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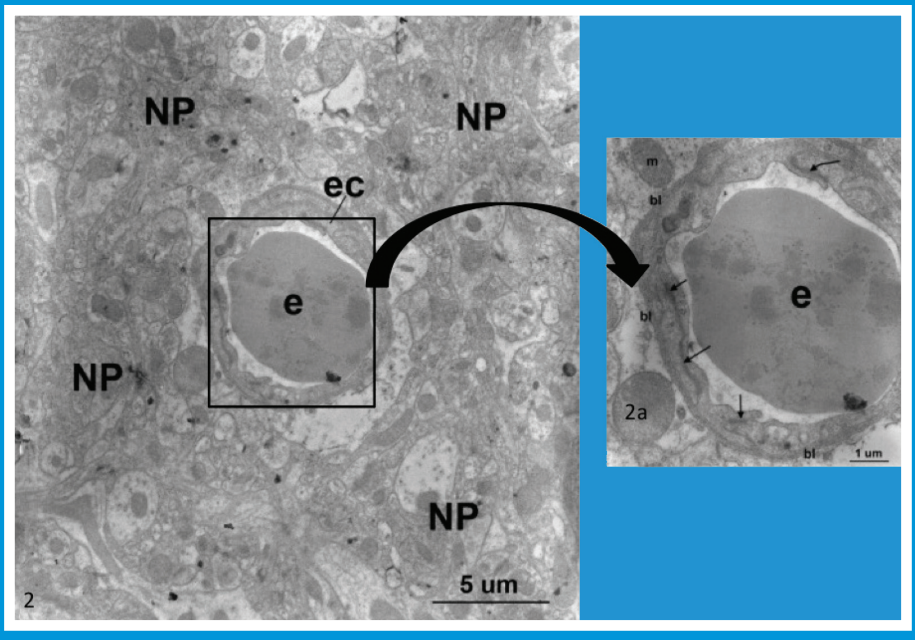

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Original Scientific Article

NO EVIDENCE OF AVIAN INFLUENZA VIRUSES IN BACKYARD POULTRY FROM RISK AREAS IN R. MACEDONIA (Virological and molecular surveillance 2009)

Iskra Cvetkovikj[1]*, Aleksandar Dodovski[1], Ivanco Naletoski[2], Slavco Mrenoski[1], Dine Mitrov[1], Kiril Krstevski[1], Igor Dzadzovski[1], Aleksandar Cvetkovikj[1]

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Summary: Until 2009 no data are available for the circulation of avian influenza viruses (AIV) in backyard poultry in Republic of Macedonia. In 2009 virological and molecular surveillance was conducted in backyard poultry in the risk areas of the country according the National Annual Program for AI and Commission Decision 2007/268/EC. A total number of 2151 samples were analyzed. Two thousand and twenty cloacal swabs (sampled from backyard poultry - poultry sector 4 from the risk areas in Republic of Macedonia i.e., areas where migratory waterfowl gathers) and 131 organs/cloacal swabs (for daily routine diagnostics) were tested by virological and molecular methods. The virological diagnosis was performed by isolation on embryonated chicken eggs, followed by macro hemagglutination, hemagglutination on microtiter plate and inhibition of hemagglutination. The molecular diagnosis was performed by RRT-PCR for M-gene. All samples were negative for AIV. However, 4 samples were positive on avian paramyxovirus 1 (Newcastle disease virus). The wild birds, as a reservoir for AIV were not included in the surveillance which on the other hand does not ensure complete information for the AIV circulation in R. Macedonia and should be further investigated. This fact imposes the need for overall national action to ensure the concept of early detection and response in case of emergence of avian influenza.

Key words: avian influenza viruses; backyard poultry; cloacal swabs; Republic of Macedonia; surveillance

Introduction

In the last 10 years the field of avian influenza (AI) has undergone something of a revolution. According to Alexander [1], this happened not just because of the actual zoonoses and the potential threat of a pandemic emerging, which has highlighted public awareness, but also because many aspects of the epidemiology of AI infections in poultry and other birds appear to have changed dramatically.

The number of outbreaks of the highly pathogenic avian influenza (HPAI) disease has increased alarmingly in the last 10 years and, even more noticeably, the impact in terms of the number of birds involved and the costs of disease control have dramatically escalated [2]. In addition, the apparently unprecedented emergence and spread of the HPAI H5N1 virus in Asia and beyond has brought AI to the forefront of important animal diseases [1].

Influenza viruses are spherically or longitudinally shaped enveloped particles with an up to eight-fold segmented, single-stranded RNA genome of negative polarity. Influenza viruses hold generic status in the Orthomyxoviridae family and are classified into types A, B or C based on antigenic differences of their nucleo- and matrix proteins. AIV belong to type A [3].

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The main antigenic determinants of influenza A and B viruses are the hemagglutinin (H or HA) and the neuraminidase (N or NA) transmembrane glycoproteins, capable of eliciting subtype-specific and immune responses which are fully protective within, but only partly protective across, different subtypes. Because of the antigenicity of these glycoproteins, influenza A viruses currently cluster into 16 H (H1 - H16) and nine N (N1 - N9) subtypes. These clusters are substantiated when phylogenetically analysing the nucleotide and deducted amino acid sequences of the HA and NA genes, respectively [4].

To date, only viruses of the H5 and H7 subtypes have been shown to cause HPAI. It appears that HPAI viruses arise by mutation after low pathogenic avian influenza (LPAI) viruses have been introduced into poultry by several mechanisms [5]. AIV have been shown to infect birds and mammals. The main factor that influences susceptibility to infection is the receptor conformation on host cells (AIV bind preferentially to SA-α2,3-Gal and human IV to SA- α2,6-Gal terminated saccharides [5].

Influenza viruses have been shown to infect a great variety of birds [6, 7], including free-living birds, captive caged birds, domestic ducks, chickens, turkeys and other domestic poultry.

All available evidences suggest that in the conventional situation the primary introduction of LPAI viruses into a poultry population is a result of wild bird activity, usually waterfowl [5]. The greatest threat of spread of AIV is by mechanical transfer of infective faeces in which virus may be present at concentrations as high as 107 infectious particles/g and may survive for longer than 44 days [8].

The fact that AI represents one of the greatest concerns for public health that has emerged from the animal reservoir in recent times [5], leads to the considerable public and media attention having in mind its zoonotic potential. This situation has given rise to the fear that the virus might acquire the capacity of sustained human–to-human transmission and thus cause global influenza pandemic.

This fear has been the main driving force behind the international commitment to global HPAI control, to date amounting to an estimated US$1.02 billion disbursed funds and US$1.68 billion committed funds as of June 2007 [9], which in turn has significantly influenced national responses to outbreaks of H5N1. The latter have involved destruction of birds on a massive scale, regulation of poultry production and trade and, in some countries, large-scale vaccination campaigns. These responses often come at considerable cost to poultry producers.

HPAI, like other highly contagious animal diseases, affects animal production via three main pathways: direct losses, public impact and market reactions [10].

Firstly, HPAI causes direct losses to producers and other actors interlinked to the production and marketing of poultry through morbidity and mortality and the private costs associated with ex-ante risk mitigation (e.g. investment in animal housing) and/or ex-post coping measures during periods of downtime (e.g. bridging loans if the enterprise carries significant borrowings) and the need to reinvest in replacement birds. Secondly, animal diseases that are 'notifiable' can have severe impacts through government intervention, which carries a cost borne by the public at large and affects producers (and associated up- and downstream actors), irrespective of the disease status of their flocks. These costs include public investment in animal health infrastructure and epidemic preparedness. Thirdly, disease impacts arise through market reactions, which can be particularly severe on the demand-side for diseases that are associated with a public health risk. Analogous to disease control measures affecting producers even if their flocks have not contracted HPAI, market reactions can occur, irrespective of whether or not avian influenza has actually occurred in the country.

Geographically, R. Macedonia belongs to the Black sea - Mediterranean migratory flyway, surrounded by Albania, Greece, Bulgaria, Serbia and Kosovo. Three of these neighboring countries at some point have reported to OIE outbreaks of HPAI H5N1 (Albania 2006 - 1 case, Greece 2006 – 1 case, Bulgaria 2006 – 1 case and 2010 – 1 case). In the wider region, Romania has reported 109 cases of H5N1 in 2006, 1 in 2007 and 2 cases in 2010. Turkey has also reported 2 cases in 2005, 200 in 2006, 17 in 2007 and 7 cases in 2008.

As pointed by the claims of EU, surveillance of AIV for poultry and wild birds is mandatory for every Member state and comprises the implementation of surveillance programs according the EU legislative guidelines.

The National Annual programme for AI surveillance includes conducting surveillance programs

in poultry and wild birds. Unfortunately, until now according to the National Annual program no surveillance has been conducted. This is a great disadvantage giving the fact that there is no available data for the previous years and it is anybody's guess if there is circulation of AIV or not.

Although knowing the fact that 3 of the surrounding countries have reported outbreaks of HPAI H5N1, the question of AIV circulation in R. Macedonia remained unanswered and thus emphasized the need of investigation due to the possibility of spreading the disease to Macedonia.

Therefore, the aim of this study was to assess the AIV situation in the poultry sector 4 in R. Macedonia, i.e., to affirm the presence and distribution and/or to confirm the absence of AIV in backyard poultry.

Materials and methods

The cloacal swabs were sampled from backyard poultry (poultry sector 4) from the risk areas in R. Macedonia i.e., areas where migratory waterfowl gathers. The poultry sector 4 defines backyard poultry production in villages with minimal biosecurity measures (birds are kept out most of the day with high possibility of contact with other chicken, ducks, domestic birds and wildlife). Those characteristics define the poultry sector 4 as the most prone sector for AI emergence, especially in locations near lakes and flooding areas where migratory waterfowl gathers.

The distribution of the sampled villages in the risk areas is given in Table 1, and their location is given in Figure 1. The distribution of samples per poultry species is given in Table 2.

As pointed in the Annual program for AI surveillance [11] and Commission Decision 2007/268/EC [12], the sampling was representative for the whole state.

A total number of 213 holdings in 59 villages (1-6 holdings per village) were sampled. According to the previously defined representative number for cloacal swabs per holding (n-11), 11 cloacal swabs were sampled wherever possible. In smaller holdings, the number of sampled cloacal swabs varied from 1 to 11 depending on the number of poultry reared in the holding. Besides these samples, the survey included 131 samples from domestic and wild birds submitted for daily routine diagnosis in the laboratories of the Faculty of veterinary medicine in Skopje (FVMS) (Table 3). Sample processing was performed according to the guidelines in Commission decision 2006/437/EC [13] and OIE [14].

The extraction of RNA was performed with RNEasy Mini kit according to the producer's manual [15].

Detection of M-gene was conducted on Bio-Rad's IQ5 RRT-PCR. The method was performed as pointed in the protocol of the Waybridge reference laboratory [16].

The following reagents were used:

1. Primers and probes:
   - Sep 1 AGA TGA GTC TTC TAA CCG AGG TCG (Operon)
   - Sep2 TGC AAA AAC ATC TTC AAG TCT CTG (Operon)
   - SePRO FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA (Operon)
2. Real time PCR master mix: Qiagen Onestep RT-PCR kit

The virological methods (virus isolation on embryonated chicken eggs - ECE and identification by hemagglutination inhibition - HI test) were performed according to the Commission decision 2006/437/EC [13] and OIE [14]. These methods are not specific for AIV and are used for detection of other avian viruses that can be propagated on ECE and identified by HI test. The HI test was performed with specific antisera for avian paramyxovirus 1, AIV H5, AIV H7 and avian adenovirus.

Table 1: Distribution and number of sampled poultry in risk areas

Risk area	Sampled villages	N° of samples
Artificial lake Mavrovo	6	188
"Sini virovi" Ohrid	4	164
Ohrid lake	20	690
Prespa lake	7	270
Fishery Bel Kamen	3	124
Fishery Bukri	2	68
Dojran Lake	4	139
Flooding area Monospitovsko blato	5	209
Flooding area Katlanovsko blato	5	103
Artificial lake Veles	3	65
TOTAL	**59**	**2020**

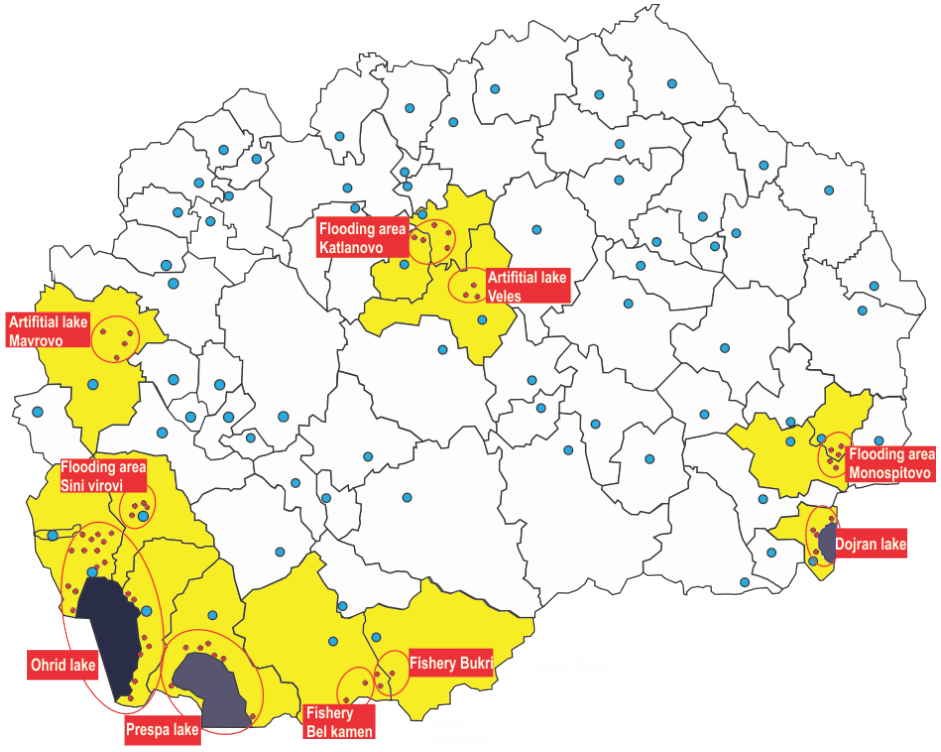

Figure 1: Location of the sampled villages in the risk areas of R. Macedonia

Table 2: Sampled poultry species

Poultry species	No of samples
Chickens	1847
Ducks	124
Geese	21
Turkeys	23
Guinea fowl	2
Pheasants	3

Table 3: Samples for daily routine diagnosis

Species	Sample	No of samples
Chickens (Holding with high rate mortality)	Carcass	12
Gulls, herons, mallard ducks (Unspecified number per each species)	Cloacal and tracheal swabs	37
Mallard ducks	Organs	11
Accipiter	Organs	2
White stork	Organs	1
Ostrich	Organs	2
Common quail	Carcass	2
Geese	Organs	1
Pigeons	Carcass	12
Chickens	Carcass	51
TOTAL		131

Results

The 2020 analyzed cloacal swabs gave negative result for the presence of AIV by both virological and molecular diagnosis.

The 131 analyzed samples for daily routine diagnosis also gave negative result for the presence of AIV by VI and PCR. Four samples (three from pigeons and one from poultry) were positive on avian paramyxovirus 1 (Newcastle disease virus).

Discusion

Until 2009, no molecular or virological surveillance was conducted to determine the presence and circulation of AIV i.e., no data were available for assessing the AI situation in R. Macedonia.

The research of Dodovski et al. [17] from September 2008 till March 2009, showed seropositive flocks by ELISA but negative on hemagglutination inhibition test for H5/H7, which may be due to circulation of non H5/H7 viruses. So, this fact imposed the question: If seropositive flocks are present - is there a circulation of the virus? Following this fact, Cvetkovikj et al. [18] in 2009 performed virological and molecular surveillance in the commercial poultry sector resulting with negative results for circulation of AIV.

According the 2009 surveillance there is no evidence of AIV subtypes circulating among backyard poultry population, which of course does not represent a constant result and imposes the need of further permanent surveillance.

So, what are the possible reasons for the negative results? There are several possible issues that may lead to this situation and are addressed below.

R. Macedonia is defined by the World Bank [19] as a country with low or moderate risk for AI outbreak, which contributes the fact that the surveillance yielded with negative result.

According to World Animal Health Information Database (WAHID) Interface [20] in 2009, in the Black Sea-Mediterranean flyway AI was reported only in 4 countries with 6 outbreaks - 3 for Low Pathogenic Notifiable Avian Influenza (LPNAI) and 3 for Highly Pathogenic Notifiable Avian Influenza (HPNAI). On the other hand, in 2008, 9 countries with total number of 141 outbreaks for HPNAI were reported and no LPNAI was reported. This fact shows the trend of decreasing prevalence of Notifiable Avian Influenza (NAI) in the countries belonging to the Black Sea - Mediterranean flyway.

The poultry sector 4 in R. Macedonia is defined as a sector with low biosecurity level and high risk for AI occurrence. In R. Macedonia the backyard poultry is reared mostly for egg production and kept in closed and covered fences, thus minimizing the possibility of contact with the wild birds or their faeces.

Besides that, R. Macedonia does not possess large water areas attractive for the migratory waterfowl – representing the potential danger of input and spread of AIV in poultry.

This surveillance was based on sampling cloacal swabs from backyard poultry. So, did the cloacal swabs influence the negative result?

For most bird species it is prudent to collect both tracheal/oropharingeal and cloacal swabs for testing, or only tracheal/oropharingeal when gallinaceous birds are subject of the surveillance. However, usage of cloacal swabs in surveillance of AI in backyard poultry is not uncommon.

Buscaglia et al. [21] in the surveillance in Argentina from 1998 to 2005, demonstrated no positive result in both serological and virological surveillance in backyard poultry using sera and 18.000 tracheal and cloacal swabs (only 10% tracheal swabs per year and 90% cloacal). After 2006, (isolation of H5N1 virus from mallard duck near Zagreb) Croatia has a regular monitoring for avian influenza in wild birds and poultry (especially in the backyard flocks using cloacal swabs). All samples were HPAI virus negative but LPAI viruses (H2N3, H3N8, H5N3 and H10N7) were isolated from wild birds [22]. Racnik et al. [23] also demonstrated negative result in backyard poultry using cloacal swabs as samples in the first Slovenian surveillance for avian influenza in migratory birds and backyard flocks.

However, to estimate the ecology of AIV in non commercial poultry, most surveillance strategies are based on sampling both tracheal and cloacal or only tracheal swabs.

Knowing the fact that gallinaceous birds typically shed AI viruses in respiratory secretions, a tracheal or oropharyngeal swab is the primary source of virus detection from chickens and turkeys. As pointed in the Diagnostic manual for avian influenza (2006/437/EC), for investigation of a holding suspected of being infected with the AI virus, the standard set of samples for virological testing is a combination of cloacal and tracheal/oropharyngeal swabs.

