53	24.10^{a} ± 0.54
Protein (%)	30.74^{b} ± 0.95	27.86^{a} ± 1.09
Protein in dry matter (%)	59.66^{a} ± 0.44	61.17 ^{b} ± 0.59
Lactose (%)	2.62^{b} ± 0.14	1.91^{a} ± 0.05
Ash (%)	4.97^{b} ± 0.11	4.32^{a} ± 0.08
NaCl (%)	1.62^{a} ± 0.19	1.39^{a} ± 0.19
Ca (mg/100g)	1104.10^{b} ± 34.13	894.32^{a} ± 63.44
Mg (mg/100g)	56.02^{b} ± 1.74	48.58^{a} ± 2.61
Na (mg/100g)	715.46^{a} ± 61.04	609.08^{a} ± 50.12
K (mg/100g)	87.3 ^{a} ± 5.43	86.52^{a} ± 4.47

$^{\alpha, b}$: Means in the same row with the same superscript did not differ significantly (P>0.05)

erage yield of reduced-fat cheese by the M technology was 15.95% compared with 12.86% for the T technology. This significant difference is mainly attributed to the higher moisture content of reduced fat cheese made by the M technology (54.51%) compared to that by T technology (48.70%) and secondly to the fact that in the case of M technology more proteins are transferred to the cheese (proteins in dry matter 61.17%) compared to that by T technology (proteins in dry matter 59.66%). The latter can be attributed to the incorporation of denatured serum proteins (mainly the β-lactoglobulin) with κ- and α_{S2}-caseins in casein micelles via sulphur bridges (Walstra and Jenness, 1984) during the heat treatment of milk (75°C/10min) in M technology.

The recovery of milk constituents (i.e. the percentage of milk constituents converted into cheese) was computed from the following equation:

$$Z \text{ component recovery (\%)} = \frac{\text{Cheese weight (kg) x \% } Z \text{ content in cheese x 100}}{\text{Milk weight (kg) x \% } Z \text{ content in milk}}$$

The recovery of fat, protein and total solid residue were 89.64, 74.58 and 50.21% respectively for T technology and 93.86, 84.00 and 54.50% respectively for M technology. Fat recovery was the highest of the three main solid components because milk fat is transferred almost entirely to the cheese mass. In contrast, the total solids recovery was the lowest of the three main solid components because a significant part of the water-soluble ingredients of the solids (lactose, soluble salts and serum proteins) were transferred to whey. No significant *($p > 0.05$)* differences were observed between the two types of cheese concerning the recovery of fat and total solids. In contrast, protein recovery was significantly higher in reduced-fat cheese made by M technology (84%) than in that made by T technology (74.58%). The higher protein recovery in reduced-fat cheese made by M technology can be attributed to increased retention of serum proteins during high pasteurization of milk than in reduced-fat cheese made from raw milk by T technology.

Physicochemical Properties of Reduced-fat Cheeses

Significant differences (*$P<0.05$*) in acidity (Table 1) between the two types of reduced-fat Halloumi were observed, with acidity being higher in the cheeses made by T technology than in those made by M technology. This difference is attributed to the partial reduction of lactic acid and lactose contents in cheese made by the modified technology, where part of the whey was replaced with brine. Drake and Swanson (1995) reported that the washing of curd decreases and limits the acidity of cheese.

Similar differences (*$P < 0.05$*) in the pH of the reduced-fat Halloumi-type cheeses were observed (Table 1) with T- cheeses having a lower pH than M-cheeses due to the increased acidity of the former.

The moisture content of the cheeses differed significantly ($P < 0.05$). The cheeses made by M technology had a higher moisture content (54.51%) than those made using T technology (48.70%). This difference was due to the six modifications (bold letters in Figure1) made for the production of reduced-fat Halloumi type cheese by the modified technology, which enhanced the cheese moisture content. The mean moisture content of reduced-fat Halloumi type cheese made by the M technology was approaching to that reported by Economides (2004) in the Lite bovine Halloumi cheese (56.06%) and was similar to that reported by Leif et al. (2009) in the reduced-fat Halloumi cheese (53.93%).

The dry matter content of each type of reduced-fat Halloumi cheese followed a trend that was the reverse of that relating to moisture. The cheeses made using T technology had a higher dry matter content (51.30%) than that made by M technology (44.79%). This difference arose from the higher temperature used to heat the curd in T-cheese (90°C for 30min compared with 80°C for 30min for M-cheese), which increased water evaporation from the open vat, resulting in a significantly higher concentration ($P < 0.05$) of total solids in the T-cheese. The mean fat content of reduced-fat Halloumi type cheese made by the M technology (10.66%) was approaching to that reported by Economides (2004) in the Lite bovine Halloumi cheese (9.75%) and the mean fat content of reduced-fat Hal-

loumi type cheese made by the T technology (12.58%) was similar to that reported by Leif et al. (2009) in the reduced-fat Halloumi cheese (12.83%).

Although the two types of cheese were produced from milk with the same fat content (1.8%), the cheese made by M technology had a lower fat content than that made by T technology (Table 1). This is attributed to the difference in moisture content between the two types of cheese. However, when fat was expressed on a dry matter basis, no statistically significant differences were observed (Table 1).

Similarly, the lower protein content of reduced-fat Halloumi type cheese produced by M technology (Table 1) compared with that of cheese produced by T technology is attributed to the difference in moisture content of the two cheeses (Table 1). In contrast though, when protein was expressed on a dry matter basis, we observed that the protein in dry matter of the reduced-fat M-Halloumi cheese was statistically significantly higher than that of T- cheese (Table 1) This difference could be attributed to denatured serum proteins in the pasteurized milk, since heating of the milk at high pasteurization denatured some β-lactoglobulin, which may have associated with κ- and α_{S2}-casein in the milk through disulfide bond formation (Walstra and Jenness, 1984), thus coagulating with casein during cheese making.

The lactose and ash content (Table 1) of reduced-fat Halloumi type cheese produced by M technology was statistically less than that produced by T technology. On the one hand, this difference could be attributed to the higher moisture content of this cheese, while on the other hand to the partial removal of water soluble lactose and salts during the replacement of part of the whey by brine in the M technology (Fig. 1).

No statistically significant differences ($P > 0.05$) in the NaCl content of the two types of reduced-fat Halloumi cheese were observed despite the different methods of salting. In the modified technology, NaCl was added as 10% sterilized brine containing 15% NaCl plus 1.5% dry salt to the cheese surface, whereas in the traditional technology dry salt only (2.5%) was added to the cheese surface.

There were statistically lower percentages ($P < 0.05$) of Ca, Mg and K in the reduced-fat type Halloumi cheese produced by the M technology compared with that produced by T technology. These differences suggest that the cheese production process plays a key role in the distribution of these elements (Kandarakis *et al*., 2004); for example, the removal of some of the whey (amounting to 30% of the milk used and the addition of sterilized brine solution (15% NaCl) to an amount of 5 % of the milk used and at the same temperature. A similar reduction in Ca was observed by realised also the researcher Rodríguez (1998), who reported that discarding the wastewater of the curd decreased the levels of soluble Ca in the cheeses with a decreased fat content. Economides (2004) also reported that reduced-fat Halloumi cheese has less Ca than traditional Halloumi. Furthermore, the increase in Ca content observed in reduced-fat Halloumi type cheese produced by T technology (heating the curd at 90◦C for 30 min) compared with that in reduced-fat Halloumi type cheese produced by M technology (heating the curd at 80◦C for 30 min) may result from more colloidal calcium phosphate at the higher cooking of the curd (Walstra and Jenness, 1984).

In these experiments, reduced-fat Halloumi type cheese produced from ovine milk by both M and T technologies is considered to be a rich source of calcium, 894 mg/100 g cheese and 1104 mg/100 g cheese, respectively. These amounts compare favourably with the lower calcium level present in reduced-fat Halloumi type cheese produced from bovine milk, 738 mg /100 g (Lteif *et al*., 2009), as well as in Slim Halloumi cheese, 690 mg/100 g cheese, and Lite Halloumi cheese, 320 mg/100 g cheese, on the Cyprus market (Economides, 2004). Similarly, the magnesium content of reduced-fat Halloumi type cheeses produced from ovine milk by M and T technology (49 mg/100 g cheese and 56mg/100 g cheese, respectively) was richer than that recorded for Slim Halloumi cheese (30 mg/100 g cheese) and Lite Halloumi cheese (20 mg/100 g cheese) from the Cyprus market (Economides, 2004).

No statistically significant differences ($P > 0.05$) in Na content between the two types of cheese were recorded, since the concentration of Na depended mainly on the amount of NaCl added to cheese.

Enumeration of Microorganisms in Reduced-fat Cheeses

The T reduced-fat Halloumi type cheese had a lower total bacterial count of 3.81 log cfu/g cheese

than that the M reduced-fat Halloumi cheese, which had a total bacterial count 4.40 log cfu/g cheese . This could be attributed to the higher temperature of curd cooking in the former (93-95^{0}C for 30 min) compared with the lower temperature (80^{0}C for 30 min) used for the production of M- reduced-fat Halloumi type cheese. The microorganisms that survived the high heat treatment could be thermotolerant microorganisms such *Lactobacillus brevis, Lactobacillus pentosus, Lactobacillus plantarum, Enterococcus faecium* and *Lactobacillus cypricasei* (Lawson et al., 2001). Coliforms and yeasts -moulds were not detected in either cheese since these are susceptible to the heating time of 30 minutes.

Textural Assessment of Reduced-fat Cheeses

The results of the textural assessment are presented in Table 2. Statistical analysis of the data showed that the reduced-fat Halloumi type cheese made by T technology exhibited higher hardness, elasticity adhesiveness, gumminess and chewiness than M-reduced-fat Halloumi type cheese.

In this study, the higher hardness of T-reduced-fat Halloumi type cheese was due to the higher total solids and ash content, the lower pH value and lower moisture content compared with that of M-reduced-fat Halloumi type cheese (Table 1). It is already known that a low pH results in the hardest cheese (Creamer and Olson, 1982).

The reduction of cohesiveness observed in cheese prepared by M technology could be attributed to serum proteins incorporated into it after the high pasteurization of the milk. Intense heat treatment of milk (M-cheese) has the effect of creating a weak cheese curd and looser paracasein network.

The lower elasticity observed in reduced-fat Halloumi cheese prepared by M technology could be attributed to serum protein incorporation after high pasteurization and their retention by caseins to form a more inelastic gel. Also, calcium content influenced positive the cheese elasticity. This appears to be in agreement with Prentice et al. (1993) that the rheological role of casein in cheese is to provide a continuous elastic framework for the individual cheese granules.

As shown in Table 2, the M-reduced-fat Halloumi type cheese had a lower adhesiveness value than T-reduced-fat Halloumi type cheese. The M-reduced-fat Halloumi type cheese had a lower gumminess value than T-reduced-fat Halloumi type cheese. The M-reduced-fat Halloumi type cheese had a lower chewiness value than T-reduced-fat Halloumi type cheese.

Organoleptic Evaluations

The results of the taste panel assessment (Table 3) showed that the reduced-fat Halloumi type cheese made by M technology received significantly ($P < 0.05$) higher scores for taste and flavour, texture and appearance than the reduced-fat Halloumi type cheese made by traditional technology. M- reduced-fat Halloumi type cheese had a softer texture, whiter color and was liked more. The stronger white coloring of cheese made by M technology is due to the coagulation of the serum proteins and their incorporation into

Table 2. Rheological characteristics of reduced-fat Halloumi type cheeses produced from ovine milk by traditional (T) and modified (M) technology. (Means of 5 trials ± standard error of mean).