This point could easily take us to the answer that maybe the negative result was influenced by sampling only cloacal and not both cloacal and tracheal/oropharyngeal swabs.

Avian influenza has had an enormous economic and social impact on affected countries and the risk of this animal disease situation evolving into a global human influenza pandemic is of great public concern. Therefore, it is of interest of all countries, developed or developing, to invest in the containment of AI because there is continuing risk of AI spreading that no poultry-keeping country can afford to ignore. [24]

The establishment of efficient preparedness and control of AI has to be substantiated by activities at national, regional and global level [24].

1. At national level, each state has to strengthen Veterinary Public Health Services to better manage national surveillance systems, to improve early detection and response, define strategies and monitor their implementation. Likewise, disease prevention and control programmes have to be implemented including stamping out, biosecurity and vaccination as appropriate as well as developing and implementing better diagnostic tools.

2. At regional level, activities should be aimed to strengthen regional co-operation for harmonization of the surveillance and control strategies as well as promoting the regional diagnostic and surveillance networks etc.

3. Globally, the activities are aimed at donor support to provide the scientific and technical advice required by countries.

In this context, R. Macedonia needs to implement surveillance programmes mostly aiming on strengthening the national preparedness plan that can only be efficient with collaboration of veterinary services, national reference laboratory, ornithologists, etc.

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NI DOKAZOV O OBSTOJU VIRUSA PTIČJE GRIPE PRI DVORIŠČNI PERUTNINI NA OGROŽENIH OBMOČJIH V R. MAKEDONIJI (Virološki in molekularni nadzor 2009)

I. Cvetkovikj, A. Dodovski, I. Naletoski, S. Mrenoski, D. Mitrov, K. Krstevski, I. Dzadzovski, A. Cvetkovikj

Povzetek: Do leta 2009 nimamo podatkov o razširjenosti virusa ptičje gripe (AIV) pri dvoriščni perutnini v Republiki Makedoniji. V skladu z nacionalnim letnim programom nadzora AI in odločbe Komisije 2007/268/ES je bil v letu 2009 izveden virološki in molekularni nadzor dvoriščne perutnine na ogroženih območjih države. Skupno smo analizirali 2151 vzorcev. Dva tisoč in dvajset kloakalnih brisov (vzorci dvoriščne perutnine perutninskega sektorja 4 iz ogroženih območij v Republiki Makedoniji, med njimi iz območij, kjer so zbirališča vodnih ptic selivk) in 131 organskih/kloakalnih brisov (za vsakodnevno rutinsko diagnostiko) je bilo testiranih z virološkimi in molekularnimi metodami. Virološka diagnoza je bila izvedena z izolacijo oplojenih kokošjih jajc, kateri je sledila makrohemaglutinacija, nato hemaglutinacija na mikrotiterski plošči in zaviranje hemaglutinacije. Molekularna diagnostika je bila izvedena z RRT-PCR za M-gen. Vsi vzorci so bili AIV negativni, medtem ko so bili 4 vzorci pozitivni na ptičji paramiksovirus 1 (virus Newcastle). Divje ptice kot primarni gostitelj AIV niso bile vključene v nadzor, kar ne zagotavlja popolne informacije o razširjenosti AIV v R. Makedoniji in jo je treba še dodatno raziskati. To pomeni, da se je treba lotiti izdelave koncepta nacionalnih ukrepov za zgodnje odkrivanje in odziv ob pojavu ptičje gripe.

Ključne besede: virus ptičje gripe; dvoriščna perutnina; kloakalni bris; R. Makedonija; nadzor

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Original Scientific Article

SOMATIC CELL COUNTS AND TOTAL BACTERIAL COUNT IN BULK TANK MILK OF SMALL RUMINANTS

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Summary: Total bacterial count (TBC) is a good tool for monitoring the hygienic environment of small ruminants. TBC is significantly correlated with the number of somatic cells in bulk milk (BMSCC). Programs to improve the hygienic quality of bulk milk are more effective if you take into account both variables (TBC and BMSCC). A significant impact on BTSCC in ewe bulk milk was found for the following factors: herd/flock, sampling month, dry therapy practice, breed of sheep, and type of milking. Factors associated with the mean standard plate count (SPC) included the month of bulk milk sampling and the number of milkings contained in bulk tank milk, as well as the adopted milking technique. In fresh sheep chesses the casein fractions (αs1-1-CN and β-CNs) were significantly lower, when BTSCC was high. These cheeses have lower fat contents and as a result an increased pH and fat acidity. Yoghurts made from milk with a high BTSCC were characterized by a higher protein content and the highest level of syneresis. To improve the udder health status of small ruminants it is necessary to ensure hygienic conditions of animal maintenance and optimization of milking machine standards and parlor systems.

Key words: small ruminants; somatic cell count; total bacterial count; bulk tank milk

Introduction

Total bacterial count (TBC) can serve as a tool in monitoring hygiene of dairy sheep flocks and can also provide a basis for payment systems for milk (4, 8, 15). A statistically significant correlations was found between TBC and the number of somatic cells in bulk milk (BMSCC). Programs to improve the hygienic quality of bulk milk are more effective if you take into account both variables (8). More recent results indicate that predictors of quality milk for cheese production include effectiveness of lactose, casein % and somatic cell count (SCC) of bulk milk (15). Milk microbiological standard stipulated by the European Council Directive 92/46/EEC may be used as a basis to develop specific quality parameters in the standard system for assessing milk of small ruminants, as it has been the case in Switzerland (27). In recent years, the Single Strand Conformation Polymorphism (SSCP) method proved to be a powerful tool for distinguishing milks and classifying them on the basis of the balance between different bacterial populations (26). In goats apocrine secretion mechanisms cause in milk the presence of cytoplasmic particles (CP), which are mostly anuclear and part of physiological secretions (22). Application of flow cytometry facilitates a rapid differentiation of somatic cells; however, it is necessary

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to use DNA-specific fluorescent dyes to avoid overlapping of SCC and CP (2). This new method allows a differentiation between viable and non-viable polymorphonuclear neutrophils (PMN), macrophages, and lymphocytes.

The aim of the study is to review the scientific literature in the field of hygienic and microbiological quality of bulk milk and the quality of products (cheese and yogurt) made from the milk of small ruminants.

Bulk milk somatic cell counts related to bulk milk total bacterial counts

The base level of SCC in bacteria free udders of goats (~ 300,000 cells/ml) and sheep (~ 200,000 cells/ml) is generally higher than in dairy cows (~ 70,000 cells/ml); hence, the level of SCC in animals with udder infection is usually much higher in small ruminants than in dairy cows (24). Intramammary infection (IMI) is a major cause of an increased SCC in milk of dairy ruminants (7, 8, 9, 13, 14, 21, 22). The innate immune system plays a major role, especially polymorphonuclear neutrophils (PMN), that appear in the mammary gland in response to invading pathogens (21). However, non-infectious factors are the cause of the variability of SCC in goat milk and some species of bacteria cause different levels of somatic cells as well as varying degrees of *mastitis* (22). Similarly in sheep, the increase in SCC is related to the stage of lactation and parity, but mainly results from intramammary infections (21). Many authors emphasize that the use of SCC is a good indicator in the assessment of sanitary conditions in herds/flocks, especially in herds with maintained control of IMI (8, 9, 22). In herds of Murciano-Granadina goats several animals infected with mycoplasma do not cause an increase in BTSCC, provided that basic rules of hygiene are kept in the herd, and infected animals are rapidly removed from the herd (6). From the 1068 collected goat milk bulk-tank samples, 84 (7.9%) were positive for the presence of *Mycoplasma* species (4). Most of the isolated species were *Mycoplasma agalactiae*, which were found in 69 samples of milk (82%), and in 14 samples (17%) was detected *Mycoplasma mycoides* subsp. *Mycoides* large colony type (*MmmLC*) in the herd. Higher BTSCC levels (1,176,000 cells/ml) were recorded in bulk-tank samples containing mycoplasmas in comparison to the count recorded for samples testing negative (875,000 cells/ml), and these differences were statistically significant. Bulk goat milk generally has a high SCC level; however, less than 50% of milk supplied by producers comply with the standards, i.e. 1 x 10^6 cells/ml (7). In a high percentage of milk samples neutrophils and variable bacterial counts, and a lack of correlation with SCC indicate problems with milk contamination. These problems may be caused by residual lactation and low milk production, but further research needs to be carried out on the high percentage of neutrophils reported in goat milk. In a study by the year 2010 in 53 herds of dairy goats 3 bulk milk samples were taken at 2-week intervals to assess how different bacterial groups in bulk milk are related to BTSCC, TBC and bulk milk standard plate counts (SPC) (13). It was found that the staphylococcal count was correlated to SCC (r = 0.40), TBC (r = 0.51) and SPC (r = 0.53). Coliform count was correlated only to TBC (r = 0.33); however, *Staphylococcus aureus* was not correlated to SCC. The share of the staphylococcal count in the SPC was 31%, while coliform count was only 1% of the SPC. Somatic cells in goat bulk milk showed a moderate correlation to the bacterial counts, and Koop et al. (14) hypothesized that IMI is an important factor affecting SCC in bulk milk. However, many other factors associated with the management of the herd, as well as nutritional factors and diseases, of which an important role is played by *mastitis*, affect SCC in bulk milk. In the literature there is little information showing an association between bulk tank total bacterial count (TBC) and bulk tank SCC (BTSCC) in dairy sheep (8, 10, 18). Dry therapy practice and milking the ewes in parlor systems were associated with low TBCs (8). A study on milk quality in Assaf ewes indicated that antibiotic residue (AR), SCC and TBC in bulk tank milk can be used as variables to monitor mammary health, milk hygiene, and safety in dairy ewes (10). High SCCs were associated with an increased AR occurrence. It was confirmed that the prevalence of AR in the milk of sheep is inversely proportional to milk yield and the highest degree of AR incidence was in the autumn. The milk from small ruminants, similarly as milk of other species in the EU countries cannot contain antibiotic residues (5). In raw goat and ewe bulk tank milk in Switzerland a wide distribution of subclinical infection with *Mycobacterium avium ssp. Paratuberculosis* (MAP) was found, amounting to 23% and 24%, respectively (18).

Risk factors associated with a deterioration of bulk milk quality

A basic condition for good quality milk from small ruminants, especially in case of small farms, is the observance of hygiene on the farm, mainly to maintain hygiene equipment and utensils for milking in good condition (17, 20). The IMI that cause subclinical and clinical *mastitis* has been estimated at 5 - 30% and 5%, respectively (5, 16). In problematic flocks mastitis incidence is much higher than 30 - 50% and may reach up to 70%. Many pathogens can cause *mastitis*; however, *Staphylococcus* spp. are the most commonly diagnosed microorganism of IMI in goats and sheep (5). Manual milking was associated with a 62% risk of bacterial infection of milk compared to mechanical milking, while mechanical milking with portable devices was associated with a 40% higher risk of bacterial infection compared to mechanical milking with fixed plants (16). In Switzerland for goat milk median SPC was 4.68 log cfu/ml and mean SPC 6.92 log cfu/ml, whereas for ewe milk median SPC was 4.79 log cfu/ml and mean SPC was 6.05 log cfu/ml, respectively (27). Factors associated with SPC included the month of bulk milk sampling, while among the four evaluated months (April, May, June and July) the highest median SPC (5.24 log cfu/ml) and mean SPC (7.13 log cfu/ml) were found in June. Differences in SPC results between the months of sample collection were significant. The number of milkings contained in bulk tank affected SPC (log cfu/ml). The highest median value of the SPC (5.70 log cfu/ml) and mean SPC (7.24 log cfu/ml) were found in bulk tank milk containing four milkings. According to Zweifel et al. (27) also milking technique affected SPC value, the highest median SPC (5.06 log cfu/ml) and mean SPC (6.90 log cfu/ml) were found for bucket milking without a parlor; however, the hand milking technique showed the lowest SPC results (median SPC 4.48 log cfu/ml, and mean SPC 6.87 log cfu/ml). Flock size also affected SPC value, as the highest median SPC (4.85 log cfu/ml) and mean SPC (6.86 log cfu/ml) were found in larger flocks (> 25 animals). In case of BTSCC in ewe bulk milk a significant impact was found for the following factors: flock/herd, sampling month, dry therapy practice, breed of sheep (Spanish Assaf, Awassi, Churra, Castel), type of milking (hand vs. machine, and type of installations used for machine milking (milking buckets and parlors: looped type milkline, dead-milkline ended, midlevel and low-level systems) (9).

Effects of somatic cell counts and contamination on quality products from goat milk and sheep milk

Bulk milk from ewes of three breeds, i.e. Churra, Castelana, and Assaf, with different levels of somatic cells was used to produce hard cheese (23). Chesses were produced from bulk milk of three classes: low (< 500,000 ml^{-1}), medium (1,000,000 – 1,500,000 ml^{-1}) and high (>2,500,000ml^{-1}) SCCs. A significant increase has been shown in proteolysis with an increase in SCC levels. In fresh cheeses the casein fractions (α_{s1}-1-CN and β-CNs) were significantly lower as SCC values increased. In cheeses made from milk with a high SCC a reduced fat content was recorded, and it followed an increase of pH and fat acidity. Yoghurts were made from milk of the Baluchi breed with two SCCs, i.e. <200,000 ml^{-1} and >750,000 ml^{-1} (19). Different levels of SCC are associated with changes in milk composition and properties of yoghurt; the SCC had no effect on acidity, while for pH of the yoghurt after 24 h this effect was significant after 168 h of storage. Somatic cell count had no effect on total solids and fat content of the yoghurt. A high SCC was associated with a higher protein content and the highest level of syneresis. In Norway the presence of potential-poisoning bacteria was evaluated during the process of small scale cheese production using caprine and bovine raw milk (12). The prevalence of *Staphylococcus aureus* was varied during the manufacturing process of cheese from the milk of both species. The prevalence of *Staphylococcus aureus* in caprine cheese ranged from 91.8% in milk at 0 h and 95.9% at 24 h, and reduced to 42.9% after 30 days. The prevalence of *Staphylococcus aureus* in bovine cheese was 47.2% at 0 h, peaked to 80.8% after 5 - 6 h, and amounted to 24.7% after 30 days. The highest contamination levels of *Staphylococcus aureus* were observed in both caprine and bovine cheese after 5-6 h. Somatic cell count in bulk goat milk below the admissible limit according to the current Pasteurized Milk Ordinance regulation (PMO) amounting to 1,000,000 cells/ml in grade. A goat milk, did not affect milk composition and semisoft cheese yield (3). However, the high SCC in goat milk resulted in a lower texture score as well as a

lower total sensory score. Cheeses made with high SCC milk exhibited higher lipolysis during ripening compared with cheeses made from low SCC milk. The high levels of SCC in goat and ewe milk are associated with milk yield losses, changes in milk composition end affect the quality and stability of cheese (22).

Control and prevention strategies

In a study by Berthelot et al. (1) was demonstrated that in ewe milk individual SCC (iSCC) represent a useful tool for the detection of subclinical mastitis in dairy ewes. The authors propose a classification as a healthy udder (specificity = 75%), if throughout lactation iSCC are lower than 0.500 x 10^6 cells/ml, and infected (sensitivity = 82%) if at least two iSCC are higher than 1 or 1.2 million cells/ml. However, the decision should be adapted to different control strategies for SCC in bulk milk. Antibiotic dry-off therapy was found to significantly reduce IMI incidence in small ruminants (5, 9, 11, 25). This treatment significantly decreased BTSCC from log BTSCC 6.24 and the geometric mean of 1,725 x 10^3 cells/ml in the pretreatment lactation to log BTSCC of 6.15 and 1.422 x 10^3 cells/ml in the subsequent lactation (11). Some authors are of a different opinion that the dry treatment did not result in reduced SCC in the subsequent lactation (25). Ewes with a history of high SCC during the previous lactation (i.e. more than 3 monthly tests ≥ 400,000 cells/ml) were more likely to have IMI in the post parturition period. Treatment with antibiotics requires veterinary supervision to ensure proper hygiene, because some *mastitis* outbreaks originated from contamination of the syringe (5). Dry therapy is generally associated with improved milking hygiene in the subsequent lactation, thus reducing IMI and improving milk quality. This practice and milking in parlor systems were associated with low TBC, and a significant reduction of BTSCC (8, 9, 10). More pulsations (180 cycles/min) and low vacuum levels (34 to 36 kPa) are optimal for udder health during milking of ewes (9).

Conclusion

BTSCC of small ruminants depends mainly on TBC, that cause infection of mammary glands. Factors that may reduce the risk of IMI include the ability to keep milking hygiene and adherence to correct milking parameters. Cheeses made from milk with a high BTSCC had a reduced fat content and increased pH and fat acidity. In yoghurt high BTSCC was associated with a higher protein content and the highest level of syneresis. Further research is needed to determine the exact thresholds of BTSCC in the milk of sheep and goats related to dairy products.

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ŠTEVILO SOMATSKIH CELIC IN SKUPNO ŠTEVILO BAKTERIJ V ZBIRALNICAH MLEKA DROBNICE

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Povzetek: Skupno število bakterij (TBC, angl. total bacterial count) v mleku je dober pokazatelj higienskih razmer pri reji drobnice. TBC je povezan s številom somatskih celic v zbiralnicah mleka (BMSCC, angl. number of somatic cells in bulk milk), če pa upoštevamo obe spremenljivki, TBC in BMSCC, dobimo še zanesljivše rezultate. Pomemben vpliv na BMSCC v zbiralnicah ovčjega mleka imajo naslednji dejavniki: čreda, mesec vzorčenja, uporaba suhe terapije, pasma ovc in način molže. Na povprečno vrednost celic na plošči (MSPC, angl. mean standard plate count) pa vplivajo: mesec vzorčenja mleka in število molž v zbiralnici kakor tudi način molže. V svežem ovčjem siru so bile kazeinske frakcije (αs1-1-CN in beta-CNS) precej nižje, medtem ko je bila vrednost BMSCC visoka. Ti siri vsebujejo manj maščob in imajo zato višji pH in maščobno kislost. Jogurti, narejeni iz mleka z visoko vrednostjo BMSCC, imajo značilno višjo vsebnost beljakovin in visoko stopnjo sinereze. Potrebno je zagotoviti ustrezne higienske pogoje pri ravnanju z živalmi in določiti higienske standarde za molzne stroje in molzišča, da bi izboljšali zdravje vimen malih prežvekovalcev.