Rheological characteristics	Reduced-fat ovine Halloumi type cheeses	
	Traditional technology (T)	Modified technology (M)
Hardness (N)	21.65 ^{b} ± 2.18	13.71 ^{a} ± 1.44
Cohesiveness (N.mm)	0.51 ^{a} ± 0.014	0.47 ^{a} ± 0.015
Elasticity (mm)	0.96 ^{b} ± 0.007	0.92 ^{a} ± 0.010
Adhesiveness (N.mm)	37.89 ^{b} ± 4.76	21.83 ^{a} ± 2.48
Gumminess (N.mm)	11.09 ^{b} ± 1.79	6.36 ^{a} ± 0.65
Chewiness (N.mm)	10.66 ^{b} ± 1.80	5.87 ^{a} ± 0.63

$^{a, b}$: Means in the same row with the same superscript did not differ significantly (P>0.05)

Table 3. Organoleptic evaluation of reduced-fat Halloumi type cheeses produced from ovine milk by traditional (T) and modified (M) technology. (Means of 5 trials ± standard error of mean).

Organoleptic characteristics	Reduced-fat ovine Halloumi type cheeses	
	Traditional technology (T)	Modified technology (M)
Taste and flavour(10)	$7.02^{a} \pm 0.72$	$7.86^{b} \pm 0.24$
Texture (10)	$6.67^{a} \pm 0.24$	$7.86^{b} \pm 0.11$
Appearance (10)	$7.09^{a} \pm 0.32$	$8.42^{b} \pm 0.24$

[a.b] Means in the same row bearing a common superscript did not differ significantly *(P>0.05)*.

the cheese curd. In contrast, the reduced-fat Halloumi type cheese made by T technology it was very hard and more yellowish in appearance.. The application of high temperatures (90◦C for 30 min) during the cheese curd cooking in T technology resulted in the formation of compounds that darken the colour of the cheese as a result of the Maillard reaction due to lactose reacting with its amino compounds, and cause increased evaporation of water from the open vat.

CONCLUSION

The results obtained enable us to draw the following conclusions: The novel reduced-fat ovine Halloumi type cheese produced by M technology had a higher yield due to higher moisture content and a higher percentage recovery of milk constituents in cheese. Also, M-reduced-fat ovine Halloumi type cheese had a softer texture, whiter color, higher sensory scores, and protein (caseins & serum proteins) in dry matter content, increasing the quality of this type of cheese compared with the T-reduced-fat ovine Halloumi type cheese. Therefore, it is proposed to utilise the M technology for the manufacture of good quality ovine Halloumi cheese with reduced-fat content. In contrary, T-reduced-fat ovine Halloumi type cheese had a higher acidity, calcium and phosphorus concentrations compared with the M-reduced-fat ovine Halloumi type cheese.

Further in this study the ovine cheeses obtained by these both technologies had a higher nutritional value (Ca and Mg content) than those of reduced-fat bovine Halloumi cheese currently available in the Cyprus market than that recorded for Slim Halloumi cheese and Lite Halloumi cheese from the Cyprus market (Economides, 2004).

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CONFLICT OF INTEREST STATEMENT

I have no conflict of interest to declare.

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Research article
Ερευνητικό άρθρο

Is rumenocentesis a safe technique to collect rumen fluid in dairy cows?

N. Panousis[1], G. Kitkas[1], G.E. Valergakis[2]

[1*]*Clinic of Farm Animals, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

[2]*Laboratory of Animal Husbandry, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

Είναι η παρακέντηση της μεγάλης κοιλίας ασφαλής μέθοδος για τη λήψη στομαχικού περιεχομένου στις γαλακτοπαραγωγούς αγελάδες;

Ν. Πανούσης[1], Γ. Κίτκας[1], Γ.Ε. Βαλεργάκης[2]

[1]*Κλινική Παραγωγικών Ζώων, Τμήμα Κτηνιατρικής, Σχολή Επιστημών Υγείας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης, Θεσσαλονίκη*

[2]*Εργαστήριο Ζωοτεχνίας, Τμήμα Κτηνιατρικής, Σχολή Επιστημών Υγείας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης, Θεσσαλονίκη*

ABSTRACT. Subacute ruminal acidosis is a major issue in dairy cattle and a definite diagnosis is only established by measuring the rumen fluid pH, most credibly collected by rumenocentesis. However, due to its invasive nature, there is still some debate whether it is a safe method or poses risks for cows' health and welfare. The aim of the study was to retrospectively evaluate the safety of rumenocentesis as a technique to obtain rumen fluid in dairy cows. Results were derived from 2 studies. In study 1, rumenocentesis was performed in 153 Holstein cows from 12 herds, once, between 10 and 90 days in milk (DIM). In study 2, 83 Holstein cows from a dairy farm were repeatedly subjected to rumenocentesis at 30, 90 and 150 DIM. From the 83 cows that were initially enrolled, 8 were culled before the end of the study for reasons irrelative to rumenocentesis; therefore, 236 rumenocenteses were actually performed in study 2 (3 times in 75

Corresponding Author:
N. Panousis, Clinic of Farm Animals, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece.
E-mail: panousis@vet.auth.gr

Αλληλογραφία:
Ν. Πανούσης, Κλινική Παραγωγικών Ζώων, Τμήμα Κτηνιατρικής, Σχολή Επιστημών Υγείας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης, 541 24 Θεσσαλονίκη.
E-mail: panousis@vet.auth.gr

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cows, twice in 3 cows and once in 5 cows). All cows were monitored for 10 days after rumenocentesis for presence of complications. In addition, daily milk yield was automatically recorded for each cow in study 2 to detect any possible post-rumenocentesis short-term reduction of milk yield. Minor only complications were recorded in 7 cases: in 4/153 (2.61%) and 3/236 (1.27%) rumenocenteses in studies 1 and 2, respectively. Small diameter abscesses in 6 cows (3 in study 1 and 3 in study 2) and a larger one in 1 cow in study 1 were observed. The 3 small abscesses in study 2 were all recorded after the 1st rumenocentesis, at DIM 30. All 7 cases were resolved spontaneously within two weeks. Moreover, short-term daily milk yield of study 2 cows was not affected by rumenocentesis. The conclusion is that rumenocentesis is a safe technique to collect small volume of rumen fluid for SARA diagnosis, which does not compromise cows' health and welfare when appropriately performed.

Keywords: rumenocentesis, safety, complications, ruminal fluid, dairy cows

ΠΕΡΙΛΗΨΗ. Η υποξεία δυσπεπτική οξέωση είναι πολύ σημαντικό νόσημα των γαλακτοπαραγωγών αγελάδων και η διάγνωσή της στηρίζεται στη μέτρηση του pH του περιεχομένου της μεγάλης κοιλίας. Η παρακέντησή της είναι η μέθοδος επιλογής για τη λήψη του, αλλά επειδή αποτελεί μια μικρής έκτασης χειρουργική τεχνική, υπάρχουν ακόμη διαφωνίες ως προς την ασφάλειά της και τον κίνδυνο εμφάνισης επιπλοκών στις αγελάδες. Στόχος της εργασίας ήταν η διερεύνηση της ασφάλειας της παρακέντησης της μεγάλης κοιλίας ως μέθοδος λήψης στομαχικού περιεχομένου. Τα αποτελέσματα προέκυψαν από 2 μελέτες. Στην πρώτη, πραγματοποιήθηκε παρακέντηση σε 153 αγελάδες φυλής Holstein από 12 εκτροφές, μία φορά, μεταξύ της 10ης και της 90ης ημέρας της γαλακτικής περιόδου (DIM). Στη 2η μελέτη χρησιμοποιήθηκαν 83 αγελάδες Holstein από 1 εκτροφή, με στόχο να παρακεντηθούν 3 φορές η κάθε μία, στις 30, 90 και 150 DIM. Λόγω πρόωρης απομάκρυνσης 8 αγελάδων για λόγους άσχετους με την παρακέντηση, έγιναν τελικά 236 παρακεντήσεις: 3 φορές σε 75 ζώα, 2 φορές σε 3 και μία φορά σε 5 αγελάδες. Όλα τα ζώα παρακολουθούνταν επί 10 ημέρες μετά την κάθε παρακέντηση για την παρουσία επιπλοκών. Επιπλέον, στη μελέτη 2 καταγραφόταν καθημερινά η ατομική γαλακτοπαραγωγή για την καταγραφή τυχόν ελάττωσης της παραγόμενης ποσότητας λόγω της παρακέντησης. Επιπλοκές παρατηρήθηκαν μόνον σε 7 περιπτώσεις: σε 4/153 (2.61%) και 3/236 (1.27%) παρακεντήσεις από τις μελέτες 1 και 2, αντίστοιχα. Αφορούσαν σε αποστήματα μικρής (6 ζώα, από 3 στην κάθε μελέτη) και μεγαλύτερης (1 ζώο στη μελέτη 1) διαμέτρου. Τα 3 περιστατικά με αποστήματα της μελέτης 2 καταγράφηκαν μετά την 1η παρακέντηση (στις 30 DIM). Όλες οι 7 περιπτώσεις αποστημάτων υποχώρησαν αυθόρμητα εντός 15 ημερών. Το ύψος της γαλακτοπαραγωγής δεν επηρεάστηκε από την παρακέντηση. Συμπερασματικά, η παρακέντηση είναι ασφαλής μέθοδος για τη λήψη υγρού περιεχομένου από τη μεγάλη κοιλία των αγελάδων όταν πραγματοποιείται κατάλληλα.

Λέξεις ευρετηρίασης: παρακέντηση μεγάλης κοιλίας, επιπλοκές, γαλακτοπαραγωγές αγελάδες

INTRODUCTION

Animal health and welfare are of increased interest in recent years (Thompson, 2005; Lusk and Norwood, 2008). Diagnostic testing procedures could induce pain and fear, responses that could affect welfare status. Therefore, it is appropriate to investigate the potential of diagnostic techniques to adversely affect animal health and welfare relative to the benefit of obtaining diagnostic results (Gianesella et al., 2010).

Subacute ruminal acidosis (SARA) is an issue of major economic and physiological concern in dairy cattle (Nocek, 1997; Enemark, 2008), characterized by ruminal fluid pH between 5.0 and 5.5 (Kleen, 2003). Due to the lack of pathognomonic clinical signs, a definite SARA diagnosis in clinical practice is only established by measuring the rumen's fluid pH, either at a specific time-point after feeding (rumen fluid is collected by stomach tubing or, more credibly, by rumenocentesis) (Duffield et al., 2004) or continuously

(using rumen boluses and pH monitoring systems). The latter is the best method, but still too expensive.

Rumenocentesis is more accurate than rumen tubing because the collected rumen fluid samples are not "contaminated" with varying amounts of saliva (Dirksen and Smith, 1987), which results in inaccurate pH measurements (Nordlund and Garrett, 1994; Oetzel and Nordlund, 1998). Some practitioners may consider the procedure too difficult to use during clinical investigations but it is easy to perform by an experienced clinician. However, it remains a minor surgical procedure and, due to its invasive nature, there is still some debate whether it is a safe method or poses risks for cows' health and welfare. Some authors stated that rumenocentesis can cause complications in the area of the puncture site (Hollberg, 1984; Strabel et al., 2007) and therefore is not an appropriate procedure for obtaining rumen fluid from cows (Strabel et al., 2007), while others that it is a safe procedure with minimal adverse effects for health and milk production (Kleen, 2004; Nordlund, 2007; Gianesella et al., 2010).

Considering the significance of SARA and given the above controversy, the aim of the study was to retrospectively evaluate the safety of rumenocentesis as a technique to obtain rumen fluid in dairy cows.

MATERIALS AND METHODS

This research was conducted with the approval of the review board of the Faculty of Veterinary Medicine, Aristotle University of Thessaloniki. The farmers gave informed consent for the cows to be included in the study and the testing procedures.