Ključne besede: drobnica; število somatskih celic; skupno število bakterij; zbiralnice mleka

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Original Scientific Article

THE EFFECT OF THYME OIL ON THE SHELF LIFE OF CHICKEN BALLS DURING STORAGE PERIOD

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Summary: The effects of thyme essential oil on the shelf life of chicken balls during refrigerated storage for 12 days were examined. Treatments examined in the present study were as follows: A (control samples, untreated), B (final concentration of thyme essential oil; 0.4% w/w, added to ball), C (final concentration of thyme essential oil; 0.4% w/w, on surface of ball). The shelf-lives of samples were determined by means of microbiological, chemical, sensory, colour and texture analyses. Microbial populations in B group samples were determined to be reduced at the end of the storage period. Lactic acid bacteria (LAB) and Enterobacteriaceae were the most sensitive groups among the microorganisms examined. B group treatment samples resulted in lower microbiological counts in comparison to the control (A) and C group samples, but LAB was found to be lower in group C samples. Salmonella spp. and Staphylococcus spp. were not detected in all samples. TBA values for C group samples remained lower than 1mg MDA/kg throughout the 12 day storage period. The pH values varied between 6.4 (day 0) and 5.9 (day 12). The C group samples were found to be desirable (organoleptically acceptable) following sensory analysis. Based primarily on sensory data (taste attribute) B and C groups samples extended the product's shelf-life by ca. 4 and 6 days, respectively, as compared to the control sample. The results of the analyses of chicken balls showed no significant differences ($p>0.05$) in colour and texture among all samples.

Key words: thyme oil; chicken ball; microbiology; chemical and sensory quality; colour and texture analysis

Introduction

Poultry meat is very popular in the world and nowadays it is consumed increasingly in many countries. Chicken meat has a low cost of production, low fat content and high nutritional value. However, poultry products are pathogenic and spoilage microorganisms (*Salmonella*, *Yersinia enterocolitica*, such as) and remains a significant concern for consumers and public health officials worldwide. High consumption of poultry products leads to concerns in the industry about shelf-life extension. *Escherichia coli* and *Staphylococcus aureus* have been used in poultry products to assess microbiological safety, sanitation conditions during processing and the retention of the quality of the product (1, 2). Natural food preservatives are used to ensure protection from both spoilage and pathogenic microorganisms (3). Recently, herb and spice extracts have been a particular focus. These extracts have been used to improve sensory characteristics and to extend the shelf-life of foods (4). There has been a great interest within the food industry during the last decade regarding the antimicrobial substances used in foods, such as thyme oil (5). Thyme (Thymus vulgaris L.) is used both as a spice and a condiment. The essential oil of thyme (Thymus vulgaris L.) has a significant rate of fungal and antibacterial activity with

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strongly inhibited lipid peroxidation and high off radical scavenging (6, 7).

This study was carried out to investigate the effects of thyme oil (on the surface of and as an additive to chicken balls) on the quality of chicken balls during storage.

Material and methods

Preparation of chicken balls

Chickens were purchased from a local market. After washing, the skin and the apparent fat and bone tissues were removed. The meat was treated using a kitchen food processor with a pore size of 5 mm. The chicken ball included 85% chicken mince, 2% salt, 0.5% cumin, 0.5% red pepper, 10% onion and 2% garlic. The ingredients were homogenized with a kitchen blender. The spices were purchased from Bağdat Company (Istanbul, Turkey). The chicken meat batter was shaped into balls using stainless steel equipment (approximately 20 g).

Preparation of experimental groups

The following lots of samples were prepared: The first lot of samples comprised the controls (aerobic packaging, group A). Lot two consisted of samples with thyme oil (Sigma, Germany) 0.4% (B and C group). The B group which thyme oil %0.4 added batter chicken meatball (considering amount of batter meat ball). The preparation of C group, thyme oil was surface balls (final concentr ations equal to 0.4% w/w). Thyme oil was added undiluted using a micropipette. The thyme oil was massaged onto the product, so as to get even distribution of the oil using gloved fingers (to avoid cross-contamination of samples and also transmission of food poisoning organisms). After three groups of chicken balls were produced, they were refrigerated at 4 ± 1 °C in straphor trays covered with aluminium foil for testing microbiological, chemical, sensory, colour measurement and texture analysis on 0, 3, 6, 9 and 12 days of the storage.

Microbiological analysis

Approximately 25 g of the chicken meat balls were sampled using sterile scalpels and forceps, immediately transferred into a sterile stomacher bag, containing 225 ml of 0.1% peptone water (pH 7.0), and homogenized for 60 s in a Lab Blender 400 Stomacher at room temperature. Microbiological analyses were conducted using standard microbiological methods (8). The amount of 0.1 ml of these serial dilutions of chicken homogenates was spread on the surface of dry media. Total viable counts (TVC) were determined using Plate Count Agar (PCA, Merck code 1.05463), after incubation for three days at 30 °C. *Pseudomonads* were determined on cetrimide fusidin cephaloridine agar (Oxoid code CM 559, supplemented with SR 103) after incubation at 25 °C for 2 days (9, 10). For members of the family *Enterobacteriaceae*, 1.0 ml sample was inoculated into 5 ml of molten (45 °C) Violet Red Bile Glucose Agar (Oxoid code CM 485). After setting, a 10 ml overlay of molten medium was added and incubation was carried out at 37 °C for 24 h. The large colonies with purple haloes were counted. Lactic acid bacteria (LAB) were determined on de Man Rogosa Sharpe Medium (Oxoid code CM 361) after incubation at 25 °C for 5 days. Yeasts and moulds were enumerated using Rose Bengal Chloroamphenicol Agar (RBC, Merck code 1.00467) after incubation at 25 °C for 5 days in the dark. Isolation of *Salmonella* spp. was carried out in four stages. After incubation at 35–37 °C for 16–20 h for the pre-enrichment step, 0.1 and 1 ml of the homogenate was transferred to RV (Rappaport Vassiliadis, Merck) and Selenite Cystein Broth (Merck) for selective enrichment with an incubation period of 42 and 35 °C for 24 h, respectively. After incubation, a loopful from each tube was streaked on Brillant Green Phenol Red Lactose Agar (Merck) and Bismuth Sulfite Agar (Merck). These plates were incubated for 20–24 h at 35 °C and checked for typical colonies. Five colonies were selected for biochemical tests and were grown in Nutrient Agar (Oxoid) at 35 °C for 18–24 h (11).

Baird Parker Agar (Merck) was used for the estimation of *Staphylococcus* spp. counts. After incubation at 35 °C for 45–48 h, typical colonies were tested for the detection of coagulase production. (12). Duplicate plates were spread from each dilution of 0.1 ml. All plates were examined for typical colony types and morphology characteristics associated with each growth medium.

Chemical analysis

The pH value was recorded using a pH meter. Chicken samples were thoroughly homogenized

with 10 ml of distilled water and the homogenate used for pH determination (13). Thiobarbituric acid (TBA) was determined according to the method proposed by Pearson (14).

Sensory analysis

The door and taste of cooked chicken balls were evaluated by a panel of seven judges, experienced in chicken ball evaluation on each day of sampling. Chicken ball samples were cooked individually in an oven, for 5 min and were then immediately presented to the panellists. The scale points were: excellent, 5; very good, 4; good, 3; acceptable, 2; poor (first off-odour, off-taste development), 1; a score of acceptability (15).

Colour measurement

Colorimetric measurements of cooked chicken balls were determined in triplicate using a Colorimeter (Minolta spectrophotometer CM 3500d, Japan). The colour reading includes lightness (L), redness (a) and yellowness (b). The equipment was standardized with a white colour Standard. Five replicate measurements were taken for each sample, following the guidelines for colour measurements from American Meat Science Association (16).

Texture profile analysis (TPA)

Texture measurement of cooked chicken balls were used a Texture Analyzer (TA.XTPlus Stable Micro Systems, UK). Texture Profile Analysis (TPA) was used to determine hardness, cohesiveness, chewiness and springiness (17). This test was carried out by using compression plate with a diameter of 75 mm. The mean of five measurements was taken for each hardness, cohesiveness, chewiness and springiness.

Statistical analysis

Analysis of the data was conducted using a Statistical Analysis System (SAS) packages software. Values between groups and within groups-between days were compared. Data were subjected to variance analysis in accordance with 3 x 5 x 3 x 2 factorial design and in terms of fix effects and inter-variable interactions so that "repetition number x sampling time x test groups x number of samples examined at one instance from each test group". According to the General Linear Models (GLM) procedure, the Fisher's smallest squares average (LSD) test was used. Standard deviation figures of all averages were calculated (18). The alpha value was determined as 0.05.

Result

Microbiological changes are given in Fig.1. The thyme oil treated groups had a lower count TVC. The control samples reached a TVC count of upper 7 log cfu/g storage day. Listeria spp. and *Salmonella* spp was not detected in any of the groups. Whereas on the 3rd day of storage, the number of *Pseudomonads* in group B was 1 log cfu/g, no reduction was determined in the *Pseudomonads* values of the C group ($P > 0.05$). On day 9 of storage, group B was 3.11 log cfu/g ($P<0.05$), while groups C and A were determined as 5.11, 7.37 log cfu/g. Initial populations of LAB were ca. 3.42 and increased to reach counts of 7.13 log cfu/g on day 9 of storage, group A samples ($P<0.05$). However, significantly lower LAB counts ($P < 0.05$) were recorded for C samples during the entire storage period under refrigeration. The *Enterobacteriaceae* counts were determined by 7.56 log cfu/g, 2.13 log cfu/g and 2.59 log cfu/g group A, B and C on day 9, respectively. The yeast and mould counts were initially (0 day) determined as 2.78 log cfu/g all samples. In the A group of samples, the yeast and mould count was shown to have increased steadily during storage time (2.78, 4.26, 4.6 and 5.13 log cfu/g, 0, 3, 6 and 9 day, respectively). B and C group samples were found to contain a yeast and mould count of <10 log cfu/g, on the third and sixth days of storage. B group samples were not observed on day 9 while the yeast and mould counts were found to be 2.56 log cfu/g, at the end of storage. C group samples were found to be 2.19, 2.43 log cfu/g, on days 9 and 12, respectively.

Changes of pH values are shown in Fig. 2a. Values of pH initial were recorded for groups A, B and C as 6.17, 6.21 and 6.19, respectively. The TBA values for all chicken ball treatments are given in Fig. 2b. The TBA values for the control group varied between 0.3 and 1.8 mg MDA/kg. A very low lipid oxidation value of between 0.3 and 0.9 mg MDA / kg was determined for the treatment group.

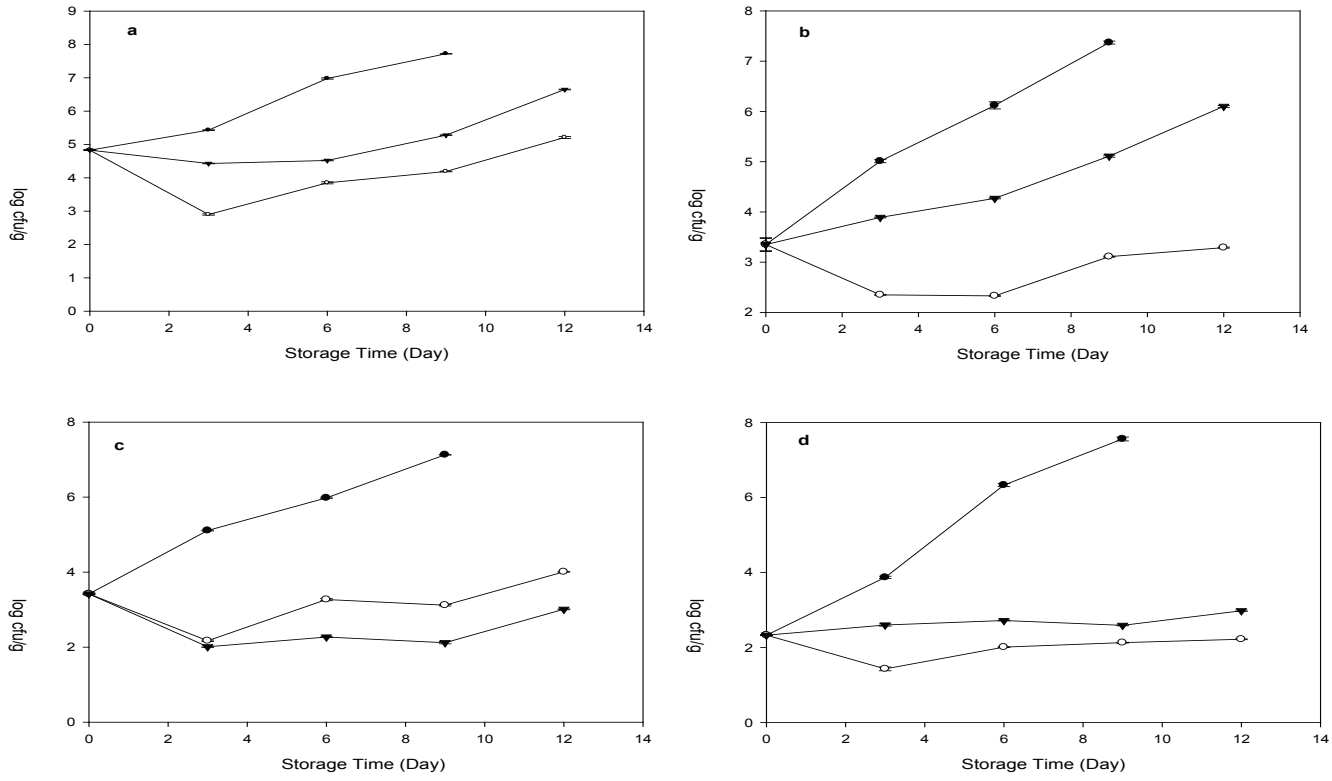

Figure 1: Changes (log cfu/g) in (a) Total Viable Counts (TVC); (b) *Pseudomonas* spp.; (c) LAB and (d) *Enterobacteriaceae*, control (A), batter with added thyme EO 0.4% (B) and ball surface thyme EO 0.4% (C). Each point is the mean of three samples taken from two replicate experiments (n: 3 x 2: 6). Error bars show SD. —●— A —○— B —▼— C

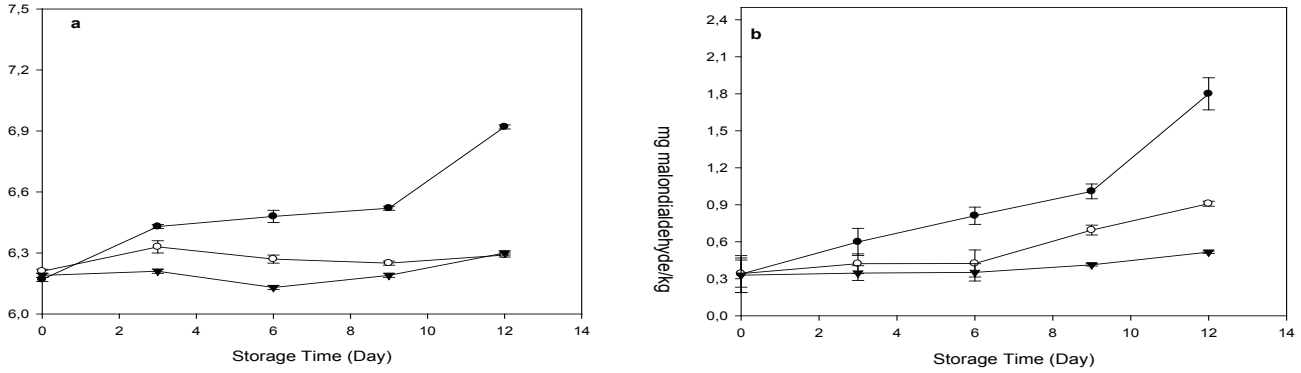

Figure 2: Changes of pH (a) and TBA (b) values of chicken balls, control (A), batter with added thyme EO 0.4% (B) and ball surface thyme EO 0.4% (C). Each point is the mean of three samples taken from two replicate experiments (n: 3 x 2: 6). Error bars show SD. —●— A —○— B —▼— C

The sensory properties (odour and taste) of the cooked chicken balls are given in Fig. 3a-b. Sensory analysis was carried out until the 6th day of storage for control group samples and until the 12th day of storage for treatment group samples. Due to the microbiological quality of the control group samples, exceeded limit values were not subject to sensorial evaluation. Taste attribute has usually been a more sensitive parameter than odour; therefore, taste attribute was used for the sensory evaluation of the thyme products and the determination of sensorial shelf-life in the present study. On day 0 of storage, the cooked chicken balls had a pleasant taste and odour. The presence of thyme oil (on ball surface) in the samples produced a very pleasant taste ($P<0.05$).

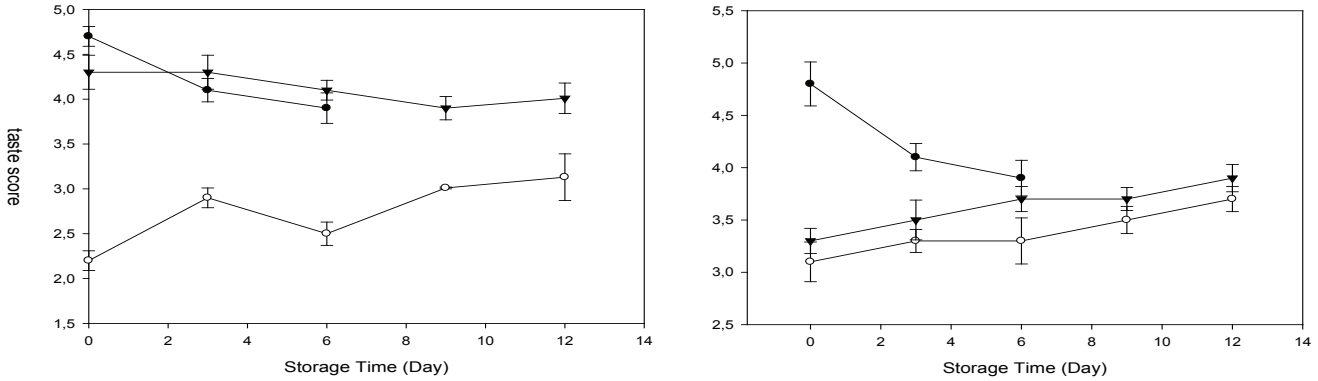

Figure 3: Changes in the taste (a) and odour (b) of chicken balls, control (A), batter with added thyme EO 0.4% (B) and ball surface thyme EO 0.4% (C). Each point is the mean of three samples taken from two replicate experiments (n: 3 x 2: 6). Error bars show SD. —●— A —○— B —▼— C

Table 1 shows the colour measurement results of chicken balls. All chicken balls varied insignificant in their L, a and b values ($p>0.05$).