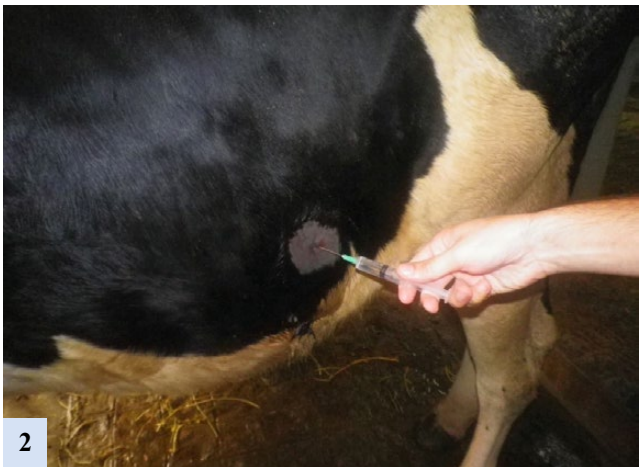
Data were derived from 2 studies. In study 1, rumenocentesis was performed in 153 Holstein cows from 12 herds of Central Macedonia region - Greece, once, between 10 and 90 days in milk (DIM), in order to collect rumen fluid for SARA prevalence assessment (Kitkas et al., 2013). Commencement of study 2 took place approximately 10 months after the completion of study 1. In study 2, which aimed to evaluate SARA prevalence as well as its effect on rumen fatty acids and milk parameters in different stages of lactation, 83 Holstein cows from a dairy farm located in the region of Thessaloniki, Greece, were overall used and repeatedly subjected to rumenocentesis at 30, 90 and 150 DIM. Farm selection was based on history of high SARA prevalence (farm #11, Kitkas et al., 2013). From the 83 cows that were initially enrolled, 8 were culled before the end of the study due to mastitis and/or lameness; therefore, 236 rumenocenteses were actually performed in study 2 (3 times in 75 cows, twice in 3 cows and once in 5 cows).

All cows were randomly chosen as long as they were clinically healthy, based on recent history and a clinical examination prior to each rumenocentesis. The same author performed all 389 rumenocenteses (153 in study 1 and 236 in study 2).

Rumenocentesis was consistently performed 5-8 hours after the morning feeding. Cows were restrained without sedation. The puncture site was located 10-15 cm, according to cow size, behind the last left rib on the horizontal line passing through the stifle. A small area (10 cm x 10 cm) was shaved and disinfected with 7.5% iodine povidone scrub solution (Figure 1).

Local anaesthesia was performed prior to rumenocentesis by injecting 4 mL of 2% Xylocaine

1



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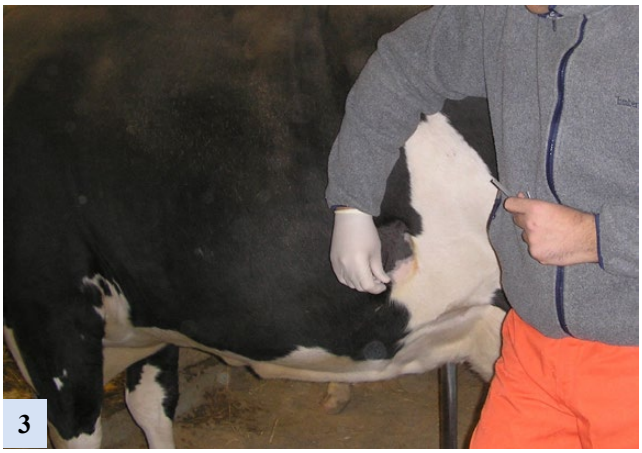

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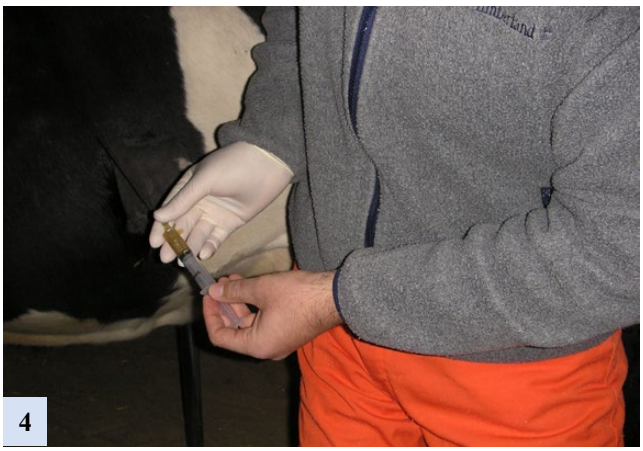

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(AstraZeneka, Athens, Greece), containing 20 mg/mL lignocaine hydrochloride, at the puncture site (2 mL subcutaneously and 2 mL intramuscularly) (Figure 2).

Then, a 1.65 x 130 mm stainless steel needle (H. Hauptner & Richard Herberholz GmbH & Co. KG, Solingen, Germany) was inserted through the skin into the rumen (Figure 3). During this procedure, an assistant was raising the cow's tail vertically to her body for better restraint, while in extremely stressed animals a nose holder was additionally applied.

Approximately 2-3 mL of rumen fluid were carefully aspirated, within 20 sec, into a 5 mL plastic syringe (Figure 4).

All cows were monitored for 10 days after rumenocentesis for the presence of rumenocentesis-related complications like peritonitis, hematoma or abscess formation at the puncture site and any other health issue. In case of complication(s), monitoring period was extended until all lesion(s) resolved. In addition, daily milk yield was automatically recorded for each individual cow in study 2, using an automatic milk yield recording system (AfiFarm Herd Management Software®, Afimilk Ltd., Kibbutz Afikim, Israel), to detect any post-rumenocentesis short-term reduction of milk yield during the monitoring period.

RESULTS

Minor only complications were recorded in 7 cases: in 4/153 (2.61%) and 3/236 (1.27%) rumenocenteses in studies 1 and 2, respectively. Small abscesses (<3 cm in diameter) in 6 cows (3 in study 1 and 3 in study 2) and a larger one (ca. 10 cm in diameter) in 1 cow in study 1 were observed. The 3 small abscesses in study 2 were all recorded after the 1st rumenocentesis, at DIM 30. All 7 cases were resolved spontaneously within two weeks. Moreover, short-term daily milk yield of study 2 cows was not affected by rumenocentesis.

DISCUSSION

The objective of the current study was to evaluate whether rumenocentesis, the most accurate method to evaluate rumen pH values for SARA diagnosis, poses health risks for the cows. The disadvantage associated with the technique is that it is quite invasive and could theoretically result in peritonitis (Nordlund and Garrett, 1994; Kleen, 2004), haematoma and abdominal or ruminal wall abscessation at the puncture site (Nordlund and Garrett, 1994; Abdela, 2016).

Hollberg (1984) pathologically examined 47 cows that were slaughtered 1-6 days after rumenocentesis; hemorrhages in the ruminal wall, peritoneal adhesions, peritonitis and hematomas were diagnosed in the area of the puncture site in 41 out of the 47 (87%) cows. However, rumenocentesis in this study was done with a large needle (2.4 x 150 mm) with two side fenestrations at the tip, and a large volume of fluid (200 mL) was collected over a prolonged period (>100 sec). On the contrary, in the present study rumenocentesis technique was performed in a different way: the size of the needles was smaller (1.65 x 130 mm), they had not any side fenestrations, volume of the collected rumen fluid (2-3 mL) was smaller and the duration of the aspiration (<20 sec) was shorter. These differences could explain the low incidence and severity of complications recorded in our research.

In a Swiss study, the clinical status of 11 dairy cows that were subjected to rumenocentesis (using 1.8 x 130 mm needles) was daily evaluated; the preparation of the

puncturing site, the needle size, the technique applied to collect rumen fluid, and its quantity were almost similar with the present study. All cows were slaughtered and necropsied 7 days after rumenocentesis in order to detect possible complications (pathologic lesions) at necropsy (Strabel et al., 2007). The following abnormal clinical signs were diagnosed: increased respiration depth (3 cows), transient episode of hyperthermia (2 cows), increased tension of the abdominal wall (8 cows) and positive foreign body tests (3 cows). One cow was culled on day 7 because of severe generalized septic peritonitis, spreading from the site of rumenocentesis, and hematoma in the area of the puncture site was found in 9 out of 10 cows.

The authors concluded that the severe complications recorded do not legitimate rumenocentesis as a routine procedure for collection of rumen fluid in cows under Swiss conditions. However, besides the unfortunate case of generalized peritonitis, the severity of other signs and lesions recorded could be disputed. The results of the present study that included a large number of cows kept under field conditions showed only minor health complications, in only a few ones, and no adverse effects on milk production.

In contrast to the above studies, Nordlund and Garrett (1994) reported an incidence of subcutaneous abscess formation of only 1-2%, which is in accordance with the present findings of 1.79% abscessation incidence (7 abscesses in 389 rumenocenteses).

Kleen et al. (2004) collected rumen fluid with rumenocentesis from 164 Holstein cows of 19 farms in the Dutch province of Friesland. Local anesthesia with 2 mL of 2% lidocaine was provided to 119 out of the 164 cows. Puncturing site selection and preparation as well as restraint were similar with that applied in the present study. Rumenocentesis was made with 2.1 x 80 mm size needles. The average volume of the aspirated rumen fluid was 14.8 mL (SD 3.6). The proportion of cows not showing complications due to rumenocentesis was significantly higher in animals that received local anaesthesia. Concerning complications, swelling, hematomas and small diameter abscesses were diagnosed in 5/164 (3%) cows. Health status of other 3/164 (1.8%) cows was impaired as they showed depression and reduction of dry matter intake; 1 of those 3 had also elevated rectal temperature and another 1 of the 3 was diagnosed with left abomasal displacement (LDA) the following of the rumenocentesis day. However, whether LDA is due to rumenocentesis is questioned even by the authors. The lower incidence of complications recorded in our study might be due to the smaller outside diameter of the needles (1.65 vs. 2.1), the smaller volume of aspirated fluid or the experience of the person performing the rumenocenteses.

Duffield et al. (2004) performed rumenocentesis in 16 Holstein cows repeatedly (4 times), at weeks 6, 8, 10 and 12 after calving, in order to compare ruminal pH value collected with different techniques. The puncturing site (left flank, at the level of the stifle and approximately 15 to 20 cm caudoventral to the costochondral junction of the last rib) was surgically prepared and the animals were sedated with xylazine, without application of local anesthesia. Rumenocentesis was done with 1.6 x 125 mm size needles and the aspirated ruminal fluid volume was 2-5 mL. Complications of rumenocentesis were limited to the development of 1 to 2 small (1 to 2 cm) nodular swellings at the puncturing site in approximately 33% of the cows, without any short- or long-term adverse consequences. The finding of the present study, including much more rumenocenteses and cows, that rumenocentesis is a safe technique to collect ruminal fluid is in agreement with the above.

In Italy, Gianesella et al. (2010) performed rumenocentesis in 6 cows and used another 6 as controls, to evaluate the effect of the technique on health, welfare status and milk production of lactating dairy cows. The puncturing site of all cows in both groups was sheared and disinfected, and the rumenocentesis was made without prior local anesthesia, with 2.4 x 105 mm needles. Milk yield and rectal temperature were daily recorded and they were not affected by rumenocentesis. Average superficial skin temperature at the puncturing site increased by 1.0 °C immediately after rumenocentesis and returned to normal level after 48 h, where it remained constantly until the end of the study. These results suggest that rumenocentesis has minimal adverse effects on cows health and production, which is in accordance with our findings.

It seems that the small needle size and volume of collected rumen fluid (enough to determine pH and ruminal fatty acids concentration), as well as the short duration of aspiration were the main underlying factors

responsible for the scarce post-puncture complications recorded in the present study. Additional supporting factors were the skills and experience of the person performing the technique and, possibly, the application of local anesthesia. The fact that this is the only accessible study in literature that uses data from so many observations (389 rumenocenteses in 236 dairy cows), including repeated ones in 75 cows, increases the soundness of the findings.

CONCLUSIONS

Rumenocentesis is a safe technique to collect small volume of rumen fluid for SARA diagnosis, which does not compromise cows' health and welfare when appropriately performed.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

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Short communication
Σύντομη δημοσίευση

Stability of HCT, HGB and RBC values in the Mute Swan *(Cygnus olor)* blood stored at 4°c and 24°c differs between traditional and multi-parameter automated methods

M.A. Trzeciak, W. Meissner

Avian Ecophysiology Unit, Department of Vertebrate Ecology & Zoology, University of Gdańsk, Gdańsk, Poland.

ABSTRACT. The objective of this study was to evaluate the influence of time of sample storage, method of analysis, and storage temperature on stability of HCT, HGB, and RBC in avian blood samples. Blood samples from mute swans were stored at 24°C or 4°C. Analyses of HCT, HGB, and RBC were carried out after 5 and 25 hours after collection of blood using both traditional and Cell-Dyn 3700 analyzer methods. Storage temperature had no significant influence on HCT, HGB, and RBC values of mute swan blood. However hematological parameters obtained by the traditional method were significantly lower than the results from the analyzer. Time of storage had a significant influence on values obtained traditionally for HCT, HGB, and RBC, but did not affect results that were obtained by the multi-parameter automated method. These results indicate that the mute swan blood can be stored at both room and refrigerator temperature. However, the analysis should be performed as soon as possible, especially in the case of traditional methods. Reference intervals for this species should always include information about the method used for hemogram determination and time elapsed since the blood collection.

Keywords: Avian blood, Cell-Dyn 3700 analyzer, Mute Swan, storage time, temperature.

Correspondence: Włodzimierz Meissner,
Avian Ecophysiology Unit, Department of Vertebrate Ecology & Zoology,
University of Gdańsk, WitaStwosza 59, 80-308 Gdańsk, Poland.
E-mail: w.meissner@ug.edu.pl

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INTRODUCTION

The key parameters included in the hemogramare: packed cell volume (HCT), hemoglobin (HGB), and red blood cell count (RBC) (Ihedioha et al. 2007; Owen 2011). It is known that the number of preanalytical factors, such astime of storage before analysis, method of analysis, and storage temperature of the sample, may influence the final value of hemogram parameters (Hadzimusic et al. 2010). Although it has been suggested that avian blood should be stored in a refrigerator before the analysis (Hadzimusic et al. 2010; Owen 2011), there have been no reports of studies on artifactual changes in avian hematological parameters determined either by traditional (manual) ormulti-parameter automated analysis. This study evaluated the influence of storage time, storage temperature, and analysis method on the variation in RBC, HCT, and HGB values in avian blood.

The model bird species chosen for our study was the mute swan *Cygnus olor*. This species is often treated in wildlife rehabilitation centers, both in Europe and in North America, where diagnostic CBC tests are performed in order to evaluate their condition status, especially before releasing them into the wild (e.g. The Swan Sanctuary: http:// www.theswansanctuary.org.uk, Wings Wildlife Rehabilitation Centre: http:// www.wingsrehab.ca). Mute swans weighing 4–16 kg are a good model for this CBC study, as it is possible to obtain large quantities of blood from one individual, which is necessary to carry out different hematological analyses.

MATERIALS AND METHODS

Sample collection

Blood samples were obtained from 10 adult males and 10 adult females of mute swans wintering along the municipal Baltic coast of Gdańsk, Sopot, and Gdynia on the Polish Baltic coast. Body masses of birds used in this study were in the range of those given in the literature for the non-breeding period (7.47–11.73 kg in females, and 8.06–12.03 kg for males) (Wieloch et al. 2004).Thus, it was assumed that blood samples were obtained from mute swans that were in good condition. Within 10 minutes of the swans beingcaught, blood (2 ml) was collected from the metatarsal vein into a test tube containing EDTA anticoagulant. Capture of birds and blood collection were allowed based on a Permit of the Local Ethics Committee (no. 61/2012). Blood samples were collected in the morning hours from no more than four birds at a time. The exact time of blood collection from a particular individual was recorded. All samples were then transported to the laboratory (time of transit ranged from 30 to 40 minutes). All blood samples were divided into two aliquots: one was intended for traditional analyses, while the other was used for multi-parameter automated analysis.

Laboratory analysis

Both traditionally and automatically analyzed samples were then split further into twoequal parts: one part was kept on the work-topin the laboratory at room temperature (~24°C), while the other part was stored in a refrigerator (4°C).Hematologic analyses were carried out for all samples at 5 and 25 hours after collection of blood using both traditional and multi-parameter automated methods.

The only parameter determined for ambient temperature only, and not for refrigerator temperature,was traditional HCT obtained 3 hours after blood collection. This exception was due to the fact that a pilot study showed that at~4 hours after collection, swan blood very often becomes hemolysis in the microhematocrit centrifuge and hence, these results become unreliable. After 4 hours from collection of blood, samples stored at 4°C and those at 24°C did not differ and 80% of them had no signs of hemolysis. After 5 hours, only 15% (samples stored at 24°C) and 5% (samples stored at 4°C) had no signs of hemolysis. At 24°C hemolysis occurred more slowly (G test, P=0.003). After 10 hours at least a partial hemolysis was observed in all samples. 70% of the samples stored at 24°C and 80% of the samples stored at 4°C have been completely hemolysis after 10 hours (Fig. 1). However, in the case of determination made by the analyzer, such a phenomenon has not been observed.

Hematologic determinations were carried out following standard traditional laboratory procedures for avian bloodand in hematological analysis.[9] In traditional method HCT was determined by the microhematocrit method (10640 x g; Microhematocrit Centrifuge Type 346, Unipan, Warsaw 00-818, Poland).The HGB concentration was obtained using the cyanmethemoglobin method (elimination of nuclei: centrifugation

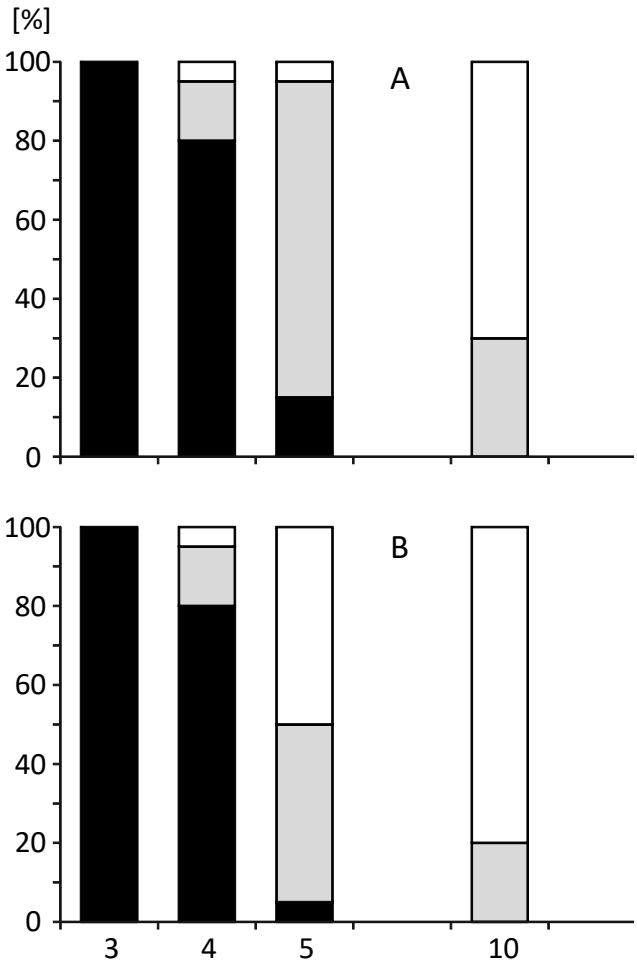

Fig 1:. Comparison of swan blood hemolysis in the microhematocrit centrifuge after: 3, 4, 5 and 10 hours from sample collection (A – samples kept at 24°C, B – samples kept at 4°C). Black bar – no signs of hemolysis, grey bar – partial hemolysis, white bar – complete hemolysis.

at 1000 x g for 10 min): Drabkin's solution (Analab, Warsaw 02-055, Poland) and spectrophotometer (7 Series Spectrophotometer Model 722, Hinotek, Ningbo, Zhejiang 315040, China) with wavelength 540 nm. RBC was determined using a hemocytometer (an improved Neubauer counting chamber). Before the count, blood was diluted 200 timeswith saline solution. Diluted blood was applied to the Neubauerchamber (Marienfeld Superior, Lauda-Königshofen, Bavaria 97922, Germany). Erythrocytes were counted in all squares exactly 3 min after application (magnification x400, microscope Ken-A-Vision, Kansas City, MO 64133, USA). To obtain the final RBC in 1μl, the sum of cells counted in all squares was multiplied by 10 (as the volume of the chamber, 0.1μl, multiplied by 10 gives 1μl) and finally the result was multiplied by the dilution (x200). Counting of every sample was repeated twice. The final result was the average of the two counts. Differences between the two results obtained for counting of the same sample did not exceed 5%.

Automated determinations were carried out using hematological analyzer Cell-Dyn 3700 (Abbott, Abbott Park, IL60064, USA) and the Veterinary Package Program was applied. Before analyses, the analyzer was configured on the basis ofpre-programmed configuration files designed for domestic (Pekin) duck (DUCK MODE). The appropriate analyzer settings were computed for a newly created file intended for swans by running blood samples from the mute swan on the analyzer. The position of each scattergram and histogram (COMPLEXITY, LOBULARITY, RBC, and PLT) were then compared to the reference scattergrams and histograms (included in the analyzer'smanual). Adjustment factors were computed from these data for electronic gain settings. Final configurations of gain settings for mute swan used in this study were as follows: WOC 0D: 2896, WOC 10D: 1500, WOC 90D: 2996, WOC 90DP: 2278, RBC: 1200, PLT: 2727, WIC: 3325.

HGB is directly measured by the Cell-Dyn 3700 analyzer using a modified hemoglobin hydroxylamine method, in which a filtered LED with a wavelength of 540 nm is the light source. A photodetector measures the light that is transmitted, and the HGB concentration is expressed in grams of hemoglobin per deciliter. RBC is counted using electrical impedance. HCT is calculated by the analyzer according to the obtained values of RBC and MCV.

Statistical analysis

Repeated measures ANOVA was used for a comparison of mean RBC and HGB values at 5 and 25 hours after bleeding. The applied method (traditional or Cell-Dyn) and temperature (room temperature and refrigerator) was used as discrete factors.In the case of HCT measurements, two different analyses were conducted. The results obtained from Cell-Dyn were compared using repeated measures ANOVA with two levels of temperature as the discrete factor. Microhematocrit results were compared only with the first reading (after 5 hours) by the Cell-Dyn by a single factor ANOVA. Assumption of sphericity was not violated in all repeated measures

ANOVA analyses (Mauchly'sphericity test, p>0.05 in all cases), andhence, univariate analysis was selected. The Tukey test was used for post-hoc comparisons after ANOVA.All calculations were conducted using Statistica 10 software (StatSoft Inc., Tulsa, OK 74104, USA). Significance level was set at p<0.05.

RESULTS

There were no statistically significant differences among mean HCT values obtained after 5 and 25 hours in analyzer (p=0.43). Temperature had no influence on mean HCT (p=0.96). Mean HCT obtained by microhematocrit method was significantly lower than both mean results from analyzer (p<0.001) (Fig. 2).

Mean HGB was significantly higher in the case of the Cell-Dyn method compared to the Drabkin's method after both 5 and 25 hours (p<0.001, Fig.3). Bythe Drabkin'smethod, there was a statistically significant decrease in mean HGB between measurements taken at 5 and at 25 hours after bleeding (p=0.04).

In the case of RBC, count method (p<0.001) and time (p<0.001) had a significant influence on the obtained results with a statistically significant interaction between these factors (p<0.001). Storage temperature had no significant effect on RBC count result (p=0.08). There were no statistically significant differences between mean Cell-Dyn counts after 5 and 25 hours (p=0.91), but mean RBC traditional counts decreased gradually with time (p<0.001), while Cell-Dyn count remained at the same level (Fig. 4). Ateach time-point,

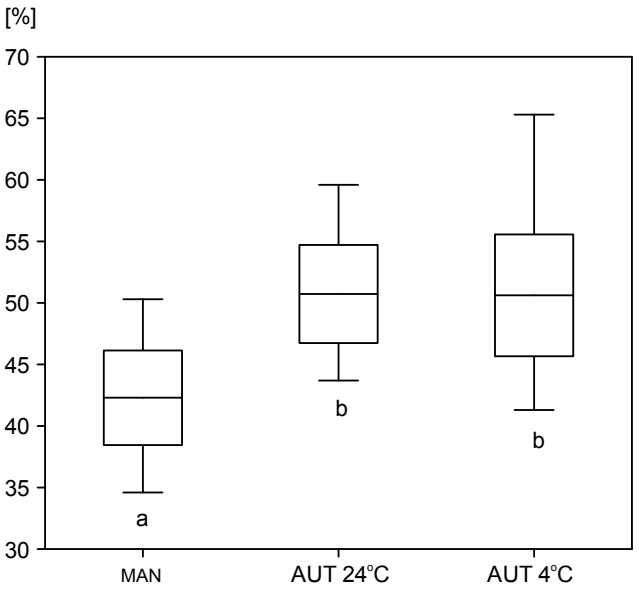

Fig 2:. Comparison of mean HCT obtained by traditional method (MAN) and by multi-parameter automated method after storing of the sample in room temperature (AUT 24°C) and in refrigerator (AUT 4°C). Horizontal line – mean, rectangle – standard deviation, vertical line – range. Letters designate means not significantly different from each other (p<0.05).