Table 1: Colour properties of experimental chicken balls

Group	Storage time (day)					
	Analysis	0	3	6	9	12
A	L*	75.42±0.46 ^{a,z}	75.42±0.33 ^{a,z}	75.33±0.56 ^{a,z}	75.56±0.41 ^{a,z}	75.33±0.39 ^{a,z}
	a*	0.90±0.09 ^{a,z}	0.90±0.06 ^{a,z}	0.91±0.4 ^{a,z}	0.93±0.21 ^{a,z}	0.90±0.36 ^{a,z}
	b*	16.77±0.25 ^{a,z}	16.77±0.21 ^{a,z}	16.81±0.56 ^{a,z}	16.85±0.43 ^{a,z}	16.77±0.21 ^{a,z}
B	L*	75.33±0.19 ^{a,z}	76.16±0.26 ^{a,z}	76.01±0.31 ^{a,z}	75.19±0.18 ^{a,z}	75.96±0.16 ^{a,z}
	a*	0.91±0.04 ^{a,z}	0.90±0.02 ^{a,z}	0.96±0.05 ^{a,z}	0.91±0.04 ^{a,z}	0.93±0.04 ^{a,z}
	b*	17.13±0.22 ^{a,z}	17.09±0.19 ^{a,z}	17.33±0.24 ^{a,z}	17.13±0.19 ^{a,z}	17.21±0.21 ^{a,z}
C	L*	76.01±0.56 ^{a,z}	76.81±0.54 ^{a,z}	76.16±0.42 ^{a,z}	76.21±0.36 ^{a,z}	76.33±0.29 ^{a,z}
	a*	0.93±0.01 ^{a,z}	0.91±0.06 ^{a,z}	0.94±0.02 ^{a,z}	0.90±0.01 ^{a,z}	0.92±0.03 ^{a,z}
	b*	16.36±0.12 ^{a,z}	16.22±0.21 ^{a,z}	16.43±0.23 ^{a,z}	16.21±0.19 ^{a,z}	16.23±0.21 ^{a,z}

Texture analysis results for chicken balls are shown as in Table 2. Results regarding hardness, cohesiveness, springiness and chewiness value during storage time were determined to be similar for all groups, therefore, the mean values have been given.

Table 2: Textural properties of experimental chicken balls.

Group	Hardness (kg)	Cohesiveness(mm/mm)	Springiness (mm/mm)	Chewiness (kg/mm)
	M ± SD			
A	3.73±0.22	0.67±0.01	12.56±0.42	31.33±1.56
B	3.96±0.19	0.65±0.04	12.93±0.46	35.21±1.41
C	3.52±0.28	0.69±0.02	12.87±0.52	32.56±1.82

Discussion

The changes in total viable count (TVC) of the chicken meat balls are given Fig. 1a. The initial value of TVC (day 0) for fresh chicken meat was ca. 4.3 log cfu/g, indicative of good quality chicken meat (19). TVC reached a value of 7 log cfu/g, considered as the upper microbiological limit for good quality fresh poultry meat, as defined by the ICMSF (2), on day 6 for the control samples. Samples treated with thyme oil in groups never reached the limit of 7 log cfu/g during the 12 day storage period. The group B samples were determined to be 4.83 log cfu/g 0 day. This value decreased about 2 log cfu/g on day 3 of storage and reached 5.21 log cfu/g at the end of storage. There was no significant change to the C groups sample during storage. Thyme oil in the additive ball was more effective than in the surface ball on reducing TVC. Thyme oil resulted in a shelf-life extension of six days as compared to the control samples. In related studies, Giatrakou (7) mentioned a shelf-life extension of 2 days after the application of thyme oil 0.2% v/w on chicken, while Ergezer (20) reported that TVC in chicken balls was reduced with whey powder stored at -18 °C for three months.

Listeria spp. and *Salmonella* spp were found in one of the samples. They are also in agreement with those reported by Yavaş et al. (21), who reported no determination in ground chicken balls after 21 days of storage under modified atmosphere pressure (MAP).

Pseudomonads (Fig. 1b) are Gram-negative bacteria, comprising the main spoilage microorganisms in meat (22). In other studies, the combined use of thyme oil on chicken products stored aerobically at 4 °C, resulted in about 8.0 log cfu/g of the final population of pseudomonas (6). Deans and Richie (23) showed that thyme oil was very effective against *Pseudomonas aeruginosa*, in a study where the inhibitory properties of ten plant essential oils were tested using an agar diffusion technique. This is in agreement with Elgayyar et al. (24) who reported that oregano essential oil was less effective in inhibiting P. *aeruginosa* compared to other microorganisms.

Lactic acid bacteria (LAB) (Fig. 1c) facultative anaerobic species were found to be members of the microbial flora of the chicken product. Of all the antimicrobial treatments in this study, thyme oil on surface of ball treatment proved to be the most effective in inhibiting the growth of LAB, almost stable during the storage period. For Georgantelis et al. (25), the combined use of rosemary essential oil and chitosan on fresh pork sausages stored aerobically at 4 °C, resulted in a 2.0 log cfu/g reduction of the final population of LAB. In another study, LAB count reduced by 0.2% in the chicken balls with thyme oil during storage (6).

With respect to *Enterobacteriaceae* (Fig. 1d), considered as a hygiene indicator (26), the initial counts of 2.33 log cfu/g were indicative of good quality chicken meat. *Enterobacteriaceae* grew under essential oil applications at a slower rate than others. This is in agreement with the results of Choularaia et al. (27), who reported that oregano oil had a strong effect in the reduction of *Enterobacteriaceae* counts. On day 9 of storage, the use of thyme oil had practically no effect on *Enterobacteriaceae* counts ($p > 0.05$). On the same day, the *Enterobacteriaceae* counts were determined by 7.56 log cfu/g, 2.13 log cfu/g and 2.59 log cfu/g in groups A, B and C, respectively. Giatrokau et al. (7) reported that *Enterobacteriaceae* of chicken products were inhibited in the presence of thyme oil packaging.

As a result, the initial population of yeasts and moulds were very low (2.78 log cfu/g) and no population was determined on day 3 and 6 of storage for B and C group samples. The balls with added thyme oil were a lot more effective in reducing yeast populations in comparison to surface added balls ($P<0.05$). Yeast and mould were not determined in B and C group samples on day 9 of storage, whereas 5.3 log cfu/g was determined for the A group samples on the same day. Conner and Beuchat (28) reported a strong inhibitory action of oregano and thyme oils on the growth of yeast. The above results are in agreement with those of Giatrakou et al. (7), who reported that thyme oil 0.2% significantly lower yeasts-moulds counts than the control samples during the storage.

The microbiological analysis was performed on B and C groups for day 12, while group A was done until day 9.

Kayışoğlu et al. (29) reported a pH value of 6.1 for raw chicken doner kebab, this situation is similar to our finding. The addition of thyme oil resulted in a slight increase in pH values. The group A samples increased pH value during storage ($P<0.05$). Georgantelis (25) reported higher pH values for Greek-style sausages, containing chitosan and rosemary extract.

Choluaria et al. (27) who reported TBA values of 0.1–0.9 mg MDA / kg meat for chicken product after 25 days of storage. Thyme oil significantly affects the degree of lipid oxidation under present experimental conditions, given the antioxidant properties of this essential oil. Kim et al.(5) reported TBA values of 0.13–0.68 mg MDA/ kg meat for turkey and pork after seven days of storage, and these figures are similar to our findings. The thyme oil application of samples showed slightly higher values of TBA. Botsoglou et al. (4), who reported a threefold reduction in the degree of lipid oxidation (0.6-0.2) in turkey meat treated 200 mg/kg oregano oil, this is in contrast our results.

According to Choluaria et al.(27) the addition of thyme oil to chicken products results in a more acceptable odour and flavour as compared to the untreated samples.

Colour parameters (L*, a*, and b*) and TPA values in samples containing the thyme oil were not affected during storage. Changes in colour parameters and TPA values were statistically insignificant (P>0.05) in all samples.

As a result, it was determined in this study that thymol prolongs the shelf life of chicken meat balls. The effect of essential oil is already known. However, thyme oil added to chicken balls is as an effective method as applying it to the surface. Moreover, the taste of chicken balls with added thyme oil on the surface was probably better. This method may be preferred because it is safe, practical and a positive sensory feature. This method may be used to protect other foods as well.

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VPLIV TIMIJANOVEGA OLJA NA ROK UPORABNOSTI PIŠČANČJIH KROGLIC V OBDOBJU SKLADIŠČENJA

Ö. Pelin Can

Povzetek: Ugotavljali smo učinke timijanovega eteričnega olja na rok uporabnosti piščančjih kroglic v 12-dnevnem obdobju skladiščenja. Uporabili smo naslednje vzorce: A (kontrolni vzorci, netretirani), B (0.4% koncentracija eteričnega olja timijana dodana kroglici), C (0.4% koncentracija eteričnega olja timijana, nanesena na površino kroglice). Obstojnost vzorcev smo ugotavljali s pomočjo mikrobioloških in kemičnih analiz ter z zaznavo barve in teksture. V skupini B se je mikrobna populacija na koncu obdobja skladiščenja zmanjšala. Med opazovanimi mikroorganizmi so mlečnokislinske bakterije (LAB, angl. lactic acid bacteria) in enterobakterije najbolj občutljive. V skupini B je bilo ugotovljenih manj mikroorganizmov kot v kontrolni skupini (A) in skupini C, vendar je bilo ugotovljeno, da je vrednost LAB nižja v skupini vzorcev C. *Salmonella* spp. in *Staphylococcus* spp. nista bili odkriti v nobenem vzorcu. Skupno število bakterij vzorcev C je ostalo pod 1 mg MDA / kg v celotnem 12 dnevnem obdobju shranjevanja. pH vrednosti so se gibale med 6.4 (dan 0) in 5.9 (dan 12). Po izvedeni senzorični analizi je bilo ugotovljeno, da je skupina vzorcev C organoleptično sprejemljiva. Na osnovi senzorične analize (okus) je bilo ugotovljeno, da se je vzorcem skupin B in C podaljšal rok uporabnosti za 4 oz. 6 dni v primerjavi s kontrolnimi vzorci. Analiza piščančjih kroglic vseh vzorcev ni pokazala značilnih razlik ($p > 0,05$) v barvi in teksturi.

Ključne besede: timijanovo olje; piščančje kroglice; mikrobiologija; kemična in senzorična kakovost; analiza barve in teksture

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Original Scientific Article

DETERMINATION OF SEX HORMONES IN RAT HAIR AFTER ADMINISTRATION OF TESTOSTERONE PROPIONATE AND ESTRADIOL VALERATE

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Summary: 17ß-testosterone (testosterone) and 17ß-estradiol (estradiol), together with their synthetic analogues, have been banned in the EU as growth promoters for fattening purposes. A simple, reliable method for their detection in animals is therefore required. Levels of testosterone and estradiol in hair and plasma of Wistar rats have been determined, following intramuscular (i.m.) administration of testosterone propionate and estradiol valerate, using commercial ELISA kits. Experiments were performed on prepubertal female rats for the detection of hair testosterone and on prepubertal male rats for the detection of hair estradiol. A significant increase of testosterone in hair was observed 3, 6 and 9 days after the administration of testosterone propionate, with no significant change in plasma content. Estradiol valerate administration also resulted in a significantly increased concentration in hair on the 3rd and 9th days, and in plasma on the 9th day. Thus, detection of testosterone and estradiol in hair after i.m. testosterone propionate and estradiol valerate administration can be achieved using commercial ELISA kits. Up to 0.80 % of the administered testosterone and 0.06 % of the administered estradiol were present in hair. This method could therefore serve as the basis for establishing a pre-screening tool for detecting abuse of anabolic steroids in farm animals.

Key words: testosterone; estradiol; hair; plasma; rats; ELISA

Introduction

17β-testosterone (testosterone) is the principal endogenous androgen synthesized by Leydig cells in the testes. Small amounts are also produced by the adrenal cortex. 17β-estradiol (estradiol) is produced mostly in the ovary and placenta. Sex steroids are transported in the blood from the steroidogenic tissues to target cells by blood where they are bound to sex hormone-binding globulin. They diffuse readily across the cell membrane and bind to intracellular receptors in the cytoplasm. Their activity is achieved through interaction of the receptor-hormone complexes to the hormone response elements on DNA that act as transcription factors, activating or inhibiting gene expression (1).

Testosterone has androgenic and anabolic activities. It regulates the male reproductive system by binding to androgen receptors present in particular in the reproductive organs, as well as interacting with receptors in muscles and fat tissue. Testosterone is also converted to estradiol and the more potent androgen dihydrotestosterone. In veterinary medicine testosterone and its synthetic analogues have been used for treating aplastic anaemia in dogs and cats, myeloproliferative disease and enhancing athletic performance in horses (1, 2).

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Estradiol plays an important role in the estrous cycle and in late pregnancy. Therapeutically it is used to prevent uterine infections and accelerate uterine involution in postpartum cows, for estrus synchronization in heifers and for induction of parturition in mares (1). Treatment of perianal gland adenoma in dogs with estradiol has also been described (3).

Although testosterone and estradiol, as well as their synthetic analogues, have been banned in the EU as growth promoters for fattening purposes (4, 5) they are still in use in many countries. According to 'The 2011 Prohibited List' of the World Anti-Doping Code (6), anabolic androgenic steroids belong to class S1.1 and are prohibited in and out of competition, including, notably, athletes and racing horses worldwide. For fattening and doping purposes steroids are usually administered as synthetic steroid esters. In the organism they are rapidly hydrolysed into natural steroids and free fatty acids. Therefore, exogenous steroid esters are difficult to detect in body fluids. Following oral intake of testosterone undecanoate, the unmetabolized ester could be detected in plasma of athletes only for 6 hours (7).

Sex steroids of endogenous and exogenous origin are inactivated in the liver and excreted mostly in the bile and urine. Estimation of reproductive status in various species by measuring faecal androgens, estrogens and progestins has been described (8, 9, 10, 11). Besides their presence in urine and faeces, steroids are also present in saliva, milk, different tissues, and hair (12, 13, 14, 15). Steroids and steroid esters can be incorporated into the hair fibre from blood via the hair follicles (15, 16, 17) and via sweat and sebum excretion at the surface of the skin, followed by diffusion into the hair fibres (15, 18). Some individual differences in steroid metabolism rates also occur (18). Hair steroids can be detected and quantified by gas or liquid chromatography coupled to mass spectrometry (17, 18, 19, 20, 21), desorption electrospray ionization mass spectrometry (22) and enzyme-immunoassay (23). Although commercial ELISA kits are useful for determining steroids in various matrices, their detection in hair by this means has not been described. The aim of the present study was therefore to explore whether commercial ELISA kits can be used for detecting testosterone and estradiol in hair. The study was performed by measuring testosterone and estradiol concentrations in rat hair after intramuscular administration of testosterone propionate and estradiol valerate. Testosterone and estradiol concentrations were also determined in plasma. The differences in hair steroid content between treated and non-treated rats could serve as a base for establishing the use of ELISA for detecting steroids in farm animals, where abuse of anabolic steroids can occur. To avoid the influence of endogenous testosterone and estradiol, experiments were performed on prepubertal female rats for the detection of hair testosterone and on prepubertal male rats for the detection of hair estradiol. The cross-reactivity of testosterone propionate and estradiol valerate with commercial ELISA kits was also checked.

Materials and methods

Experimental design for testosterone detection in hair

15 female Wistar rats aged 46-48 days at the beginning of the experiment were used in the study. The animals were divided into a control group of 7 and an experimental group of 8 animals. The environmental conditions were: 21°C, 12h light-dark regime, standard rat feed and water *ad libitum*. Seven days after housing, the animals from the experimental group were treated i.m. with 20 mg of testosterone propionate (T1875, Sigma – Aldrich, St.Louis, USA) per kilogram of body weight. Testosterone propionate injection solution was prepared by dissolving testosterone propionate in sterile olive oil (20 mg/mL) one day before treatment. Rats from the control group were treated with placebo (0.2 mL of olive oil i.m.).

Experimental design for estradiol detection in hair

15 male Wistar rats aged 47-48 days at the beginning of the experiment were used in the study. The animals were divided into a control group of 7 and an experimental group of 8 animals. The environmental conditions were the same as in the testosterone experiment. Seven days after housing, the animals from the experimental group were treated i.m. with 10 mg of estradiol valerate (E 1631, Sigma – Aldrich, St.Louis, USA) per kilogram of body weight. Solutions were prepared by dissolving estradiol valerate in sterile olive oil (10 mg/mL) one day before treatment. Rats from the

control group were treated with placebo (0.2 mL of olive oil i.m.).

The study protocol was approved by the Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia; license number 34401-11/2011/4.

Sampling regime

Hair samples were collected by cutting with electric scissors from the rat's back. They were collected before treatment and on the 3rd, 6th and 9th day after treatment and stored at -20 ^{0}C until extraction.

Blood samples were collected from the ophthalmic plexus using heparinized glass micro-haematocrit tubes. Plasma was prepared by centrifugation at 2.500 g for 30 minutes at room temperature and stored at -20 ^{0}C until analysis. Blood samples were collected before and on the 9th day after the treatment. At the time of blood sampling the rats were anaesthetized with carbon dioxide.

Steroid extraction from hair

Approximately 0.15 g of hair was put in a mortar and 20 mL of liquid nitrogen was added to freeze the hair. The sample was pulverized by grinding for two minutes. 0.1 g of hair powder was weighed and placed into a plastic tube. 0.6 mL absolute methanol (Cat. No.1.06007., Merck, New Jersey, USA) and 0.5 mL bi-distilled water were added (24). The tube contents were shaken at 500 RPM for 30 minutes, centrifuged at 2500 g for 20 minutes at 4 ^{0}C and the supernatant decanted. The extract (supernatant) was stored in plastic tubes at -20 ^{0}C until analysis.

ELISA procedure

Testosterone and estradiol concentrations in intact plasma and in hair extracts were determined using commercial Testosterone ELISA and 17beta – estradiol ELISA kits (both IBL, Hamburg, Germany) following the instruction manuals. Absorbances were measured with a microtiter plate photometer Multiskan FC (Thermo Fisher Scientific, Waltham, USA). The concentrations of testosterone and estradiol in hair extracts were expressed as ng per g of hair.

Partial validation of the ELISA kits, which included determination of intra-assay and inter-assay coefficients of variation (CV) for hair testosterone and estradiol, was performed. Samples with low (control group) and high (experimental group) concentrations of testosterone and estradiol in hair extracts were run in triplicate in each ELISA test and repeated in the next ELISA tests.

Intra-assay CVs for hair testosterone were determined as 4.7 % and 4.3 %. Inter-assay CVs for testosterone were 8.2 % and 7.7 %. Intra- and inter-assay CVs for hair estradiol were 5.1 % and 9.3 % (intra-assay CVs) and 6.7 % and 11.4 % (inter-assay CVs).

Commercial testosterone and estradiol ELISA kits were also tested for cross-reactivity with testosterone propionate and estradiol valerate. 100 µg of testosterone propionate (T1875, Sigma – Aldrich, St.Louis, USA) or estradiol valerate (E 1631, Sigma – Aldrich, St.Louis, USA) were dissolved in 1 ml of absolute methanol (Cat. No. 1.06007., Merck, New Jersey, USA). Serial dilutions with phosphate buffer were used for preparing 10 ng/mL and 1 ng/mL of testosterone propionate and estradiol valerate solutions respectively, which were used for testing the commercial testosterone ELISA kit for cross-reactivity with testosterone propionate and the commercial estradiol ELISA kit for cross-reactivity with estradiol valerate.

Statistical analysis

Data were analysed using SPSS 17.0 (Chicago, USA) commercial software. Means and standard errors of the mean (mean ± SE) were calculated for plasma and hair testosterone and estradiol concentrations. Repeated measures ANOVA with Bonferroni correction was used for testing for statistically significant differences of testosterone and estradiol concentrations in hair between different sampling times in the control and experimental groups of animals. At each sampling time, hair testosterone and estradiol concentrations between control and experimental group were compared by independent t-test, using Bonferroni correction for multiple tests. In the control and experimental groups, paired t-test was used to compare plasma testosterone and estradiol concentrations before and after treatment. Independent t-test was used to compare plasma estradiol concentrations in the control and experimental groups before and after treatment. Pearson's correlation coefficient analysis was performed to determine if there is a statistically significant correlation between plasma and hair testosterone and estradiol levels. A value of $P < 0.05$ was considered significant.

Results

Testosterone concentrations in hair and plasma

Hair testosterone concentrations in the control and experimental group are presented in Figure 1. Before treatment the mean hair testosterone concentration (± SE) did not differ significantly between the control and experimental groups, 7.11 ± 1.09 and 10.17 ± 2.82 ng/g, respectively. In the control group testosterone concentrations remained unchanged throughout the experiment, while in the experimental group, mean hair testosterone concentration increased, reaching the highest value (182.47 ± 32.92 ng/g) three days after treatment. On the 6th day of the experiment, mean testosterone concentration (150.01 ± 23.78 ng/g) was lower than that on day 3, although the difference was not significant. At the end of experiment (on the 9th day) , the mean testosterone concentration (76.76 ± 14.33 ng/g) was significantly lower than on days 3 and 6 ($P < 0.05$); however, the value was significantly higher ($P < 0.05$) than that before treatment (10.17 ± 2.82 ng/g).

The differences in hair testosterone concentrations between the control and experimental groups were statistically significant ($P < 0.01$) on days 3, 6 and 9 of the experiment.

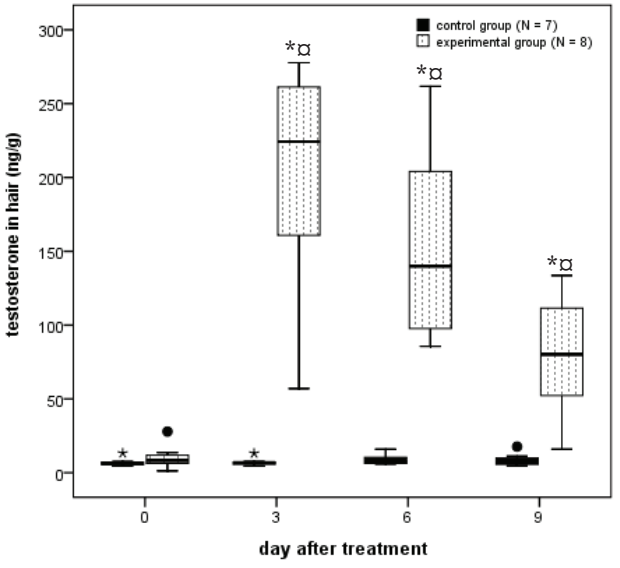

Figure 1: Testosterone concentrations in hair of the control and experimental groups

*P<0.05 (comparison of control and experimental groups)
¤P<0.05 (comparison with value before treatment)

Mean plasma testosterone concentrations (Table 1) before and on the 9th day after testosterone propionate or placebo administration were low, with no significant differences between and within the control and experimental group.

Table 1: Plasma testosterone concentrations (mean ± SE) in the control and experimental groups

plasma testosterone (ng/mL)		
	before treatment	after treatment
control group (N = 7)	0.77 ± 0.18	0.48 ± 0.09
experimental group (N = 8)	0.65 ± 0.26	0.56 ± 0.14

Pearson's correlation coefficients between hair and plasma testosterone were r = 0.10 (control group) and r = 0.24 (experimental group) and were not found significant.

Determination of cross-reactivity of the testosterone ELISA kit to testosterone propionate showed that measurements of testosterone propionate solutions in concentrations of 10 and 1 ng/mL had not achieved the detection limit (0.1 ng/mL).

Estradiol concentrations in hair and plasma

Hair estradiol concentrations in the control and experimental groups are presented in Figure 2. Before estradiol valerate or placebo administration, mean hair estradiol concentrations did not differ significantly between the control and experimental groups (0.40 ± 0.05 and 0.49 ± 0.09 ng/g, respectively). In the control group, estradiol concentrations remained unchanged throughout the experiment. In the experimental group, mean hair estradiol concentration increased significantly ($P < 0.05$) from 0.49 ± 0.09 ng/g to 2.75 ± 0.53 ng/g on the 3rd day after estradiol valerate administration. The peak value (6.07 ± 1.53 ng/g) was observed on the 6th day after treatment. On the 9th day after treatment there was a drop of mean hair estradiol (4.46 ± 0.75 ng/g). In the experimental group, no significant differences in estradiol concentrations between those measured at days 3, 6 and 9 were observed. Hair estradiol concentrations in the treated animals were significantly higher ($P < 0.01$) than on the control group on days 3, 6 and 9 after treatment.

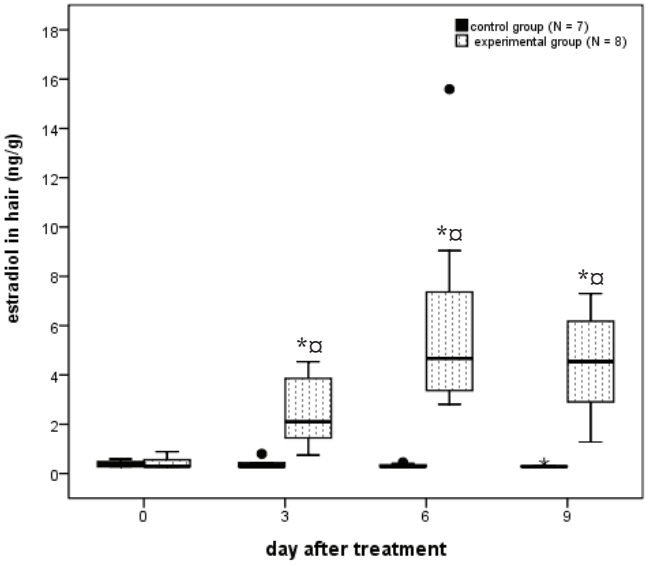

Figure 2: Estradiol concentrations in hair of the control and experimental groups

* P < 0.05 (comparison of control and experimental group)
¤ P < 0.05 (comparison with value before treatment)
β P = 0.055 (comparison with value before treatment)

In plasma, before treatment, there were no significant differences in estradiol concentrations (Table 2) between the two groups. Nine days after estradiol valerate administration, mean plasma estradiol concentration was significantly higher (P < 0.01) than the mean value before treatment, as well as than the control group value (P < 0.01).

Table 2: Plasma estradiol concentrations (mean ± SE) in the control and experimental groups

plasma estradiol (ng/mL)		
	before treatment	after treatment
control group (N = 7)	0.053 ± 0.018	0.062 ± 0.022
experimental group (N = 8)	0.091 ± 0.023	1.132 ± 0.133*¤

* P < 0.01 (comparison of control and experimental group)
¤ P < 0.01 (comparison with value before treatment)

Pearson's correlation coefficient between hair and plasma estradiol in control group (r = 0.20) was not found significant. In contrast, a significant positive correlation (r = 0.56; P < 0.05) was found between hair and plasma estradiol in the experimental group.

Determination of cross-reactivity of the estradiol ELISA kit to estradiol valerate showed that measurement of estradiol valerate solutions in concentrations of 10 and 1 ng/mL had not reached the detection limit (0.025 ng/mL).

Discussion

Commercial ELISA kits are used for routine and research purposes in endocrinology; they are accurate and sensitive, relatively cheap and easy to handle. Although mass spectrometry is considered to offer more selectivity than ELISA, mainly due to variable extents of cross-reactivity of steroids against the antibody (21), our results suggest that ELISA could serve as a pre-screening or alternative method for detecting testosterone and estradiol in hair. Our results have demonstrated for the first time that testosterone and estradiol can be detected in hair extracts after intramuscular administration of testosterone propionate and estradiol valerate using commercial ELISA kits. In Wistar rats treated with testosterone propionate or estradiol valerate, testosterone and estradiol concentrations in hair were significantly higher than in control groups from the 3rd to the 9th day after treatment. The results indicate that testosterone propionate and estradiol valerate are hydrolysed (7) in the rat's body and that, during distribution and excretion, some amounts of free testosterone and estradiol are incorporated into rat hair.

In animals treated with testosterone propionate, the content of testosterone in hair was, from the 3rd to the 9th day after treatment, significantly higher than in the control group. High testosterone values could result from hydrolysis of testosterone propionate and excretion of free testosterone onto the hair (15, 16, 18). Higher testosterone hair level on the 3rd day than on days 6 and 9 after testosterone propionate administration indicates intensive testosterone excretion via sweat, sebum and saliva immediately after administration. The slight decrease in testosterone concentrations on the 6th and 9th days after treatment could be caused by less intensive excretion onto the hair surface and eventual incorporation of previously excreted testosterone into hair fibre (15, 18).

Blood plasma testosterone concentrations before treatment and on the 9th day of the experiment were low, with no significant differences between control and experimental groups. These results indicate that, by the 9th day of the experiment, all

administered testosterone propionate has been hydrolyzed and all free testosterone excreted from the blood, which could also explain the gradual decrease of testosterone levels in hair from the 3rd to the 9th day after treatment. Non-significant correlation between hair and plasma testosterone might also be caused by the same reasons.

Similarly to testosterone, estradiol concentrations in hair increased but reached their highest mean value on the 6th day after administration although the value was not significantly higher (P = 0.055) than that before treatment. Some individual differences in metabolism and excretion rate of steroids are possible (18), which could be the reason for the wide dispersion of the hair estradiol measurements on the 6th day following treatment and consequent high standard deviation that resulted in a P value close to statistical significance.

In contrast to plasma testosterone concentrations, plasma estradiol increased significantly after estradiol valerate administration, resulting in a significant difference in estradiol concentration between the control and experimental groups. This suggests slower hydrolysis of estradiol valerate and prolonged persistence of estradiol in blood than of testosterone in the corresponding experiment. Estradiol is thus excreted onto the hair fibre over a longer period than that for testosterone, which could be the reason for the highest concentration being reached not earlier than on the 6th day after estradiol valerate administration. Additionally, the significant positive correlation between hair and plasma estradiol suggests that increased hair estradiol levels are the consequence of high plasma estradiol concentration.

The transport of steroids into hair is mediated via the hair follicle or by diffusion into the hair fibre from sweat, sebum and saliva (16, 17, 18, 25). Significantly higher hair testosterone and estradiol values on the 3rd, 6th and 9th days than in control groups confirmed that it is possible to detect prior use of testosterone and estradiol esters by measuring testosterone or estradiol concentrations in hair. According to our results of cross-reactivity testing, testosterone propionate and estradiol valerate cannot be detected by the commercial testosterone and estradiol ELISA kits used in the present study, which indicates the reliability of the chosen method, since possible contamination of the hair during administration is not detectable. Additionally, the low intra- and inter-assay CVs in testosterone and estradiol measurements, the significant correlation between hair and plasma estradiol, and the fact that the concentrations of testosterone and estradiol measured in the samples of hair extracts were above the detection limit indicate the repeatability, sensitivity and accuracy of the described method.

Comparison of the intramuscularly administered doses of testosterone propionate and estradiol valerate and the testosterone and estradiol hair concentrations indicates that up to 0.80 % of the former and up to 0.06 % of the latter is incorporated in hair. Due to their prolonged presence in hair, high levels could indicate previous use of testosterone and estradiol esters. For fattening purposes in farm animals, steroids are used in lower doses (26) than those used in our study; the described method therefore cannot be applied directly to detect hair testosterone and estradiol in farm animals. Because species-specific kinetic characteristics must also be considered, the present study can only serve as a base for establishing the ELISA method for the detection of steroids in hair in farm animals. Although detection of steroids and steroid esters is widely performed by the more selective gas and liquid chromatography coupled to mass spectrometry (16, 17, 19, 20), commercial ELISA kits could potentially serve as a pre-screening tool for the detection of testosterone and estradiol in hair after parenteral administration of testosterone and estradiol esters.

Acknowledgements

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UGOTAVLJANJE SPOLNIH HORMONOV V DLAKI PODGAN PO APLIKACIJI TESTOSTERON PROPIONATA ALI ESTRADIOL VALERATA

T. Snoj, N. Čebulj-Kadunc, A. Nemec Svete, S. Kobal

Povzetek: Uporaba 17 ß-testosterona (testosteron) in 17 ß-estradiola (estradiol) ter podobnih sintetičnih snovi z namenom, da bi se izboljšal prirast pri farmskih živalih, je v EU prepovedana. Za določanje teh snovi pri živalih bi bila uporabna enostavna in zanesljiva metoda. Z uporabo komercialnih ELISA kompletov smo v raziskavi ugotavljali vsebnost testosterona in estradiola v dlaki in plazmi podgan pasme wistar po predhodni intramuskularni (i.m.) aplikaciji testosteron propionata in estradiol valerata. Poskus ugotavljanja vsebnosti testosterona v dlaki je bil izveden na mladih samicah, estradiol v dlaki pa se je ugotavljal pri mladih samcih. Statistično značilno višjo koncentracijo testosterona v dlaki smo ugotovili tretji, šesti in deveti dan po aplikaciji testosteron propionata, medtem ko statistično značilnih razlik v krvni plazmi nismo ugotovili. Po aplikaciji estradiol valerata smo ugotovili statično značilno višjo koncentracijo estradiola v dlaki tretji in deveti dan, višja vrednost pa je bila deveti dan ugotovljena tudi v plazmi. Na podlagi rezultatov sklepamo, da je z uporabo komercialnih ELISA kompletov možno ugotavljati vsebnost testosterona in estradiola v dlaki po predhodni i.m. aplikaciji testosteron propionata in estradiol valerata. V dlaki je do 0,80 odstotka apliciranega testosterona in do 0,06 odstotka apliciranega estradiola. Opisana metoda bi se lahko uporabila kot osnova za uvedbo presejalne metode ugotavljanja zlorabe anaboličnih steroidov pri rejnih živalih.

Ključne besede: testosteron; estradiol; dlaka; plazma; podgane; test ELISA

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Original Scientific Article

BLOOD LEUKOCYTES IN CARTHUSIAN MARES WITH ENDOMETRIOSIS OR ENDOMETRITIS IN COMPARISON WITH HEALTHY MARES

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Summary: Significant changes in total blood white blood cells (WBC) and populations are found in many generalized and localized pathological conditions in veterinary medicine. Endometritis is an inflammatory process that might lead to infertility in mares and it is associated with an influx of polymorphonuclear neutrophils (PMN) into the uterine lumen. Endometriosis is a chronic degenerative endometrial disease that causes severe alterations in the connective tissues of the uterus. We took blood samples from 6 Carthusian mares with endometritis (EM1) and 8 with endometriosis (EM2) in order to check whether these changes lead to changes in hematology. Further, 6 healthy mares of the same breed and similar age were also sampled (control group, CG). An analysis of variance was used to evaluate the differences between the three groups of mares. In comparison with CG, mares of group EM1 had significantly higher hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, segmented PMNs and neutrophils/lymphocytes ratio, and lower total white blood cells and lymphocytes. Mares of group EM2 presented lower red blood cell and white blood cell counts. No morphological changes were detected in the different types of WBC when examined in the blood smear. It is concluded that some hematological parameters differ in mares with endometritis and endometriosis in comparison to healthy mares. Even though the hematological study is not useful in the diagnosis of these diseases, the results of the present research are important in order to diagnose other clinical conditions that could coexist with these reproductive diseases.

Key words: endometritis; endometriosis; hematology; horse; mare

Introduction

Leukocytes (WBC) are vital for host defense, and for the initiation and control of inflammation and immunity. Although generally protective or supportive of the host tissue, WBCs are also involved in host-harmful allergic and immune-mediated diseases. Significant changes in blood WBC numbers and/or of their different populations, with or without morphological changes are observed in many diseases and they are of pivotal help in the diagnosis and prognosis in veterinary medicine. The pattern of the WBC response depends on many factors, such as animal species, susceptibility of the patent, virulence of the pathogenic agent involved, immun status of the patient and extension of the inflammation and/or infection (localized vs. generalized) (1, 2). The WBC response to different reproductive diseases has been reported in small animals. Dogs with localized infections, such as pyometra exhibits leukocytosis with neutrophilia and sometimes, toxic changes in neutrophils and left shift, with increased band neutrophil numbers (3, 4, 5, 6).

Endometritis encompasses endometrial changes associated with acute or chronic inflammation and it is a major cause of mare infertility aris-

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ing from incapacity or failure to remove bacteria, spermatozoa and inflammatory exudates. Defects in genital anatomy, myometrial contractions, lymphatic drainage, mucociliary clearance, cervical function vascular degeneration and ageing underlie endometritis (7). It should be promptly diagnosed and resolved in order to avoid chronicity. Endometritis might be difficult to identify, because clinical signs, ultrasonographic and laboratorial findings can vary according to the cause. Some cases are associated with an influx of inflammatory cells, mainly polymorphonuclear neutrophils (PMN) and fluid into the uterine lumen (8). Although affected mares do not show clinical signs associated with systemic disease, it is plausible to think that an acute inflammation might exist. Recently, it has been reported that mares with endometritis experimentally induced by intrauterine infusion of *E. coli* had increased serum concentrations of some acute-phase proteins (e.g. amyloid A and fibrinogen). These results indicated that endometritis in mares is linked to a systemic acute phase response (9).

Endometriosis is a chronic degenerative endometrial disease involving severe alterations in the uterine connective tissues. It is characterized by destruction of the uterine gland, including cystic dilation, hyperplasia and periglandular fibrosis, glandular epithelial hypertrophy, lymphatic lacunae or lymphagiectasia, angiosis (a vascular pathological condition with associated venous congestion) and, subsequently resulting in the development of a stromal fibrosis (10, 11). Briefly, the term 'endometritis' is used to describe inflammatory changes, whereas 'endometriosis' reflects chronic changes associated with age and parity (12, 13).

When an acute phase response appears, the number and percentage and/or the morphological characteristics of the different blood WBC populations might change. The current study aims to compare the hematological profile in three groups of mares: healthy, mares with endometritis and with endometriosis. It is hypothesized that mares with endometritis will show modifications in the leukogram in response to the inflammation. Further, both of them could be chronic processes, and therefore, it is also plausible to evidence a leukogram of chronic disease.