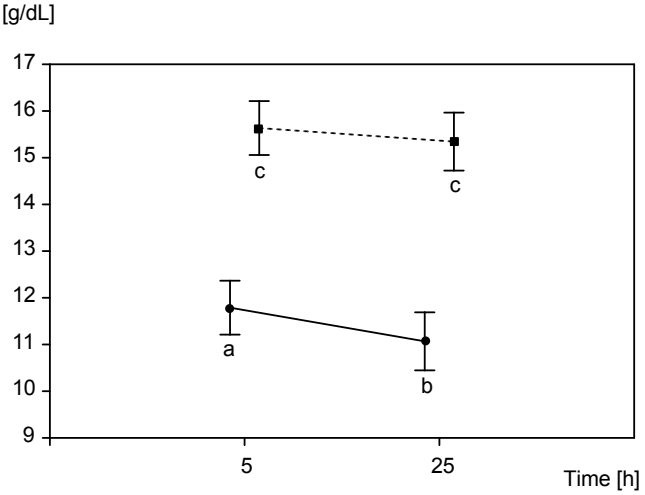

Fig 3:. Comparison of mean HGB obtained by traditional (dots, solid line) and multi-parameter automated method (squares, dashed line) after 5 and 25 h from bleeding. Vertical line shows 95% confidence limit. Letters designate means not significantly different from each other (p<0.05).

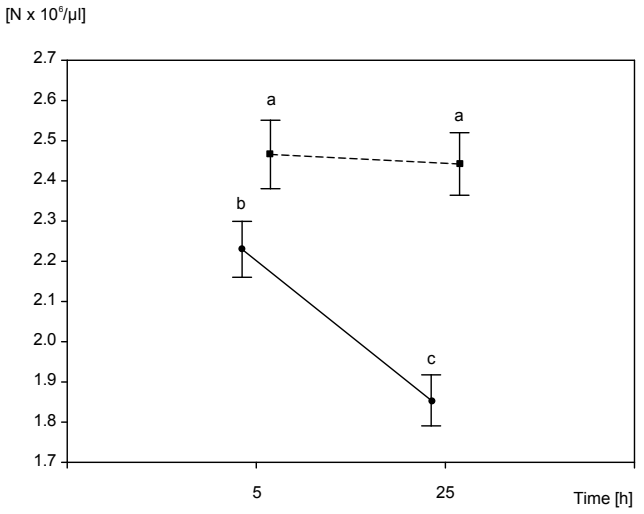

Fig 4: Mean RBC count using Cell-Dyn (dots, dashed line) and traditional method (squares, solid line) after 5 and 25 hours after bleeding. Vertical lines show 95% confidence intervals. Letters designate means not significantly different from each other (p< 0.05).

the Cell-Dyn RBC count was significantly higher than that from the traditional count $p<0.003$ in both cases).

DISCUSSION

Sample stability is the ability of its analyte to maintain the initial value of the determined parameter. Analyte stability, however, may vary due to storage temperature of the sample, time elapsed since blood collection, animal species, and method of analysis (Ameri et al. 2011).In this study, temperature of sample storage had no significant effect on analyte stability for all of the determined parameters (HCT, HGB, and RBC) regardless of the determination method (traditional or multi-parameter automated method). Based on other studies, temperature may have a different impact on sample stability in various mammalian species (Ihediohaet al. 2007). There are few data on the effect of storage temperature on the stability of avian hematological parameters. However, it was suggested that turkey's blood should be stored at refrigerator temperature before hematological analysis (Hadzimusic et al. 2010). It has also been demonstrated that storage temperature may differently affect the fragility of erythrocytes of different bird species. For example, storage of blood at 10°C for 24 hours causes a significant decrease in the fragility of pigeon *Columba livia forma domestica* and peafowl *Pavo cristatus* erythrocytes and a significant increase inthe erythrocyte fragility in domestic fowl *Gallus gallus domesticus* (Oyewale 1994). Therefore, storage temperature should be stable to provide comparable conditions for the determination of hemogram in the subsequent blood samples.

Time of storage and method of analysis had a significant influence on the stability of the mute swan HCT, HGB, and RBC. For all of the measured parameters, higher stability was observed in the case of the multi-parameter automated analysis method. Moreover, values of HCT, HGB, and RBC were higher when determined automatically. The differences between susceptibility to storage time of traditionally and automatically measured parameters are most probably due to the higher fragility of the mute swan erythrocytes in the case of traditional methods. There are numerous studies that show that the fragility of erythrocytes of different bird species varies to agreat extent (Viscor and Palomeque1982; Oyewale and Durotoye 1988; Oyewale 1992. Even within one species of birds, fragility of erythrocytes may vary significantly among different breeds, even though their hemogram parameters are identical, which was shown in three breeds of chicken (Oyewale and Durotoye1988). Interspecies differences in erythrocyte fragility with similar hemogram values suggest that red blood cells of different bird species may react differently in the same conditions. Even small differences in hemoglobin structure may result in very distinct physiological properties of erythrocytes (Oberthür et al. 1982; Habibu et al. 2013). In the case of HCT, the stability of the values obtained after traditional analysis was so susceptible to the time of storage that, after 5–6 hours since blood collection, the results obtained by the microhematocrit method were not reliable due to substantial hemolysisthat occurred in micro-capillaries. This was due to the fact that, during centrifugation of blood in micro-capillaries, the majority of the mute swan erythrocytes were destroyed duringthe longer time of blood storage, because the erythrocyte fragility increases. In the case of the multi-parameter automated method, HCT was not determined directly, but was calculated on the basis of two other parameters: MCV and RBC. Therefore, the high fragility of the mute swan erythrocytes did not affect the obtained result. These results are in agreement with other published data, where the mute swan HCT obtained traditionally was lower (21–44%) than HCT determined by the analyzer method (35–50%) (Routh and Sanderson 2010; Dolka et al. 2014).

Furthermore, RBC determined traditionally decreased significantly during the storage of samples, whereas RBC measured automatically did not change during the time of blood storage. In the case of RBC obtained by the traditional method, erythrocytes were diluted in saline solution and remained there for at least 7 minutes (mixing in saline solution, application to a Neubauer chamber, and counting). Unlike in the case of the traditional counting method, RBC values that were measured automatically were determined almost immediately after the aspiration of the blood sample. Therefore, the duration of diluent's influence on erythrocytes was much shorter (only a few seconds), and so majority of erythrocytes remained unhemolysed. This result also agrees with other data published for themute swan, where RBC values obtained by the traditional method were lower (1.72–2.43 x 10^6/μl) than values determined

by the analyzer method (1.85–2.86 x 10^6/µl) (Routh and Sanderson 2010; Habibu et al. 2013).

In the case of HGB, the significant instability of values obtained using the Drabkin's method may be connected to the interspecies differences between erythrocytes (e.g., high fragility of erythrocytes in the mute swan). Perhaps also the hemoglobin of various avian species may react differently in comparable conditions. For example, it is known, that amino acid sequence of hemoglobin's chains in the Canada goose *Branta canadensis* is very similar to the mute swan hemoglobinsequence (Oberthür et al. 1982). However, oxygen affinity of the mute swan's hemoglobin is much higher (Oberthür et al. 1982). The Drabkin's method is based on the reaction, in which hemoglobin and some of its derivatives are oxidized by $K_3[Fe(CN)_6]$ to methemoglobin, and then converted under the influence of KCN in a stable compound: cyanmethemoglobin (Drabkin 1949). It can be assumed that the hemoglobin in mute swans is in some way different from the hemoglobin of other avian species, in which the Drabkin's method was successfully used, as it is recommended for many avian species (Owen 2011). However, in the studies where the Drabkin's solution method was used to determine the mute swan HGB, the range of values was very wide. For example, in data published by Dolka et al. (2014), the range of HGB values for normal, adult mute swans was 8.5–24.6 g/dl. In comparison, the range of values obtained by other authors using multi-parameter automated methods is much narrower: 11–16.5 d/dl or 10.6–16.1 g/dl (Flinchum 2006; Dolka et al. 2014). This data are in agreement with our results, where the range of HGB determined traditionally is much wider (7.1–19.7 g/dl) than the values of HGB measured automatically (12.2–19.2 g/dl). Moreover, in our study, there was a significant difference between HGB values determined with the two different methods, even for the results obtained 5 hours after blood collection. Results of HGB that was measured automatically were not only more stable than the traditional ones, but also similar to the data published by other authors. Therefore, it is suggested that the method using Drabkin's solution and centrifugation should either not be used for the mute swan, or the results obtained by this method should only be compared with HGB values determined by the same method. Moreover, it is suggested that in the case of the mute swan hemogram reference intervals, the time of sample storage before the analysis should always be stated, at least in the case of traditional methods of determination, as previously this information has beenomitted by some authors (Dolka et al. 2014).

In conclusion, the mute swan blood can be stored both at room temperature and refrigerator temperature. However,the analysis should be performed as soon as possible, especially in the case of traditional methods. It is recommendedto determine hematological parameters of the mute swan within 3–4 hours in the case of the traditional method. It is suggested that the results obtained for the mute swan hemogram by traditional and multi-parameter automated methods should be treatedseparately. Thus, reference intervals for this and other species should always include information regardingthe method used for hemogram determination and the time elapsed since the blood collection. It is also suggested that the Drabkin's method may not be optimal for the evaluation of hemoglobin concentration in the mute swan blood stored for a longer time.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Case Report
Κλινικό Περιστατικό

Sesamoid disease in two dogs

A.F. Karkanis[1], N.N. Prassinos[1], G. Kazakos[1], M. Ktoris[2], K. Antonopoulos[3], N. Krystalli[1]

[1] Companion Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences,

Aristotle University of Thessaloniki, Greece

[2] Limassol Veterinary Clinic, Limassol, Cyprus

[3] Small Animal Clinic, Kalamata, Greece

Νόσος των σησαμοειδών στον σκύλο. Αναφορά σε δύο κλινικά περιστατικά

Καρκάνης Α.Φ.[1], Πράσινος Ν.Ν.[1], Καζάκος Γ.[1], Κτωρής Μ.[2], Αντωνόπουλος Κ.[3], Κρυστάλλη Ν.[1]

[1] Κλινική Ζώων Συντροφιάς, Τμήμα Κτηνιατρικής, Σχολή Επιστημών Υγείας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

[2] Κτηνιατρική Κλινική Λεμεσού, Κύπρος

[3] Κλινική Μικρών Ζώων, Καλαμάτα

ABSTRACT. Two young dogs, a Rottweiler and an Épagneul Breton, presented with chronic forelimb lameness. Orthopaedic examination confirmed the mild lameness and revealed pain on deep palpation at the palmar aspect of the 5th metacarpal. Radiographic evaluation revealed fracture of the 7th metacarpophalangeal sesamoid at the 5th metacarpophalangeal joint. Based on these findings, diagnosis of sesamoid disease was achieved. Both cases had been treated conservatively with restricted activity and anti-inflammatory drugs without any success. Then, resection of the affected sesamoid was performed. The outcome was good and the dogs were free of lameness 1 year post-operatively.

Keywords: dog, metacarpophalangeal joint, sesamoid disease

Corresponding Author:
Karkanis Athanasios DVM, MRCVS
Polygyrou 20, 40 Ekklisies, GR-54636 Thessaloniki, Greece
E-mail: akarkanis@hotmail.com

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ΠΕΡΙΛΗΨΗ. Δύο νεαροί σκύλοι, φυλής Rottweiler και Épagneul Breton προσκομίστηκαν με χρόνια χωλότητα σε πρόσθιο άκρο. Η κλινική εξέταση επιβεβαίωσε την ήπια χωλότητα και αποκάλυψε άλγος στην ψηλάφηση της παλαμιαίας επιφάνειας του 5ου μετακαρπίου. Διενεργήθηκε ακτινολογικός έλεγχος και διαπιστώθηκε κάταγμα του 7ου σησαμοειδούς της 5ης μετακαρπιαιοφαλλαγικής άρθρωσης. Με βάση τα παραπάνω τέθηκε η διάγνωση της νόσου των σησαμοειδών. Σε αμφότερα τα περιστατικά είχε προηγηθεί ανεπιτυχής συντηρητική αγωγή, η οποία περιελάμβανε τον περιορισμό της κινητικότητας του ζώου και την ολιγοήμερη χορήγηση αντιφλεγμονωδών φαρμάκων. Ως εκ τούτου εφαρμόστηκε χειρουργική θεραπεία με αφαίρεση του προσβεβλημένου σησαμοειδούς. Η έκβαση της χειρουργικής επέμβασης ήταν καλή και για 1 χρόνο μετεγχειρητικά η χωλότητα δεν είχε επανεμφανιστεί.

Λέξεις ευρετηρίασης: μετακαρπιαιοφαλαγγική άρθρωση, νόσος σησαμοειδών, σκύλος

INTRODUCTION

Sesamoid disease (SD) of the metacarpophalangeal (MCP) joints is an uncommon clinical condition of large breed dogs. MCP's sesamoids bones sustain traumatic or degenerative changes (Davis et al. 1969, Berg 1972, Bennett and Kelly 1985, Nickel et al. 1986, Robins 1986, Robins and Read 1993, Weinstein et al. 1995, Mathews et al. 2001, Daniel et al. 2008, Fossum 2013, Evans and de Lahunta 2013, DeCamp et al. 2016). The aetiology of SD is not clear (Read et al. 1992); however, traumatic incidents (fracture, luxation) of the sesamoid bones have recorded in racing Greyhounds in United Kingdom and Australia during the last 30 years (Bateman 1959, Davis et al. 1969, Cannon 1972). The function of the digital flexor tendons on the sesamoid bones at high impact, in which MCP joints are hyperextend, may produce sesamoid's fractures in some dogs (Weinstein et al. 1995, Mathews et al. 2001). Greyhounds and Rottweilers may manifest various disturbances in sesamoid bones and are the most clinically affected breeds by the SD. In other breeds (e.g. Labrador Retriever) these disturbances can be an incidental finding during orthopaedic examination (Bateman 1959, Davis et al. 1969, Berg 1972, Bennett and Kelly 1985, Krooshof and Hazewinkel 1986, Read et al. 1992, Robins and Read 1993, Mathews et al. 2001).

Veterinary literature has restricted information on MCP sesamoid disease. This paper presents two young dogs with forelimb lameness, due to fragmented MCP sesamoid bone, that were treated successfully by sesamoidectomy.

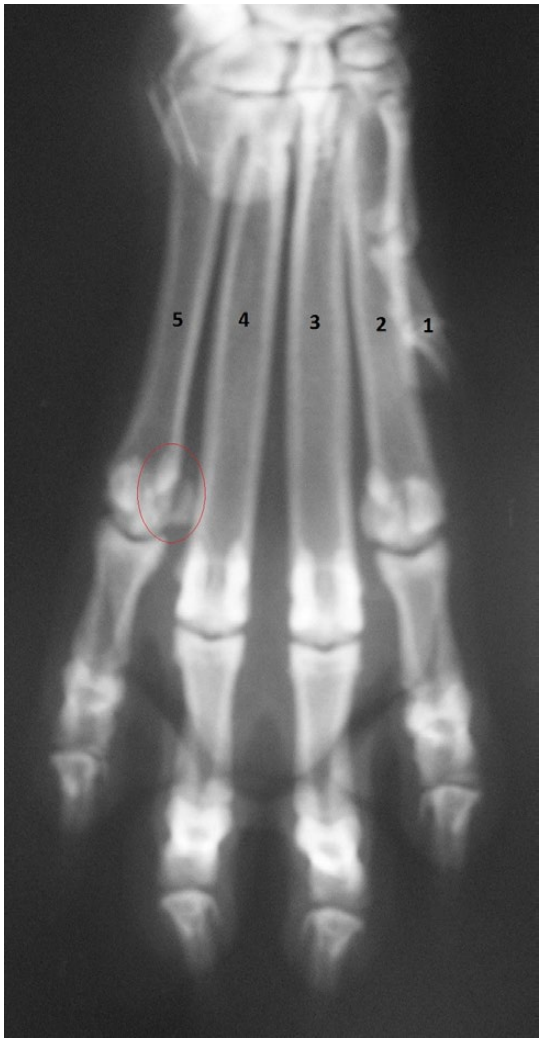

Fig 1: Dorsoventral radiographic view of a comminuted fracture with mild medial displacement of the 7th sesamoid bone at the 4th metacarpophalangeal joint (circled) (Case 1)

Anatomy

The canine forelimb has five MCP joints, one for each digit, numbered from the medial to lateral side. On each of the four main MCP joints there are two elongated, slightly curved palmar sesamoid bones on the ventral aspect and one osseous nodule on the dorsal aspect. Only a single osseous bead is located on the palmar side of the metacarpophalangeal joint of the first digit. The palmar sesamoids bones of the main digits (2^{nd} -5^{th}) are numbered from 1 to 8, from medial to lateral (Evans and de Lahunta 2013).

The shape of the palmar sesamoid bones varies. The tension that occurs on the 2^{nd} and 7^{th} sesamoid is excessive in contrast to the other sesamoids, due to the flat articular surface and the anatomic relation with the digital flexor tendons of the 2^{nd} and the 5^{th} digit (Nickel et al. 1986, Robins and Read 1993).

CASE HISTORY

Case 1

A female Rottweiler, of 37 kg body weight and 1-year-old, presented due to right forelimb lameness of 3 months duration. It was acute on onset and was getting progressively worse. Lameness improved after the administration of corticosteroids 3.5 weeks ago, due to anaphylactic reaction, but relapsed after treatment completion. The dog was being fed dry commercial food, raw meat and calcium supplements.

Orthopaedic examination revealed mild lameness (3/5) of the right forelimb and severe pain on deep palpation at the palmar aspect of the 5^{th} distal metacarpal. The dorsoventral radiograph of the paw revealed a comminuted fracture of the 7^{th} sesamoid bone with mild medial displacement (Figure 1).

The affected sesamoid was surgically removed (Figure 2) and a Robert Jones bandage was applied for 10 days. Amoxicillin plus clavulanic acid (Synulox, Zoetis, 15 mg/kg b.w., p.o., BID, for 6 days) and caprofen (Rimadyl, Zoetis, 2.2 mg/kg b.w., p.o., SID, for 5 days) were prescribed. Histopathology of the removed sesamoid showed the presence of callus consistent with old fracture(s) and no bone necrosis. This was insufficient to diagnose osteochodrosis or degenerative disease of the sesamoid bone.

Case 2

A 10-month-old, 18.5 kg body weight, male, hunting Épagneul Breton presented with left forelimb lameness. Six months ago, two episodes of acute lameness, 4 weeks apart, were recorded 2 days after hunting trial. Both were successfully treated with non-steroid anti-inflammatory drugs (NSAIDs) (Rimadyl, Zoetis, 2.2 mg/kg b.w., p.o., SID, for 15 days), but the lameness relapsed 4 months later. The dog was being fed only dry commercial food.

Orthopaedic examination revealed mild lameness (3/5) of the left forelimb and pain on the palpation at the palmar aspect of the left 5^{th} distal metacarpal bone. The dorsoventral radiograph of the paw showed fracture and lytic areas of the 7^{th} sesamoid bone with mild osteoarthritis in the 5^{th} MCP joint.

Given the chronicity and relapses of the condition, sesamoidectomy was the choice of treatment. A Robert Jones bandage was applied for 10 days. Amoxicillin plus clavulanic acid (15 mg/kg b.w., p.o., BID, for 6 days) and caprofen (2.2 mg/kg b.w., p.o., SID, for 5 days) were given post-operatively.

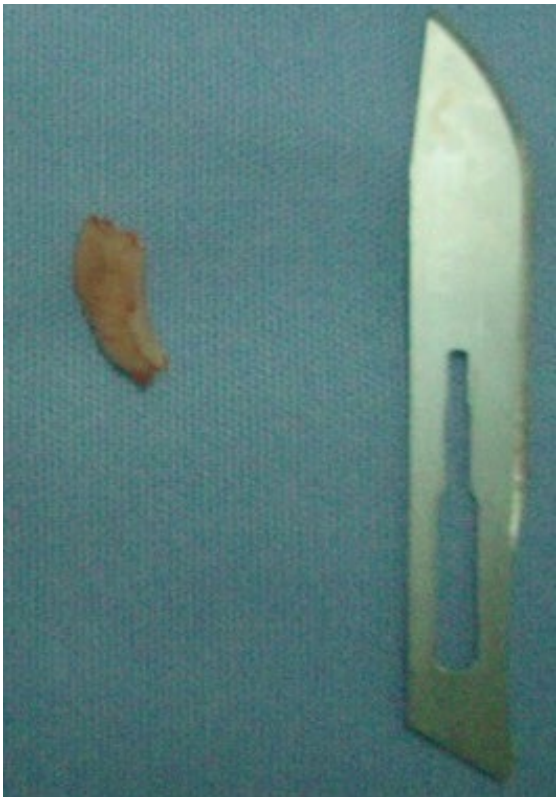

Fig 2: Fragment of the 7^{th} metacarpophalangeal sesamoid after total sesamoidectomy (Case 2).

Two weeks after surgery, both dogs used their forelimb. A low grade lameness was still present, while on the 4th week no lameness was evident. One (case 2) or two (case 1) years post-operatively the dogs remained free of lameness, even after intense exercise.

DISCUSSION

This study reports two cases of canine MCP sesamoid disease that were treated successfully with sesamoidectomy.

Both dogs were young, working and belonged to medium or large breeds. All these data was similar to that published for other cases (Bennett and Kelly 1985), except the medium breed hunting dog No 2. Maybe the use of the dog (i.e. work) and the stress applied to the sesamoid of the MCP joints, was the crucial factor for initiating the disease. The breed that has been extensively studied during the past 30 years is the racing Greyhound, but lately, the Rottweiler breed is the one bringing the most interest results in SD (DeCamp et al. 2016). In a study of 25 dogs with clinical and radiographic findings of SD (mean age: 20 months, range: 7 months – 7.5 years), the breed distribution was 21 Rottweilers, two Greyhounds, one Labrador retriever and one Queensland cattle (Robins and Read 1993). The high incidence of the condition in the Rottweilers may suggest a predisposition for SD, although this may be due to over-representation in the survey sample. Many of them presented for investigation of other orthopaedic problems, such as medial coronoid disease or hip dysplasia (Robins and Read 1993). Predisposition of SD in Rottweilers also confirmed in another study (Read et al 1992). Rarely, this condition occurs in other breeds (DeCamp et al 2016). In our study, due to the small number of cases, the authors cannot deduce the frequency of SD in certain breeds. However, this is the first report of SD in an Épagneul Breton. SD seems to occur more frequently in the right forelimb and appears to be unrelated to the animal's gender (Bateman 1959, Berg 1972, Bennett and Kelly 1985, Robins 1986, Krooshof and Hazewinkel 1986, Vaughan and France 1986, Read et al. 1992, Robins and Read 1993). Our cases were equal distributed concerning these factors. As far as the age concerns, 7% of the dogs will present clinical signs of this condition until the age of 3 months, while 50% will present clinical signs after the age of 9 months (Read et al. 1992).