Material and methods

This research was approved by the Ethic Committee for Animal Experimentation of the Cardenal Herrera-CEU University. Jugular venous blood samples were withdrawn from a total of 20 adult Carthusian mares, divided into three groups: healthy (n=6), considered as control group, CG, mares with endometritis (n=6), EM1 and with endometriosis (n=8), EM2. The 20 mares had previously foaled, although the rate of success was different. The reproductive history of the studied mares is presented in table 1. The mares were considered healthy (CG) when the echography of the reproductive system was unremarkable, physical examination did not provide any clinical indication of disease, all of them were pregnant without problems, have viable foals and had hematological parameters within the reference range established for this breed (14, 15). The diagnosis of mares EM1 was made with ultrasound (Pie Medical 480®), cytology and microbiological cultures. Mares of EM1 presented pus or fluid in the uterine lumen and endometrial edema and cytology showed predominant numbers of PMN. Diagnosis of mares of group EM2 was made by echography and endometrial biopsy. The echography showed increased echotexture in endometrial mucosa and endometrial

Table 1: Age and reproductive history of the 20 Carthusian mares included in this research (CG, control group; EM1, with endometritis; EM2, with endometriosis)

Mare groups	Age (mean±SD)	Number of previous successful foaling	Previous foal
CG (n=6)	8.345±2.45	3 foals (n=6)	Previous mating (n=6)
EM1 (n=6)	10.54±2.12	1 foal (n=3) 2 foals (n=2) 3 foals (n=1)	1 season without successful mating (n=4) 2 seasons without successful mating (n=2)
EM2 (n=8)	14.96±1.23	1 foal (n=4) 2 foals (n=4)	At least 3 seasons without successful mating

biopsy revealed a chronic endometriosis degree III (system of Kenney and Doig; 12, 13).

The mares of the three groups were sampled in the morning, before feeding. The samples were extracted in the follicular phase, 2-3 days before ovulation. Immediately after blood extraction, and directly from the syringe, without anticoagulant, a blood smear was carried out. The rest of the blood was poured into tubes with EDTA-3K for hematology and into glass tubes in order to obtain serum to measure total proteins. In all the cases, the samples remained refrigerated during the transportation to the laboratory and the analyses were performed within the first 12 hours after extraction.

The following hematological parameters were included in this research: red blood cells (RBC), hemoglobin concentration (HB), hematocrit (HT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC) and platelets (PLT). These measurements were made using a semiautomatic counter (System F820®). The differential WBC count was performed in the blood smear, fixed with ethanol and stained with May-Grünwald-Giemsa technique. The absolute number of the WBC populations was quantified by microscope (Olympus CX 21®): lymphocytes (LYMP), polymorphonuclear neutrophils (PMNs), segmented PMN (SPMN) and in band PMN (BPMN), eosinophils (EOS), monocytes (MON), basophils (BAS) and neutrophil/lymphocyte ratio (N/L). In the blood smear, we also assessed the presence of morphological changes in the PMNs, such as Döhle's bodies, basophilia, cytoplasmatic vacualization, nuclear edema and/or hypersegmentation. They were subjectively scored over 5 points (0: abscense of changes; 5: maximum morphological changes). Finally, the concentration of total serum proteins (TSP) was assessed by spectrophotometry with specific reagents (Metrolab, RAL®).

Data are presented as means±SD. Differences between the three groups were analyzed with an analysis of variance ANOVA. Statistical significance was set at $p<0.05$. The statistical program Statistica v. 7.0 for windows was used (Statsoft®, Inc, 2001. Tulsa, Oklahoma, USA)

Results

The hematological parameters for the three groups of mares (CG, EM1 and EM2) are presented in table 2. Mares of group EM1 in comparison to CG had higher HB, HT, MCV, MCHM, SPMN and N/L ratio and lower WBC and LYMP. Mares of

Table 2: Mean±standard deviation of the hematological parameters in three groups of Carthusian mares (CG, control group; EM1, with endometritis; EM2, with endometriosis). Significant differences from the CG are indicated with an asterisk. Level of significance $p<0.05$ (*RBC, red blood cells, HB, hemoglobin concentration, HT, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets; TSP, total serum proteins; WBC, white blood cells; SPMN, segmented polymorphonuclear neutrophils; BPMN, band neutrophils; LYMP, lymphocytes; EOS, eosinophils; MON, monocytes; N/L, neutrophil/lymphocyte ratio)*

Parameter and unit	CG	EM1	EM2
RBC (10^6/µl)	9.43±1.58	9.49±2.29	8.90±2.34*
HB (g/dl)	12.60±1.40	13.50±2.06*	13.06±2.77
HT (%)	46.39±6.21	49.85±12.93*	47.51±13.76
MCV (fl)	50.39±2.33	52.43±2.86*	52.82±5.54
MCHC (g/dl)	26.86±5.23	27.82±3.84*	29.55±13.65
PLT (10^3/µl)	199.1±116	143.2±104	164.1±118
TSP (g/dl)	7.35±0.30	7.72±0.46	7.50±0.49
WBC (10^3/µl)	12.44±2.28	11.40±5.97*	10.46±8.01*
SPMN (10^3/µl)	4.92±0.15	5.45±1.46*	5.09±2.17
BPMN (10^3/µl)	0.21±0.01	0.24±0.12	0.24±0.28
LYMP (10^3/µl)	5.06±0.12	3.18±0.59*	4.55±2.30
EOS (10^3/µl)	0.67±0.04	0.69±0.48	0.65±0.12
MON (10^3/µl)	0.22±0.01	0.24±0.10	0.24±0.08
N/L	1.02±0.03	1.84±0.69*	1.21±0.64

group EM2 showed lower RBC and WBC (Table 2). No morphological changes in PMN neither in LYMP were found when assessing the blood smears.

Discussion

In the present research, we have found significant differences when comparing the hematological profile in mares with localized diseases (endometritis and endometriosis) with healthy mares. Both conditions are common causes of reproductive failure in mares and they are not accompanied by systemic clinical signs. However, they can cause changes in the uterine tissues that could lead to changes in the blood profile. Thus, endometritis is associated with an infiltration of inflammatory cells, mainly PMN in the stratum compactum (16, 17). Recently, it has been demonstrated that endometritis induces a systemic acute phase reaction, with increased serum amyloid (9). Traditionally, fever and changes in WBC numbers and/or morphology have been considered to be the hallmarks of inflammation and infection, even though many reports lastly have confirmed that they are less sensitive that acute phase proteins (18, 19). Despite these ideas, it is plausible to think that endometritis could induce some variations in the hematological profile of the mare.

The higher HT of the EM1 mares seemed to result from the higher size of the erythrocytes (higher MCV), as RBC were similar in both groups. The reasons of these results are unknown. Firstly, this result was not associated with dehydration, as TSP did not exhibit significant differences between both groups. This was an unexpected finding, as chronic diseases lead to a normocytic normochromic anemia or do not exert any significant effect on the number of circulating RBCs (20, 21).

Increased PMN occurs during infectious and non-infectious inflammatory conditions (20, 21). Immature PMN, i.e. BPMN is the most sensitive differentiator of infectious inflammatory disease, but they are not seen as frequently in horses as in other animal species (21). In equids, BPMN are most commonly seen in severe acute bacterial infections or septicemic processes. This can be the explanation for the similar values for BPMN found in CG and EM1. The higher number of SPMN in EM1 is consistent with inflammation. It has been demonstrated that PMN increased in uterus exudates at 30 min after experimental endometritis, induced by infusion of *Streptococcus zooepidemicus* (16). The lower LYMP in group EM1 could be the reflex of stress of the disease. Other causes of decreased LYMP, such as viral infections or glucocorticoid administration do not appear to be important in our mares. Probably both facts i.e. increased SPMN and decreased LYMP led to the higher N/L of the EM1 group in comparison with CG.

Endometriosis is a chronic degenerative endometrial disease associated with age and parity and with the presence of fibrosis (10, 11). The lower RBC in the mares of group EM2 in our study is consistent with the chronicity and degenerative condition of the process.

Surprisingly, the other types of WBC, mainly MON, were not different between groups. Increased MON are found during disease processes with increased tissue demand for phagocytosis of particles, such as tissue necrosis and infection (20, 21), as happen in endometritis. Finally, it is interesting to indicate that HT, MCV, TSP WBC, SPMN, BPMN, LYMP, EOS and N/L ratio had greater SD in both diseased groups (EM1 and EM2) than in CG. This result has two implications. Firstly, it could have limited the evidence of statistical significance when comparing the three groups and secondly, it might indicate the clinical differences between groups.

Our results indicate that some hematological parameters are different in Carthusian mares with endometritis and with endometriosis in comparison with a control group of mares of the same age and breed. Although the hematological profile is not used for the diagnosis of these diseases, it is important to establish whether the hematological parameters vary in mares with these reproductive problems in order to diagnose or to assess other clinical conditions that could co-exist with these conditions.

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LEVKOCITI V KRVI KARTUZIJSKIH KOBIL Z ENDOMETRIOZO ALI ENDOMETRITISOM V PRIMERJAVI Z ZDRAVIMI KOBILAMI

K. Satué, M. Felipe, J. Mota, A. Muńoz

Povzetek: Pri številnih splošnih in lokaliziranih patoloških stanjih ugotavljamo znatne spremembe vseh belih krvničk (WBC) kot tudi njihovih populacij. Endometritis je vnetni proces, ki lahko vodi do neplodnosti pri kobilah in je povezan z vdorom polimorfonuklearnih nevtrofilcev (PMN) v svetlino maternice. Endometrioza je kronična degenerativna endometrična bolezen, ki povzroča resne spremembe v vezivnem tkivu maternice. Odvzeli smo vzorce krvi šestih kartuzijskih kobil z endometritisom (EM1) in osmih z endometriozo (EM2), da bi preverili, ali te spremembe povzročijo spremembe v krvni sliki. Poleg tega smo odvzeli vzorce šestim zdravim kobilam iste pasme in podobne starosti (kontrolna skupina, CG). Z analizo variance smo ocenjevali razlike med tremi skupinami kobil. Kobile iz skupine EM1 so imele bistveno višjo vsebnost hemoglobina, višji hematokrit, višjo povprečno prostornino krvnih celic, višjo povprečno vsebnost hemoglobina v krvnih celicah, več polimorfonuklearnih celic in višje razmerje nevtrofilci/limfociti ter manjšo količino belih krvničk in limfocitov v primerjavi s CG. Kobile iz skupine EM2 so imele manj rdečih in belih krvničk v krvi. V krvnih brisih pri različnih vrstah WBC nismo zaznali morfoloških sprememb. Ugotovili smo, da se nekateri hematološki parametri razlikujejo pri kobilah z endometritisom in endometriozo v primerjavi z zdravimi kobilami. Čeprav krvna študija ni uporabna pri diagnosticiranju teh bolezni, so rezultati raziskave pomembni za diagnosticiranje drugih kliničnih stanj, ki so lahko tudi prisotna pri omenjenih reproduktivnih boleznih.

Ključne besede: endometritis; endometrioza; hematologija; konj; kobila

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Original Scientific Article

EFFECTS OF ORGANOPHOSPHATE CHLORMEPHOS ON DEVELOPMENT AND FUNCTION OF TESTES AND BRAIN

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Summary: Several chemicals, known as endocrine disrupting chemicals have the capacity to interfere with hormone action in the mammalian body. The aim of our study was to establish whether long term exposure to low doses of organophosphorus compound Chlormephos affects the development and function of reproductive tract and endocrine parts of the brain. Adult male and female mice were exposed to 3.5µg/ml and 0.35µg/ml of Chlormephos in the drinking water during mating, pregnancy and lactation until weaning of the offspring. Testes development was studied in the offspring of exposed mice by monitoring daily sperm production, number of apoptotic cells and spermatogenesis. Immunoexpression of antimüllerian hormone and 3ß-hydroxysteroid dehydrogenase was assessed qualitatively in testes from 9 and 19 days old offspring, respectively. No statistically significant differences (or qualitative differences in assessment of immunoexpression) were found in any of the observed parameters, suggesting that low dose of Chlormephos does not act as an endocrine disruptor in reproductive tract. To examine whether exposure to low doses of Chlormephos affect brain development, offspring of the exposed mice were tested for anxiety like behaviour in the elevated plus maze. Mice exposed neonatally to the higher dose of Chlormephos spent significantly less time in the open arms of the elevated plus maze in comparison to mice from control group, suggesting an increase in the anxiety like behaviour. Ultrastructural analyses did not reveal any changes in brain ultrastructure, in particular in blood-brain barrier which has been reported before to be a target for organophosphorous compounds.

Key words: mice; testis; brain; organophosphorus; daily sperm production; electron microscopy; apoptosis; anxiety-like behavior

Introduction

Endocrine disruptor chemicals (EDCs) in the environment could possibly interfere with the endocrine system and mimic or depress the function of endogenous hormones. EDCs usually interfere with the endocrine system through hormone receptors such as estrogen and androgen receptors. In recent years, several studies have shown that environmental contaminants such as isomers of dichlorodiphenyltrichloroethane (DDT), some polychlorinated biphenyls (PCBs), the plasticizer bis-phenol A, and some detergents such as p-nonylphenol, can exhibit estrogenic or anti-androgenic activity (1).

Organophosphates (OPs) are a large class of chemicals, which act as irreversible inhibitors of acetylcholinesterase (AChE). OPs cause accumulation of neurotransmitter acetylcholine and they are often used in farming as insecticides and antiparasitics (2, 3). Chlormephos (*S*-chloromethyl *O,O*-diethyl phosphorodithioate) is an organophosphorous (OP), insecticide that was introduced to the market in 1973 to control soil-dwelling pests (4). Chlormephos was in use until 2006 when it was withdrawn from all EU markets.

OP could act as endocrine disruptors in reproductive system, brain development and behavior. *Sarkar et al.* reported that OP pesticide quinalphos

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disrupts hypothalamo-pituitary-gonadal axis. Sublethal chronic administration of quinalphos resulted in decreased testicular mass and AChE activity in central and peripheral organs; increased serum LH, FSH, prolactin and testosterone concentrations; decreased pituitary and increased testicular activity and severe disruption of spermatogenesis with increasing doses of pesticide (5). *Verma and Mohanthy* (6) showed that *in utero* and lactational exposure of mice to low doses of dimethoate caused reduced testis weight, reduced sperm count, histopathological changes in testes and epididymidis, lower plasma LH and testosterone and reproductive dysfunction of adult mice. Study by *Umzucu et al.* (7) demonstrated that early postnatal exposure of rats to metoxyclor resulted in reduced ovarian weight, inhibition of folliculogenesis and stimulation of anti-müllerian hormone production.

Several studies investigated effects of low-level exposure to OPs pesticides during pregnancy on neurodevelopment and behavior of newborns. Prenatal exposure of children to higher doses of chlorpiryfos resulted in Psychomotor Development Index and Mental Development Index delays, attention problems, attention-deficit/hyperactivity disorder problems, and pervasive developmental disorder problems at 3 years of age (8). Developing blood-brain barrier is highly vulnerable to single or repeated exposure to low doses of certain pesticides such as organophosphate quinalphos, pyrethroid cypermethrin and organochlorine lindane although there seems to be some species differences in vulnerability of blood-brain barrier to the pesticides (9, 10).

The aim of our study was to determine whether long term exposure to low levels of organophosphorus compound Chlormephos can affect the development and functioning of testes and brain in mice. Although there are no data about human exposure to Chlormephos, the data for exposure to other organophosphorous pesticides show that human dietary or environmental exposure is similar to doses used in our study (11, 12).

Material and methods

Animals

Sexually mature (60 – 70 days old) BALB/c male and female mice (all together 18 control females, 16 females exposed to 3.5 μg/ml and 12 females exposed to 0.35 μg/ml Chlormephos) were breed in standard conditions at 20 - 25° C, humidity 55–65%, dark : light cycle 12:12, with chow without phytoestrogens (2916, Harlan Tekland, England) ad libitum. All animal experiments were approved by the Veterinary commission of Slovenia (approval number 3440-176/2006) and were done according to the EU directive and NIH guidelines.

Mice were supplied either with drinking water containing 3.5 μg or 0.35 μg of Chlormephos per ml (10 and 100 times lower dose of LD50 for mammals) (13). As Chlormephos is insoluble in water, it was first dissolved in ethanol at concentration 1 mg/ml, and this stock solution was used for preparing final drinking water solution. Final concentration of ethanol in the drinking water was 3.5 μl per ml in group exposed to 3.5 μg of Chlormephos per ml and 0.35 μl per ml in group exposed to 0.35 μg of Chlormephos per ml. Control group received drinking water without Chlormephos, containing 3.5 μl of ethanol per ml (0.35 %, vol/vol).

The effects of Chlormephos were monitored in the offspring of exposed animals. Sexually mature male and female mice were randomly divided into control or treated groups; weighted and exposed to Chlormephos/Ethanol for seven days. After seven days, one male and one female mouse were joined in a single cage. Mice were exposed to Chlormephos/Ethanol throughout pregnancy and lactation period until offspring weaning at 21 days. Litter size was carefully monitored; pups were always counted on the day of delivery. Male pups from different litters were randomly assigned to groups for sacrifice at different postnatal age (in each age group of mice, pups from at least three different litters were included). Pups were sacrificed at 9, 19, 48 and 70 days of age, 48 and 70 days old mice were exposed to Chlormephos/Ethanol only until weaning on day 21. Nine and 70 days old mice were sacrificed by CO_2 exposure followed by cervical dislocation. Mice 19 and 48 days old were anaesthetized by a mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and perfused with Bouin's solution. Testes were isolated and postfixed in Bouin's solution for 4 to 20 hours depending on size. Subsequently, testes were processed into paraffin wax using standard procedures. At the time of sacrifice, whole mice as

well as testes and seminal vesicles were weighted. Testis weight (average of left and right testis) was expressed as a percentage of total body weight (= relative testis weight). Some offspring of exposed animals were breed in adulthood, number of pups was monitored. Anxiety tests were performed on offspring of exposed mice.

Effects of Chlormephos on daily sperm production (DSP)

Daily sperm production was examined in 70 days old male offspring of treated and control mice as described before (14). Briefly, testes were dissected, weighed and homogenized in 5ml of physiological saline containing 0.05% Triton X-100. Spermatozoa were counted in Bürker – Türk chamber under 400x magnification. Ten visual fields per sample were counted and daily sperm production was calculated using formula N x 25 x 5000 (N = number of counted cells, 25 and 5000 dilution factors), divided by testes weight and this result was divided by 4.48, thus obtaining average DSP.

Immunohistochemistry

Immunohistochemistry was performed as described before (15) using antibodies against antimüllerian hormone (AMH) in concentration 1: 200 and 3β-hydroxysteroid dehydrogenase (3β-HSD) in concentration 1:1000 using peroxidase anti-peroxidase system. Specific rabbit antibodies against AMH were gift from Natalie Josso (France), polyclonal rabbit antibodies against 3β-HSD from dr. Ian Mason (United Kingdom).

Apoptosis detection

Apoptotic cells were detected by ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA)) following manufacturer's protocol. Apoptotic cells were counted in testicular sections in 40 visual fields for each sample in testes from 48 old male mice exposed neonatally to Chlormephos. Results are presented as average number of apoptotic cells in one visual field.

Anxiety-like behavior test

In 70 to 80 days old mice of both sexes, standardized test for anxiety-like behavior using elevated plus maze was performed (16). Tests lasted 5 minutes during the beginning of the dark cycle with dim red light. Time in opened and closed arms of the maze as well as latency to enter closed or opened arms was recorded using Stopwatch+ program (version 1.5.1, Center for behavioral neuroscience, Atlanta, USA).