The causes of SD in dogs could be traumatic, ossification disorders or degeneration of the MCP joints due to uneven stress distribution, and secondary osteonecrosis because of vascular impairment (Cake and Read 1995, Daniel et al. 2008). Fractures and degeneration disease are probably part of the same clinical problem (Robins 1986). Especially for Rottweilers, fracture of the MCP sesamoid bones is caused by congenital abnormalities, degenerative disease or trauma (Mathews et al. 2001). In our study, fracture of the sesamoids seems to be the cause of the SD, based on history, and histologic (case 1) and/or radiographic findings.

Épagneul Breton (case 2) was too young (approximately 4 months old) when its sesamoid bone fractured. It occurred during a hunting trial, as it is common for hunters to test the 'hunting instinct' of their puppies next to adult hunting dogs at an early age of 5-6 months old. However, this kind of trauma is unusual considering the roughness of the training at this age. Having in mind that fracture of the MCP sesamoids bones could be caused by their congenital abnormalities (i.e. Rottweilers) (Mathews et al. 2001), and lacking of histologic findings in our case 2, we could add in the differential diagnosis that this 'trauma' could potentially be part of a genetic weakness of the sesamoid bones making them more susceptible to injury.

The common presentation of these animals is acute or chronic lameness (Bennett and Kelly 1985), but they may also have no clinical signs (Read et al. 1992). In a study, 73% of Rottweiler puppies had lesions on the sesamoid bones, although only 22% of them presented lameness due to these lesions. The authors concluded that although SD is often subclinical, it could cause clinical lameness (Read et al. 1992). The type of lameness in SD varies in duration and severity (Read et al. 1992). Traumatic lesions of the sesamoid bones can cause acute onset, moderate forelimb lameness, and also oedema and pain at the site of trauma. In contrast, degenerative lesions can cause chronic and mild lameness, especially after exercise (Weinstein et al. 1995, Harasen 2009). Fractures and degenerative disease of sesamoid bones have the same clinical signs in chronic patients (Berg 1972, Bennett and Kelly 1985, Robins 1986, Read

et al. 1992, Robins and Read 1993, Cake and Read 1995, Mathews et al. 2001). In this study, cases were classified as chronic due to the initial favourable response to conservative treatment, followed by relapse prior to referral. However, in both dogs, the onset of lameness was acute, which reinforce the traumatic cause of SD. In chronic cases, dogs are weight bearing on the affected limb during exercise and non-weight bearing after it. In acute cases, lameness is moderate with a non-weight bearing lameness of the affected limb (Berg 1972, Bennett and Kelly 1985, Robins 1986, Read et al. 1992, Robins and Read 1993, Cake and Read 1995, Mathews et al. 2001, Fossum 2013). Our dogs presented mild lameness while walking and non-weight bearing on the affected limb while standing.

Palpation of the affected MCP joint reveals the painful area, although this might be subjective to interpretation given the intolerance presented by young dogs to manipulation of the extremities (Read et al. 1992). Especially in puppies, diagnosis of SD is difficult, given their lively behaviour during orthopaedic examination (Read et al. 1992). It is worth mentioning that in chronic cases, SD may cause joint effusion, synovial distention by increasing the volume of synovial fluid and significant reduction of joint flexion angle (Bennett and Kelly 1985, Robins 1986, Read et al. 1992, Cake and Read 1995, Mathews et al. 2001, Kapatkin et al. 2012). During flexion and extension of the affected MCP joint, pain and sometimes crepitus occur (Robins 1986). The only significant orthopaedic finding in our cases was the pain response on the deep palpation of the palmar aspect of the 5th digit. It is very important to consider concurrent subclinical diseases, as these are very common. Thus, in dogs presented with forelimb lameness, it is possible the main cause of lameness to be a concurrent orthopaedic condition and not the SD (Bennett and Kelly 1985, Read et al. 1992, Cake and Read 1995, Mathews et al. 2001).

For the radiographic assessment of the sesamoid bones, two views (dorsovental and mediolateral) of the distal limb are required (Cake and Read 1995), although in our study, the dorsovental view proved sufficient to establish diagnosis. It is always advised to radiograph the contra-lateral limb as the condition is often bilateral, and to have into consideration that the disease is more obvious in dogs >12 months old (Robins 1986, Read et al. 1992, Robins and Read 1993, Fossum 2013). The abnormal findings at the orthopaedic examination may not correlate with the radiographic view before the age of 9-12 months, and so they cannot be used as markers for the progression of the SD (Read et al. 1992). The radiographic examination of the other forelimb in both cases revealed that the SD was unilateral.

Radiographic findings vary depending on the chronicity and cause of SD (Robins 1986). They might include two or more bone fragments, osteophytes, dorsal displacement of the fragments, calcification of the soft tissue (Cake and Read 1995) or bone necrosis (Robins and Read 1993). However, it is difficult to differentiate between degenerative SD and sesamoid fractures (Robins 1986). In the latter, in the acute cases, the edges of the fragments are sharp, well-defined with irregular or smooth outlines (Robins 1986). Some dogs are born with bipartite sesamoid bones as a congenital anomaly, particularly in the 2nd and 5th digit. This anomaly is not associated with lameness, but it can be misinterpreted at the radiological evaluation of the affected limb (Mathews et al. 2001, Harasen 2009). In other dogs, especially Rottweilers and Greyhounds (Robins 1986), subclinical trauma (fractures) or degenerative lesions of the sesamoid bones are incidental findings and likewise can mislead the investigation of the lameness (Harasen 2009). In our cases, the radiographic findings were focused at the 7th sesamoid bone of the 5th digit of the right forelimb. They could be incidental findings but orthopaedic examination did not reveal any other concomitant diseases or injuries to justify the lameness, and also therapeutic management was curative.

Over the last 10 years, several studies compare the results of conservative and surgical treatment of SD (Berg 1972, Bennett and Kelly 1985, Krooshof and Hazewinkel 1986, Vaughan and France 1986, Read et al. 1992, Robins and Read 1993, Mathews et al. 2001, DeCamp et al. 2016). In dogs with intermittent lameness with less than 6 months duration, the condition can be resolved with rest only and over 50% of the cases do not need any further treatment (Berg 1972, Robins and Read 1993, DeCamp et al. 2016). Also, conservative treatment is recommend-

ed for dogs with acute onset lameness, including a bandage on the affected limb, activity restriction (Berg 1972, Bennett and Kelly 1985, Krooshof and Hazewinkel 1986, Read et al. 1992, Robins and Read 1993, Mathews et al. 2001, Riere-Palou 2002, DeCamp et al. 2016) and local glucocorticosteroid injections (Riera-Palou 2002). In dogs with intermittent lameness of more than 6 months duration, the condition is unlikely to be resolved with conservative treatment and total sesamoidectomy is suggested (DeCamp et al. 2016). Also, surgical treatment is suggested for dogs with chronic lameness (Cannon 1972, Berg 1972, Bennett and Kelly 1985, Krooshof and Hazewinkel 1986, Read et al. 1992, Robins and Read 1993, Weinstein et al. 1995, Riere-Palou 2002, Harasen 2009, DeCamp et al. 2016), and when conservative treatment did not resolve the lameness or was just a temporary solution. Both cases presented in this study had received anti-inflammatory drugs before sesamoidectomy, as conservative treatment (case 2 – NSAIDs) or incidentally (case 1 - corticosteroids), but only temporary remission of lameness was achieved. Because lameness was persistent and conservative treatment had failed, sesamoidectomy was applied as definitive treatment. However, it must be pointed out that anti-inflammatory treatment of the first case was not sufficient for SD, as the medication was given for a very short period of time after the anaphylactic shock.

Generally, prognosis of SD is guarded. The results of sesamoidectomy are favourable (Berg 1972, Bennett and Kelly 1985, Robins 1986, Nickel et al. 1986, Fossum 2013, Evans and de Lahunta 2013, DeCamp et al. 2016), but even with this treatment lameness may relapse after intense exercise (Robins 1986). Failure of surgical treatment is due to adhesions in multiple ligaments of the sesamoid bones that are responsible for the MCP joint stability. As a result, osteoarthritis of the MCP joint occurs (Fossum 2013) leading to thickening of the capsule and reduced range of motion (Robins 1986). Mathews et al. (2001) suggested that sesamoidectomy of the fragmented sesamoid, gradually will cause more advanced radiographic degenerative joint changes of the affected MCP joint and less clinical improvement than the conservative treatment, where the lameness will be resolved or improved clinically in most cases. According to these authors, the successful outcome of sesamoidectomy in our cases may be associated with the short clinical and radiographic follow-up, as the development of osteoarthritic changes in the affected MCP joint are more apparent in chronic cases. Whether and when the dog will develop degenerative joint disease of the affected MCP joint after sesamoidectomy is unknown.

CONCLUSIONS

In conclusion, regardless of the chronicity of the case, SD could be treated conservatively initially and if lameness persists or relapses, sesamoidectomy can be performed usually leading to a sound dog.

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CONFLICT OF INTEREST STATEMENT

All the authors declares that there is no conflict of interest for the presented case repost.

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Case Report
Κλινικό Περιστατικό

First report of granulosa cell tumour associated with an endometrial adenoma in a crossbreed cow in Portugal

D. Moura[1], J. García-Díez[2]*, C. Saraiva[2], M.A. Pires[2]

[1]North Division of the Portuguese Food and Veterinary National Authority (DSAVRN).

Portuguese Food and Veterinary National Authority (DGAV). Portugal.

[2]CECAV – Animal and Veterinary Research Centre. University of Trás-os-Montes e Alto Douro, Portugal.

ABSTRACT. A 12.5 year-old female crossbred cow without clinical signs at ante mortem inspection was slaughtered. The post-mortem inspection revealed poor carcass condition, interstitial nephritis and generalized lymphadenitis. The reproductive tract presented an unilateral and highly vascularized yellowish-white mass, with huge dimensions (60 x 40 cm and 20 Kg, approximately) described as granulosa cell tumour (GCT) and a endometrial adenoma, after histopathological analysis.

GCT has been described as the most frequent ovarian tumour in cattle. Since clinical signs are usually unspecific, the post mortem diagnosis by histopathology examination is always necessary. The endometrial adenoma could be asymptomatic, with only absence of calving, or associated with GCT. This is, of our knowledge, the first report of a GCT associated with endometrial adenoma in a cow in Portugal.

Keywords: granulosa cell tumour, ovary, endometrial adenoma, cattle

Corresponding Author:
Juan GarcíaDíez, DVM, MSc, PhD
1CECAV – Animal and Veterinary Research Centre. Universityof Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal
Email: juangarciadiez@gmail.com

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INTRODUCTION

Granulosa cell tumour (GCT) has been described in cattle (Anderson et al., 1969, Bosu, 1977, Hosteller et al., 1997, Leder et al., 1988) as well in other species (Kennedy et al., 1998, Patnaik et al., 1987). Although tumours in cattle are scarce (Garcia-Iglesias et al., 1995), GCT is considered as the most common ovarian tumour of this species (Agnew and MacLachlan, 2017) Kennedy et al, 1998; Farin, 1993). GCT affects all breeds of cattle but implementation of reproductive programs in dairy herds may explain its higher incidence compared to beef cattle. Clinical signs are variable but commonly the condition involves reproductive disorders, in consequence of hormonal production. In some cases, a presumptive diagnosis is made based on clinical signs and rectal palpation, mainly when an ovary presented an abnormal size compared to the contralateral one, that is in most cases atrophied (Bosu, 1977). However, ultrasonography may improve the presumptive diagnosis in live animal.

Despite tumours mainly occur in older animals, some cases are reported in younger as two years (Short et al., 1963) or 9 months (Masseau et al, 2004).

Endometrial adenoma is a very rare condition in domestic animals as its malignant counterpart is considered more common in cow (Kennedy et al, 1998). Recently reports considerthis situation in cat more common that described before, and proposed to be related with hormonal dysregulation with influence of progesterone in this tumour (Payan-Carreira et al., 2013, Saraiva et al., 2015). The presence of concomitant pyometra (with the presence of a purulent exudate) is reported in a high number of adenocarcinomas in the cat (Pires et al., 2016).

The livestock management in which cattle are slaughtered after a few months of growth difficult the diagnostics of similar situation. Thus, most tumour lesions are observed in the slaughterhouses after meat inspection (Lucena et al., 2011). We intend to highlight the importance of its macroscopic diagnosissince presence of tumours lesions during meat inspection implies the whole carcass condemnation.

CASE DESCRIPTION

A 12.5 year-old female crossbred cow was slaughtered intended for human consumption without clinical signs at ante mortem inspection. The cow health record registered in the Portuguese National Cattle Database indicated 9 calving being the last one three years ago and 16 official screenings for bovine tuberculosis and brucellosis diagnostics with negative results. The compulsory food chain information form presented by the owner to the meat inspector not registered any clinical information regarding the cow.

RESULTS

The post mortem inspection revealed cachexia, whitish muscle and generalised lymphadenomegalia. The visual inspection of the offal revealed the presence of white-spotted lesions on the surface of both kidneys, and the inspection of the reproductive tract revealed a unilateral, highly vascularized, yellowish-white mass, with huge dimensions (60 x 40 cm and 20 Kg, approximately), compatible with an ovarian neoplasia presented in the right side (Figure 1). The left ovary does not present any macroscopic alteration. The cut surface presented a solid yellow mass with haemorrhagic and necrotic areas. Also, it presented macroscopic unspecific uterine lesions. According to the multi-location of the lesions, the carcass was condemned by meat-inspectors.

Samples of kidneys, lymph nodes, ovary and uterus

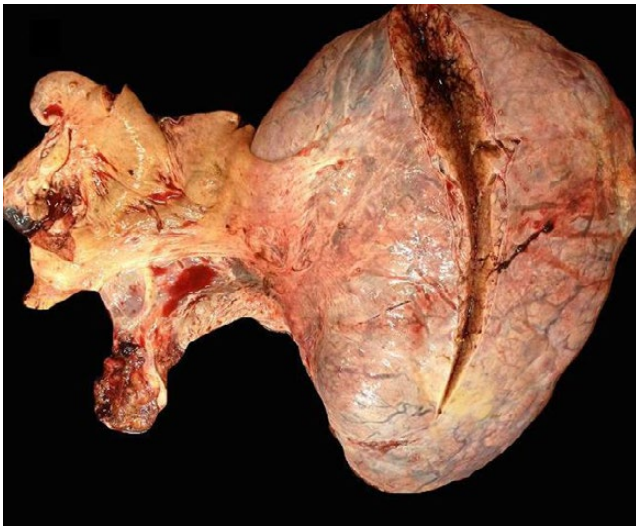

Figure 1. Enlarged wright ovary transformed in a single yellow mass. Some haemorrhagic and necrotic tissues are seem in the cut surface. Note the atrophic aspect of the contralateral ovary.

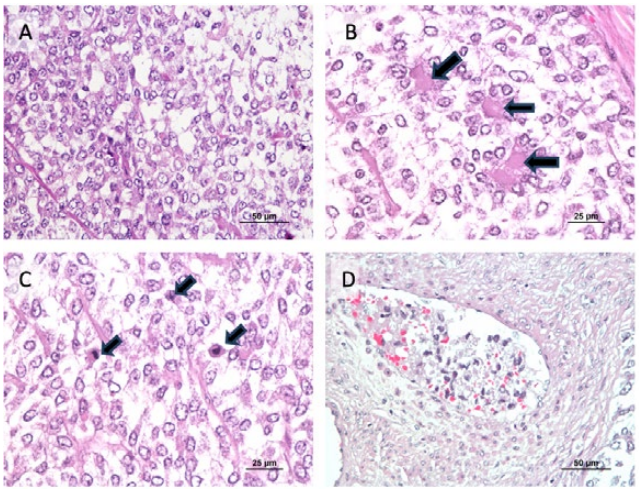

Figure 2. Histological images of the right ovary. A– Presence of granulosa cells in diffuse and solid pattern. The cells are pleomorphic with dense or vesicular nucleus, distinct nucleoli, with eosinophil or vesicular clear cytoplasm. B – Some cells are distributed in a microfollicular pattern (Call-Exner bodies, arrow) with hyaline eosinophil material inside a rosette like arrangement. C – Some mitoses isseeming (arrow) some of them anomalous. D –Vascular invasion of granulosa cells. Haematoxylin and Eosin staining.

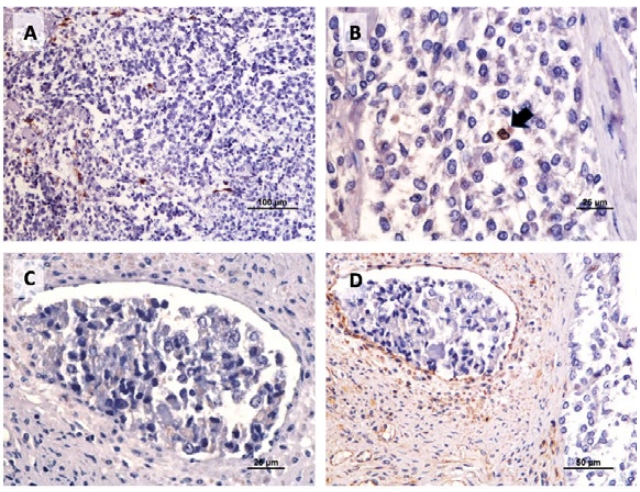

Figure 3. Immunohistochemical labelling of the granulosa cell tumour. A – Broad spectrum keratin showing a sporadic cytoplasm positivity. B - Ki67 positive nucleus (arrow). C- Cluster of CGT with weak and sporadic positivity to a broad-spectrum keratin inside a vessel. Serial section of that fig. 2D. D – Vimentin sporadic positivity of GCT cells, inside a vessel. Serial section of that fig. C. Gill's haematoxylin counterstaining.

were collected for histopathological analysis. All tissues were fixed in buffered 10 % formalin and routinely processed and embedded in paraffin wax. Sections of 3-μm thick were stained with haematoxylin and eosin (HE).

Ovary samples were 3-μm thick sectioned to Silane® coated slides and performed immunohistochemistry by the indirect avidin-biotin peroxidase complex technique, with the antibodies anti-vimentin (NCL-L-Vim-V9, Novocastra, 1:100 dilution); broad spectrum keratin (clone AE1/AE3, Dako®, Denmark, 1:50 dilution) and Ki67 (clone Mib-1, Dako®, Denmark, 1:50 dilution).

The histological evaluation of the kidney revealed the presence of interstitial infiltrate by mononuclear inflammatory cells, with hyaline cylinders and calcium in the pelvis, small cystics of distinct dimensions and fibrosis in the cortex corresponding to an old infarct, classified as interstitial chronic nephritis.

The ovary presented intense proliferation of fusiform cells with eosinophilic to vesicular/clear cytoplasm in a tubular to diffuse (and solid) pattern. Also, cell´s nucleus was vesiculated or dense and presented evident nucleolus (Figure 2 A and B). Necrosis and images of vascular invasion of neoplastic cells and low mitotic rate (one to two mitotic figures per high microscopic field-HPF- 40x, Figure 2 C, D and E).

At immunohistochemistry evaluation, rare cells were positive to vimentin and to broad spectrum keratin, and above 1% of Ki/67positive count (Figure 3 A and B). Images of vascular invasion are evidenced

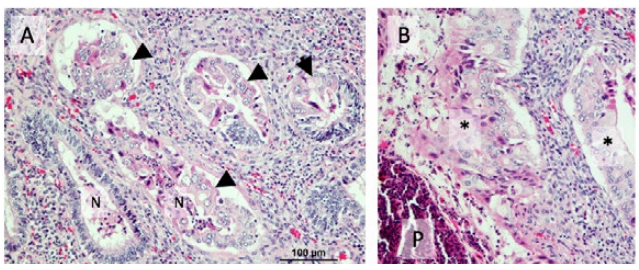

Figure 4. Images of endometrial adenoma. A - Observe the presence of neutrophils (N) inside endometrial glands (normal or with atypia - arrow). B –Presence of cellular atypia of the glands (*), purulent exudate with neutrophils and debris (P). Haematoxylin and eosin staining.

by weak positive to keratin cells inside vessels and vimentin positive vessels walls (Figure 3 C and D).

According to the morphological aspects of the lesion, it was classified as malignant granulosa cell tumour.

The endometrium presented cystic hyperplasia of glands, with neutrophil infiltrate in some cases. In addition, some of these glands presented moderate cytological atypia, clear nucleus, loss of nucleus polarity, some binucleated cells, that was compatible with an endometrial adenoma (Figure 4A and B).

The histopathological analysis of iliac lymph node revealed the presence of germinal centres, eosinophils, neutrophils and increased number of plasma cells in its medulla corresponding to hyperplasia and lymph node reactivity. No neoplastic cells were evident inside this lymph node.

DISCUSSION

Granulosa cell tumours (GCT) are specific lesions of the ovary. Although its aetiology is not completely described, some genetic mechanisms and further hormone disruptions could be involved on its development (Kitaharaet al., 2012).In animals, CGC have been described both in companion animals (Panaitk, 1987) and farm animals(Anderson et al., 1969, Kennedy et al., 1998). Adenoma of the endometrium is also described as rare entity in domestic animals (Kennedy et al., 1998).

In the consulted literature, the authors did not find the association between GCT and endometrial adenoma in cattle, and the best of our knowledge, this is the first case reported in Portugal.

GCT was described in cattle over 10 years (Bosu, 1997) in accordance with the present case. However, cases in young cattle with less than 4 years and also a case in a 9 months' calf have also been described (Hosteller et al., 1997, Kanagawa et al., 1964, Leder et al., 1988, Masseau et al, 2004, Zachary et al., 1983).

Clinical signs of GCT in cattle are variable although most of them remain asymptomatic. The cachexia and generalised lymphadenomegalia observed are unspecific clinical sings related with malignant neoplasms, in which its relationship with the GCT is not clear, but could be related with the neoplasia products released or its (huge) dimensions that could make pressing in the digestive tract and in abdomen. However, presence of dorsal jerking of the head, excessive salivation, abnormal udder development, nymphomaniac behaviour, infertility or abnormal findings by transrectal palpation or ultrasonography have been described as clinical signs related with GCT.

The diagnosis of GCT in live cattle is not frequent and usually associated to the identification of compatible images at ultrasonography in routine pregnancy diagnosis (Dobson et al., 2013). Although fine-needle aspiration or endocrine analysis are also recommended (Hosteller et al., 1997), the histopathological analysis is essential for a definitive diagnosis. In the present case, the diagnosis was performed after post mortem inspection due to the absence of clinical symptomatology. However, the absence of calfs´ registration on national cattle database suggested infertility in the last three years could be related with this tumour development as with the lesions found in the uterus.

In cases of absence of clinical symptomatology, a careful post mortem examination at necropsy or slaughterhouse inspection have been described as the only way to observe the tumour or other lesions (Kanagawa et al., 1964).

A variable size of the granulosa cell tumours, bilateral presentation and presence of metastasis are the most important features described in the literature (Kanagawa et al., 1964). GCT are malignant tumours and report of metastasis is not common (Patnaik, 1978). However, Zachary and Haliburton (1983) reported metastasis on the peritoneum and the liver. The case studied only presented a large-sized unilateral tumour with histologic evidences of neoplastic cells vascular invasion but without evidence of metastasis in the regional lymph node or in any other organs observed. The hormonal dysregulation with production of steroids by this CGT could be related to the endometrial adenoma found in the uterus as proposed by other authors (Payan-Carreira et al., 2013, Saraiva et al. 2015) and both situations should be responsible for the absence of calving in the last 3 years.

Regarding treatment, surgical removal and medical management has been described (Dobson et al., 2013)

for GCT but its successful could be conditioned by the location and size of the tumour. Since the economic cost of the treatments is not compensatory, the affected cattle are usually destined for slaughter. Nevertheless, in the present case, the endometrial adenoma, not clinically identified, will reduce the fertility even after ovarian removing.

In conclusion, this is the first case description of CGT associated with an endometrial adenoma in a cow, in Portugal, that highlights the essential role of the meat inspector at the slaughterhouse in the evaluation of macroscopic tumour-like lesions that could lead, as in the present case, to the carcass condemnation after histopathological confirmation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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