Electron microscopy

For electron microscopy, offspring from control and higher concentration Chlormephos treated group were sacrificed by carbon dioxide at 70 days of age. Part of the brain cortex from the frontal lobe was immediately fixed in 4% of glutaraldehyde for 2 hours. Samples were prepared by a standard procedure for electron microscopy with Durcupan (Fluka, Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) embedding medium. Briefly, the brain tissue fixed in glutaraldehyde was rinsed several times in a 0.05M phosphate buffer (pH=7.1-7.4, 800 mOsm), postfixed with 1% OsO_4 and dehydrated in a series of ascending alcohols. Tissue was then incubated in a mixture of absolute alcohol and acetone (1:1, 30 min.), absolute acetone (15 min.) and mixture of absolute acetone and Durcupan (Fluka). This step was followed by embedding the brain tissue into 100% Durcupan. After a polymerisation of the embedding medium in a thermostat (at 60°C, 72 hours), tissue blocks were trimmed and prepared for sectioning. Semithin sections (section thickness = 0.9 μm) were made by Power-Tome XL ultramicrotome (Bal Tec/RMC) and stained with 1% toluidine blue. The sections were analysed by Nikon binocular microscope (Eclipse E200). Based on the bright filed microscopic analysis of semi-thin sections, ultra-thin sections were made (section thickness = 70 nm), placed on copper grids, and contrasted with lead citrate and uranyl acetate. The sections were then examined by transmission electron microscope Zeiss 902A (Centre for Electron Microscopy, Medical School University of Zagreb).

Statistical analyses

Differences between groups were analyzed by two-way analysis of variance with sex and treatment as independent variables, followed by Bonferroni posthoc test to determine differences between groups. Statistical significance was determined at $p < 0.05$.

Results

Litter size, body size and testis size in control and treated groups

We have reported before in our report of preliminary data (17) that there were no differences in the litter size between control and Chlormephos exposed groups (mice that received Chlormephos through drinking water). There were also no differences in body size, absolute testis weight, relative testis weight and seminal vesicle weight between control mice and mice exposed to either concentration of Chlormephos during neonatal period through mothers (17). Similarly, when adult mice, exposed to Chlormpehos in neonatal period were mated, no statistical differences in litter size were observed with average 4.5 ± 0.9 (n=8 litters) pups in control group, 5.5 ± 0.8 (n= 4 litters) and 4.8 ± 1.2 (n=4 litters) in groups exposed neonatally to 0.35 and 3.5μg per ml Chlormephos, respectively.

Daily sperm production (DSP)

Similarly to our previously reported preliminary data (17), analysis of daily sperm production at 70 days old mice did not show any statistical differences between control and treated groups. In control group, DSP was 32,259.999 ± 1,602.126 (n=8) while it was 36,207.131 ± 539.750 (n=4) and 30,876.956 ± 5,151.737 (n=5) in groups exposed to high or low concentration of Chlormephos, respectively.

Testes histology, number of apoptotic cells and 3β-HSD and antimüllerian hormone expression

Tissue sections stained with haematoxylin and eosin from testes of all age groups of offspring were examined to determine any irregularity in spermatogenesis. No differences between control and treated groups were noted. Similarly, statistical analysis of apoptotic cells labeled with TUNEL method in 48 days old testes did not reveal any differences in the number of apoptotic cells between groups (n was 5 for each group).

Qualitative assessment of immunohystochemical staining for antimüllerian hormone (testes from 9 days old mice) and 3β-hydroxysteroid dehydrogenase (19 days old offspring) did not reveal any differences in the immunoexpression of these two proteins (Figure 1).

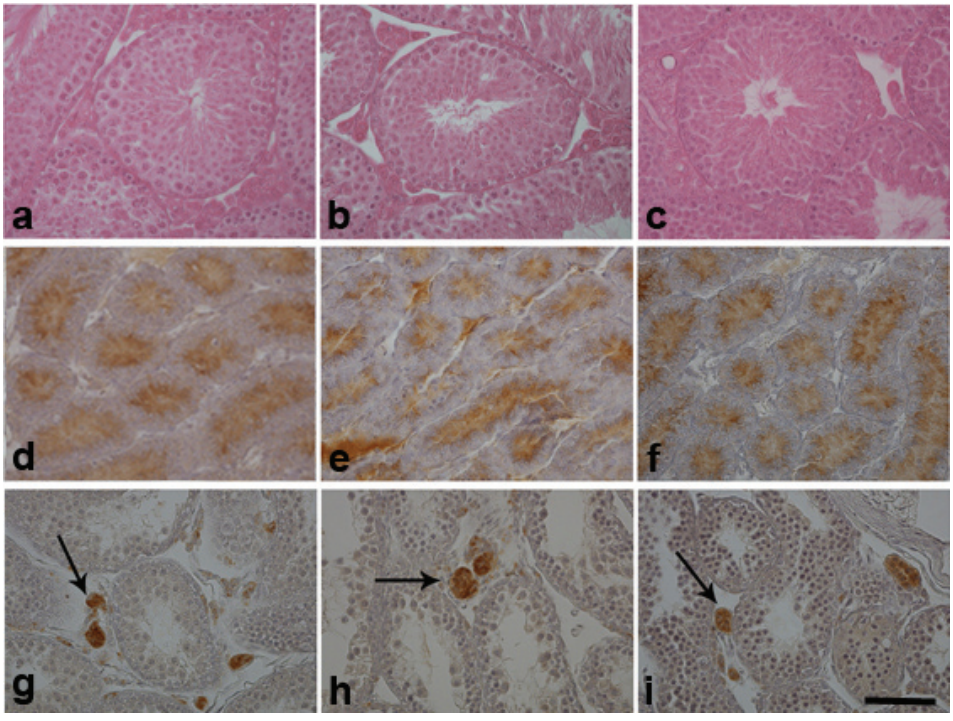


Figure 1: Histological analyses did not reveal any differences in general testis histology at 48 days of age between control (a) and groups exposed to Chlormephos through their mothers (b – 0.35 μg/ml Chlormephos; c – 3.5 μg/ml Chlormephos). Similarly, there were no obvious differences in intensity or pattern of antimüllerian hormone (d, e, f) immunoexpression in Sertoli cells in testes from 9 days old pups or 3β-HSD immunoexpression (g, h, i) in Leydig cells from 19 days old pups between control (d, g) and Chlormephos exposed mice (e, h – 0.35 μg/ml Chlormephos; f, i – 3.5 μg/ml Chlormephos). Arrows in panels g, h and i represent newly developed adult Leydig cells. Bar = 50 μm

Anxiety-like behavior test

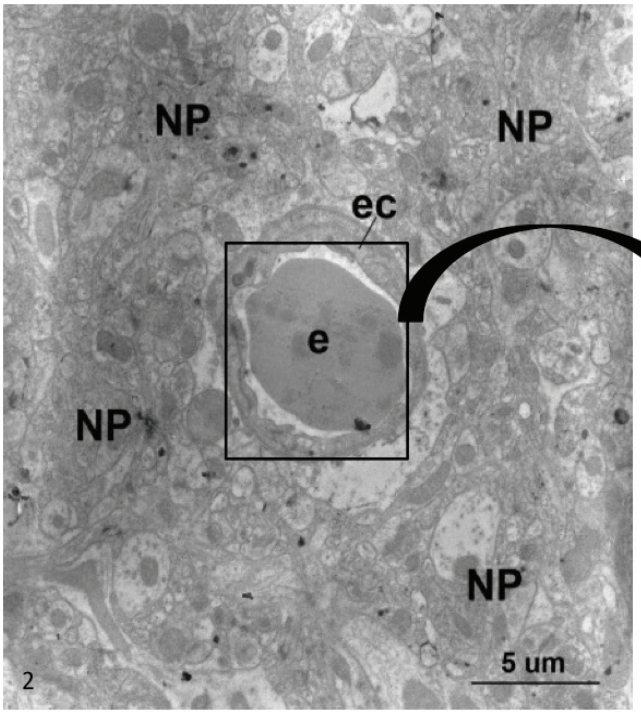
Elevated-plus maze tests at 70 days of age results revealed statistically significant differences between control mice and mice neonatally exposed to higher dose of Chlormephos. There was a significant effect of treatment in both time spent in open arms ($p < 0.05$) and time spent in closed arms ($p < 0.05$). Posthoc bonferroni test revealed significant difference between control groups and group exposed neonatally to 3.5μg per ml of water, but no difference between control group and group exposed neonatally to lower dose of Chlormephos. However, there was no significant difference between control and both Chlormephos exposed groups in latency to enter open arms or number of entrances into the open arm of elevated plus maze (Table 1).

Table 1: Statistical analysis of behavior of mice in elevated plus maze revealed significant difference (* = $p < 0.05$) between control group and group exposed to 3.5 μg/ml Chlormephos in both time spent in open arms and time spent in closed arms. There was no significant difference between groups in latency to enter open arms or number of entries to open arms, and no difference in any of parameters between control group and group exposed to 0.35 μg/ml Chlormephos. All data are presented as mean ± SEM

	Time spent in closed arms (s)	Time spent in open arms (s)	Number of entries into open arms	Latency to enter open arms (s)
Male control (n=10)	73.12 + 40.18	177.34 + 38.63	4.11 + 1.38	28.16 + 21.75
Female control (n=13)	113.19 + 22.31	124.16 + 23.18	5.13 + 1.06	53.53 + 20.84
Male Cl0.35 (n=8)	71.50 + 21.36	170.37 + 38.79	7.67 + 2.11	22.92 + 7.26
Female Cl0.35 (n=8)	113.08 + 29.87	148.65 + 37.50	5.83 + 1.54	53.68 + 32.03
Male Cl3.5 (n=10)	96.08 + 22.66*	105.19 + 29.25*	5.31 + 1.62	47.98 + 32.83
Female Cl3.5 (n=13)	162.44 + 16.88*	50.66 + 14.16*	3.45 + 0.88	84.10 + 30.67

Electron microscopy

Detailed analysis of brain – blood barrier in the cortex region from offspring of control mice and mice treated with higher concentration of Chlormephos (3 females and 3 males in each group) did not reveal any structural changes. In brains from control and treated mice, brain capillaries displayed normal morphology: endothelial cells had a flattened nucleus and an elongated cytoplasm. The euchromatin was located in the central region of the nucleus, whereas a



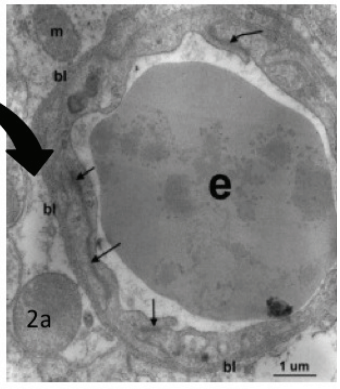


Figure 2: Blood vessel ultrastructure appeared to be intact in the brain from 70 days old mice exposed neonatally to 3.5 μg/ml Chlormephos.

NP – neuropil; e – erythrocyte; ec – endothelial cell; bl – basal lamina; m – mitochondria; arrows – junctional complexes (2 – magnification 3000x; 2a – magnification 7000x)

thick area of heterochromatin was arranged along the nuclear membrane. Within endothelial cells, one could occasionally observe a number of endocytotic vacuoles that were located either in the cell cytoplasm or associated with the cell membrane. Neighboring endothelial cells were joined by well-developed junctional complexes and laid on a continuous basement lamina. There was no damage or disruption of the basement membrane and/or endothelial cell cytoplasm. Capillaries displayed a normal perivascular space in all investigated animals. Neurons and the accompanying glial cells and neuropil preserved normal fine structure in the Chlormephos treated animals when compared to controls (Figure 2).

Discussion

Endocrine disrupting chemicals (EDC) are a group of chemicals that could potentially interfere with an endogenous hormonal system. Most EDCs could interfere with endocrine system by binding to estrogen or androgen receptors although other mechanisms such as interference with expression of activity of steroidogenic enzymes or interactions with aryl-hydrocarbon receptor were described (18, 19). Exposure of animals and human to EDCs could disturb development and function of reproductive system, and possibly of central nervous system (20). The aim of our study was to determine if Chlormephos, an organophosphorous compound, could acts as endocrine disruptor.

Several studies suggested that different organophosphorus compounds can act as EDCs. Narayana et al. (2005) reported, that treatment with metyl parathion in doses relevant for human exposure (0.5-1 mg/kg) resulted in decreased number of spermatozoa and higher number of morphological defective spermatozoa in treated mice, although this treatment did not affect the number of offspring in treated rats. Several other studies also reported effects of certain organophosphorus compounds can affect endocrine system in humans (21, 22) and animals (23, 24, 25, 26). Recio et al. evaluated relationship between OP metabolites in urine and serum levels of pituitary and sex hormones of agricultural workers exposed to mixture of different OP (methylparathion, metamidophos, endosulfan, dimethoate and diazinon) and reported that OP disrupted FSH and LH levels (22).

Okahashi et al. (2005) studied effects of organophosphorus insecticide fenithrotion in rats. Fenithrotion in high doses decreased the activity of brain cholinesterase in parental generation, but did not affect reproduction, organ weight, histopathology and semen parameters in rats treated with sub-lethal doses of fenithrotion. Furthermore, offspring of exposed mice did not differ from control groups in anogenital distance, beginning of puberty, organ weights and histopathology suggesting that fenithrotion did not act as endocrine disruptor (27). These results are similar to the results from our study where we could not find any differences between control and treated groups in any reproductive parameters studied. Our results therefore suggest, that Chlormephos does not act as endocrine disruptor and therefore does not pose a risk for endocrine system even if it would be released into the environment.

In addition to effects on reproductive organs, some endocrine disruptors could also affect central nervous system development and/or function. Some studies have suggested that different chemicals such as bisphenol A and metoxychlor, can affect sexual and non-sexual behaviors (28, 29, 30, 31). Exposure to malathion, chlorpyrifos and other OP during development can result in long-term impacts on intellectual function and delayed effects on the functioning of the central nervous system (8, 32, 33). Blood cholinesterase was also decreased in Wistar rats exposed to low doses of dichlorvos, relevant for human exposure (24).

Animals exposed to higher dose of Chlormephos in our study showed increased anxiety-like behavior in comparison to the control group. Previous studies have shown that organophosphates could negatively affect growth and terminal differentiation of nerve axons in the developing brain, thus causing long lasting changes in the brain that could be expressed only in the adult life (34, 35, 36). Organophosphates are liposoluble, so they can pass across the blood-brain barrier and come into direct contact with nerve cells, causing lasting damage to these cells. Several studies also revealed that low doses of organophosphates could have deleterious, long lasting effect on the blood-brain barrier at least in rodents (9, 10, 37). Interestingly, Song et al. (37) have shown structural damage in the blood-brain barrier in adult rats, exposed neonatally to paroxon, however, when paroxon was given to adult rats, no effect on blood-brain barrier was observed, suggesting that blood-brain barrier might be especially sensitive to paroxon during neonatal period. We exam-

ined cerebral cortex in control and treated mice using electron microscopy, but could not detect any defects in the structure of the brain tissue at the ultrastructural level. In particular, there were no differences in capillaries' walls that are part of blood-brain barrier. However, only small part of the brain was examined by electron microscopy and therefore, it is not possible to firmly conclude that blood-brain barrier was not affected perhaps in some other specific area.

In conclusion, our results showed that Chlormephos treatment of parents did not disturb development of testes and reproductive tract in the offspring of treated mice. Therefore, our study suggest that Chlormephos does not act as an endocrine disruptor in the reproductive system. However, treatment with higher dose of Chlormephos (3.5 µg/ml) produced long lasting effects on behavior as evident by increased anxiety like behavior in mice exposed to Chlormephos only in neonatal period through mothers. This study, therefore, suggests that Chlormephos as a representative organophosphorous compound could affect brain development in experimental animals and further studies are needed to examine possible long lasting effects of organophosphorous compounds on brain development in humans.

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VPLIV ORGANOFOSFATA KLORMEFOSA NA RAZVOJ IN DELOVANJE MOD IN MOŽGANOV

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Povzetek: Različne kemične snovi, pogosto imenovane hormonski motilci, lahko motijo delovanje hormonskega sistema v sesalskem telesu. Namen naše raziskave je bil ugotoviti, ali dolgotrajna izpostavljenost organofosfornemu insekticidu klormefosu lahko vpliva na normalen razvoj spolnega sistema in endokrinega dela možganov. Odraslim mišim obeh spolov smo preko pitne vode dodajali klormefos v koncentracijah 3,5 µg/ml in 0,35 µg/ml. Pari miši so bili izpostavljeni klormefosu 2 tedna pred paritvijo, ves čas brejosti in nato do odstavitve mladičev pri 3 tednih starosti. Razvoj mod pri potomcih izpostavljenih miši smo proučevali s spremljanjem teže mod, dnevne proizvodnje semenčic, apoptoze v modih ter izraženosti beljakovin antimülerjevega hormona in 3-beta hidroksisteroidne dehidrogenaze. Pri nobenem od preiskovanih parametrov nismo ugotovili statistično zanesljivih razlik med kontrolno skupino in skupinama, izpostavljenima klormefosu, kar kaže, da klormefos ne deluje kot hormonski motilec spolnega sistema.
Za ugotavljanje morebitnih škodljivih učinkov klormefosa na možgane v času razvoja, smo pri potomcih izpostavljenih miši ugotavljali obnašanje, podobno anskioznemu s testiranjem v dvignjenem labirintu. Miši, ki so bile med razvojem izpostavljene višji koncentraciji klormefosa, so kazale statistično značilno poudarjeno obnašanje, podobno anksioznemu (več časa so preživele v zaprtih krakih labirinta), v primerjavi s kontrolno skupino. Preiskava možganov z elektronskim mikroskopom pa ni pokazala nikakršnih razlik v ultrastrukturi možganov in krvno-možganske pregrade, čeprav nekatera poročila kažejo, da naj bi bila prav krvno-možganska pregrada prizadeta ob zgodnji izpostavljenosti organofosfornim snovem.

Ključne besede: miš; modo; organofosforne snovi; dnevna proizvodnja semenčic; apoptoza; elektronska mikroskopija; anksioznemu podobno obnašanje.

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Original Scientific Article

IMPROVEMENT OF SPERM SORTING EFFICIENCY AND FERTILIZING CAPACITY EMPLOYING TWO VARIATIONS OF A NEW BULL SEMEN EXTENDER (SEXCESS®)

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Summary: The aim of the present study was to analyse the effect of temporary chemical inhibition of sperm motility before and during flow cytometrical sorting on the quality and fertilizing capacity of sex sorted and frozen/thawed spermatozoa. The quality and fertilizing capacity of temporarily immobilized spermatozoa sorted in the presence of sodium fluoride (S-AO-NF) was compared with sorted semen using a standard protocol as developed in our laboratory (S-AO) and with unsorted control (C). Semen from two bulls (Holstein Friesian and Limousine) was used and in total 283 first inseminations with 2 million live, frozen/thawed sex sorted and unsorted spermatozoa were performed on 197 farms. Motility of spermatozoa was completely inhibited after addition of sodium fluoride and after sorting and centrifugation returned to a similar degree as before. Motility after thawing and incubation at 37 °C did not differ between groups. After further incubation at 37 °C for 12 h and 24 h motility was significantly higher ($P<0.001$) in both sorted groups compared to unsorted semen. The percentage of spermatozoa with acrosome abnormalities in frozen/thawed samples was significantly higher ($P<0.05$) in S-AO and C group when compared to S-AO-NF group. The same difference was found for morphological sperm abnormalities. The percentage of acrosome reacted and viable spermatozoa was analysed by FITC-PNA/PI staining. The percentage of acrosome reacted spermatozoa was significantly lower ($P<0.001$) in both groups of sorted semen when compared to unsorted control. The percentage of viable spermatozoa was significantly higher ($P<0.05$) in the S-AO-NF group when compared to groups S-AO and C. Pregnancy rates after artificial insemination with the frozen/thawed semen were not significantly different between sorted spermatozoa and controls (72.7 % vs. 73.3 % vs. 79.2 % for S-AO-NF, S-AO and C respectively).

Key words: semen; spermatozoa; flow cytometry; sorting; sex; freezing / thawing; artificial insemination; pregnancy

Introduction

Like in other domestic animal species, a broader utilisation of sexed sperm would be very beneficial for cattle industry. So far, only flow cytometry allows producing a significant shift of sex ratios. However, the technique still has its limitations in sorted sperm output (1, 2), although many improvements have helped in the recent years to start first commercial applications as shown in UK and Texas, USA.

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Especially introduction of high speed flow cytometry (3), better orientation of spermatozoa in front of the Laser beam (4) and improvements in sperm preparation before and after sorting by special media components (unpublished data) allow to minimize the sperm concentration and use liquid as well as frozen/thawed spermatozoa. However, fertilizing potential of sorted frozen/thawed spermatozoa has not reached the quality of unsorted analogues (5). The lifetime after sorting and freezing/thawing is limited and even with adapted insemination protocols, the number of calves born per AI or number of AI necessary to get the same fertility rates are critical (5). A potential solution might be to

safe as much energy as possible during processing and sorting. This might be achieved with reversible, chemical inhibition of the sperm metabolism.

Ornidazone (6), bromoydroxypropanone (7), nitric oxide (8), alfa-chlorhydrin (9), gossypol (10), Cytochalasin B (11), formaldehyde (12) and sodium fluoride (13, 14) are examples for chemicals, known to have an inhibiting effect on sperm motility. Dott et al. (12) were able to achieve pregnancies after artificial insemination in ewes and sows with formaldehyde-immobilized spermatozoa. As the toxicity of formaldehyde is high (15), specific inhibition of sperm motility may be performed by sodium fluoride (13). Sodium fluoride is a conventional inhibitor of protein-thyrosine-phosphatase (16), which is included in the process of sperm capacitation and motility (17, 18) and was reported as immobilizing substance for bull spermatozoa (19).

Flow cytometrical sorting could benefit from temporarily inhibition of sperm activity not only for the prolongation of sperm lifetime, but also to get a better orientation of spermatozoa in front of the Laser beam and to reduce individual bull effects during staining and sorting. In the present study, we analysed the effect of temporary sperm immobilization with sodium fluoride on sorting efficiency, quality and fertility of frozen thawed bull spermatozoa.

Materials and methods

L-α-Lysophosphatidylcholine (L 5004); Lectin, FITC labelled from Arachis hypogaea (peanut) (L7381); Propidium iodide (P4170), 6-Carboxyfluorescein diacetate (C5041); Bisbenzemide H 33342 (B2261), Pyruvic Acid (P-3662) and Catalase (C-1345) were purchased from Sigma-Aldrich (Taufkirchen, Germany) all the other chemicals, if not specially stated, were purchased from Carl Roth GmbH + Co (Karlsruhe, Germany).

Methods

Semen ejaculates were collected from fertility proven and performance tested Holstein Friesian bull (Bull 1, 11 ejaculates) with high fertility rate and a young Limousine bull (Bull 2, 4 ejaculates) in performance test. Ejaculates that were included into research had to reach minimum criteria of at least 70% motility and 80% of morphological normal spermatozoa.

Ejaculates were kept in a water bath at 27°C and motility was estimated under phase contrast microscope (Olympus BX 60) at 37°C. Morphology was analysed after fixation in Hancock fixative (2.784 g Tri-Natriumcitrat-Dihydrat, 4 mL 37 % formaldehyde solution and bi-distillated water to 100 mL), within 4 hours after semen collection. Concentrations of spermatozoa in ejaculates were determined with a haemocytometer (Coulter counter®). Raw semen was divided into two parts and diluted to $1x10^8$ spermatozoa/mL either with the commercially available extender Sexcess® AX (Masterrind Verden, Germany), supplemented with antioxidants and BSA fraction V (group S-AO), or Sexcess® FX additionally containing sodium fluoride (group S-AO-FX). Spermatozoa were labelled with 15, 20 and 25 µL of a 8.12 mM Hoechst 33342 solution and incubated for 1 hour at 34°C. Thereafter, labelled semen samples were kept at 22°C in the dark and sorted within 7 hours after onset of incubation. The labelled semen samples were pre-tested for maximal separation with a flow cytometer and the concentration of Hoechst 33342 stain giving the best resolution of two sperm populations was used for daily sorting. The other part of the ejaculate, which was not used for sorting, was frozen following a routine protocol of a commercial AI center and served as control. Briefly, semen was gradually diluted with TRIS egg-yolk freezing extender I to the concentration of $26.4x10^6$ spermatozoa/ml, cooled to 5°C within two hours and further diluted with TRIS egg-yolk freezing extender II containing 0.75 % detergent (Equex STM®, Nova Chemical sales, Inc., Scituate, USA) and 12.8 % (v/v) of 87 % Glycerol to a concentration of $13.2x10^6$ spermatozoa / mL or $3.3x10^6$ spermatozoa / straw, filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany), and frozen in liquid nitrogen (LN_2). Briefly, straws were placed on metal holder in nitrogen vapour 3-5 cm above LN_2 for 15 minutes. Frozen samples were then plunged into LN_2 and kept in the semen container (in LN_2) until used for analysis or artificial insemination.

Processing of the sorted spermatozoa

Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (3). Sorted spermatozoa of group S-AO were collected into collection fluid (max. 8 million spermatozoa per tube) and then centrifuged at 838xg for 20 minutes. Centrifugation of sorted spermatozoa in group S-

AO-NF allowed lower force (500xg for 15 minutes) because cells were immobilized and gave a more distinct pellet. Supernatant was discharged and the remaining sperm pellet of both groups was diluted with extender Sexcess I® (Masterrind Verden, Germany) to a concentration of $41x10^6$ spermatozoa /mL and cooled to 5°C within 2 hours. Once 5°C was reached, semen samples were further diluted with extender Sexcess II® (Masterrind Verden, Germany) to a concentration of $20.5x10^6$ spermatozoa/mL. Plastic straws (0.25 mL) (Minitüb, Tiefenbach, Germany) were pre-filled with a first segment (50 µL of a mixture made from extender I and II), and with a second segment, filled with 160 µL sorted semen (3.3 millions spermatozoa in total, equivalent to approx. $2 x10^6$ life spermatozoa). Semen samples were frozen in liquid nitrogen as already described for control samples.

Sperm analysis of frozen thawed samples

Motility analysis

Sperm motility of raw semen, of sperm samples before and after sorting and after thawing was analysed. Prior to analyses samples were prewarmed to 37°C for 15 minutes and analysed under a phase-contrast microscope (OlympusBX60) at 100x magnification, equipped with heating plate to maintain 37°C. From each sample two 6 µL drops were transferred onto pre-wormed objective glass and covered with coverslip glass. At least three fields were evaluated per drop.

Morphology analysis

Morphology was analysed after fixation of the samples in Hancock solution under a phase-contrast microscope (Olympus BX 60) at 1000x magnification. At least 200 spermatozoa were examined per sample for morphological abnormalities (MAS) and acrosome integrity. Spermatozoa were divided into two groups. Spermatozoa that had any pathological change of acrosome were included into group named damaged acrosomes (DA). Group MAS corresponds to the percentage of spermatozoa with damaged acrosomes plus spermatozoa that had any other morphological abnormalities.

Acrosome integrity and membrane stability of spermatozoa (FITC-PNA/PI)

Acrosome integrity and membrane stability were analysed with FITC-PNA/PI as described previously (20). Pre-warmed Eppendorf cups were filled with 50 µL of semen and 1 µL FITC-PNA (2 mg FITC-PNA in 2 mL PBS) as well as 2 µL PI (1 mg propidium iodide in 10 mL physiological NaCl solution) were added. Samples were incubated at 38°C for 5 minutes and supplemented with 5 µL paraformaldehyde (1 % in PBS) immediately before microscopic examination. At least 200 spermatozoa were examined under a fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400x magnification. Spermatozoa were divided into four groups: 1. PNA-negative/PI-negative (viable spermatozoa with intact acrosome); 2. PNA-negative/PI-positive (spermatozoa with damaged plasma membrane and intact acrosome); 3. PNA-positive/PI-positive (spermatozoa with damaged plasma membrane and reacted acrosome); 4. PNA-positive/PI-negative (spermatozoa with intact plasma membrane and reacted acrosome). Mean percentages of viable spermatozoa with intact membranes (group 1) and acrosome reacted spermatozoa (group 3 and 4) are presented in the results.

Artificial insemination and pregnancy control

All the straws were coded in order to make it impossible for inseminators to know the content. Straws were randomly distributed to three well experienced AI technicians within 1.5 month after sorting. For AI, semen samples were thawed at 37°C for 20 sec. and inseminated 12-24 hours after onset of natural oestrus. Technicians were advised to insert a normal AI catheter under rectal control as deep as possible into the uterine horn but without extra force or strong manipulation. In cases where it was difficult to determine the location and size of the follicle, semen was either deposited into the uterine body or the content of the straw was split for AI in both uterine horns. Pregnancies were controlled 30-60 days after insemination by transrectal examination and transrectal ultrasonography (Aloka®; 5 MHz).

Statistical analyses

Statistical analyses were performed with SIGMA STAT 2.03 for windows (Jandel Scientific Cooperation, San Ragael, CA, USA). Pregnancy rates were analysed with Chi-square test. Semen quality parameters were tested for normal distribution and either analysed with One-way ANOVA or ANOVA and Ranks and Tukey test. Data are expressed as percentages or means ± SD. Differences were considered to be significant at $P<0.05$.

Results

In the raw semen samples 72.1 ± 3.7 % spermatozoa were motile. After labelling and before sorting in the S-AO group 66.9 ± 4.6 % were motile, whereas all spermatozoa in the S-AO-NF group were immobilized. After sorting the percentage of motile spermatozoa in S-AO-NF group was 72.9 ± 4.9 %, whereas sperm motility in S-AO samples was 70.6 ± 5.6%. The difference was not significant ($P<0.05$). Sperm motility after thawing and incubation at 37°C for 6 h was not significantly different between groups. Further incubation of frozen thawed spermatozoa for 12 and 24 hours showed significant higher motility of spermatozoa in both sorted groups compared to unsorted standard processed semen (Table 1).

Table 1: Sperm motility of sorted and control groups immediately after thawing (0 h) and after further incubation at 37°C for 6, 12 and 24 h

	0 h (%)	6 h (%)	12 h (%)	24 h (%)
Control	61.5 ± 3.8	45.8 ± 9.3	$7.5 \pm 11.6^{a(L)}$	0.0 ± 0.0^{a}
S-AO	59.4 ± 4.2	45.6 ± 9.4	33.1 ± 6.5^{b}	$21.3 \pm 10.3^{b(L)}$
S-AO-NF	59.0 ± 5.9	47.9 ± 7.0	37.1 ± 4.9^{b}	$22.9 \pm 7.6^{b(L)}$

Values with different superscripts differ significantly ($P<0.001$).
Data are shown as mean ± SD. (L) - only local motile spermatozoa were observed.

After thawing the percentage of spermatozoa with damaged acrosomes and morphologically abnormal spermatozoa were significant lower in S-AO-NF group compared to S-AO group and unsorted control samples (Table 2).

Table 2: Percentage of spermatozoa with damaged acrosomes (DA) and morphologically abnormal spermatozoa (MAS)

	DA (%)	MAS (%)
Control	22.3 ± 4.0^{a}	29.0 ± 5.8^{a}
S-AO	23.0 ± 5.2^{a}	30.0 ± 6.7^{a}
S-AO-NF	14.7 ± 7.5^{b}	19.0 ± 8.1^{b}

Values with different superscripts differ significantly ($P<0.05$).
Data are shown as mean ± SD.

The percentage of acrosome reacted spermatozoa as seen after FITC-PNA staining was significantly lower in both sorted groups as compared to unsorted control samples. The percentage of viable spermatozoa was significantly higher in S-AO-NF group compared to S-AO group and unsorted control (Table 3).

Table 3: Percentage of acrosome reacted (AR) and spermatozoa with intact membranes (viable) in frozen/thawed samples, according to FITC-PNA/PI staining

	AR (%)	Viable (%)
Control	19.1 ± 4.7^{a}	61.4 ± 8.2^{c}
S-AO	10.6 ± 3.5^{b}	56.9 ± 4.7^{c}
S-AO-NF	6.7 ± 2.6^{b}	72.1 ± 5.0^{d}

Values with different superscripts are significantly different: a:b $P<0.001$; c:d $P<0.05$-
Data are shown as mean ± SD.

In total 283 inseminations were performed under field conditions on 197 Slovenian farms. Mostly it was one insemination per farm, than two inseminations were performed on 30 farms, 3 on 11 farms, 4 on 3 different farms and 5 inseminations on 1 farm. Table 4 shows the pregnancy results. No differences were found between sorted and unsorted semen samples or among sorted samples. Reanalysis of semen after thawing revealed 92.5 % average purity for X-chromosome bearing spermatozoa.

Table 4: Pregnancy rates after artificial insemination with sorted frozen/thawed spermatozoa diluted in Sexcess® AX or Sexcess® FX

	Control	Sexcess® AX (S-AO)	Sexcess® FX (S-AO-NF)
Bull 1(%)	75.5 %	73.5 %	62.1 %
Pregnant/all (n/n)	37/49	36/49	36/58
Bull 2 (%)	79.2 %	73.3 %	72.7 %
Pregnant/all (n/n)	42/53	32/44	22/30
Total (%)	77.5 %	73.1 %	65.9 %
Total (n/n)	79/102	68/93	58/88

No differences in pregnancy rates were seen among AI technicians (69.1, 70.1 and 77.9 %). Place of the semen deposition was recorded in 260 cases. It had a significant effect ($P<0.05$) on the pregnancy rates and were significantly lower after semen deposition into the uterine body and into uterine horn ipsilateral to the ovulation site compared to those inseminations where the content of the straw was divided and inseminated into both horns.

Table 5: Effect of place of semen deposition on pregnancy rates

AI place	all/pregnant	%
Uterine body	50/33	66.0[a]
Ipsilateral to ovulation	130/88	67.7[a]
both horns	80/66	82.5[b]

Values with different superscripts are significantly different: a:b $P<0.05$

Discussion

In the present study, we have demonstrated that optimal handling according to the modified Beltsville Sperm Sorting Technology (3) and protection during staining, sorting, centrifugation, diluting and freezing of the sorted spermatozoa leads to high quality of sexed semen and to pregnancy rates that are comparable with those of unsorted controls after artificial insemination under normal field conditions. However, it has to be mentioned that all experiments were performed with semen from selected bulls. Selection criteria were that non return rates were above average and that staining and sorting was homogenous between collection days in order to speed up the sorting process.

Previous studies demonstrated lower pregnancies rates after artificial inseminations with flow cytometrically sorted spermatozoa when compared to unsorted control. Probable reasons were induction of capacitation, membrane damages (21, 22, 23) and rapid loss of motility (24). In consequence, insemination protocols had to be changed and insemination had to be performed closer to ovulation.

In the present study, lifetime of sorted spermatozoa was at least as long as for those in the control group. The percentage of motile spermatozoa was even higher in both groups of sorted semen compared to unsorted controls after incubation at 37°C for 12 and 24 h. This was probably caused by utilization of antioxidants, preventing generation of reactive oxygen species, oxidation of membrane lipids, and loss of motility due to oxidation (25). Addition of sodium fluoride caused a complete, reversible inhibition of sperm motility and seemed to reduce interference of mitochondria in the mid piece of the sperm tail with the high voltage electric field, necessary to separate both sperm populations. Sorted spermatozoa regained their motility after sorting and centrifugation completely, which shows the complete reversibility of motility inhibition with sodium fluoride.

Chinoy and Naraya (26) reported toxic effects of sodium fluoride on human spermatozoa, causing irreversible damages like elongated heads, deflagellation, acrosome loss and coiling of the tails. However, in the present study, concentration of sodium

fluoride was much lower and enabled spermatozoa to completely regain motility. So far, no negative effects of sodium fluoride were seen. In opposite, the highest sperm membrane integrity was found in those samples that were diluted with Sexcess® FX. In addition, these samples also showed better sperm quality after sorting, which might mainly be due to shorter centrifugation time and force.

Laboratory results were confirmed by pregnancy data that were obtained from an AI field trial in heifers. No significant differences were seen between inseminations with sorted vs. unsorted semen.

The handling was as easy as with unsorted semen and no significant effect on the insemination results were found among AI technician. However, the site of semen deposition in the female genital tract had significant effect on pregnancy rates. Williams et al. (27) reported reduced fertility after cervical deposition of the semen compared to uterine body or bicornual insemination, and Senger et al. (28) saw significantly more pregnant animals after bicornual insemination compared to insemination into the uterine body. Accordingly, in the present study significantly more animals became pregnant after bicornual compared to uterine body insemination. This is probably because more spermatozoa were lost during transport to the fertilization site after they were deposited into the uterine body. It is also interesting that lower pregnancy rates were obtained after semen was deposited into the ipsilateral uterine horn compared to the bicornual insemination. We believe that the reason could be due to the failure of correct diagnosis of ovulation site and consequently more inseminations were performed into the wrong uterine horn. It is also possible that the ovary (follicle) was damaged when the inseminator diagnosed the site of follicle growth.

In conclusion, the use of Sexcess® extender to optimize bull sperm quality has successfully been approved and improved the fertilizing ability of sexed spermatozoa to a comparable status of unsorted frozen/thawed bull semen. Further studies will have to analyse more intensively bull effects to broaden the number of males to be included in a sperm sorting based breeding programme.

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IZBOLJŠANJE USPEŠNOSTI LOČEVANJA IN OPLODITVENE SPOSOBNOSTI PO SPOLU LOČENIH SEMENČIC Z UPORABO DVEH OBLIK NOVEGA RAZREDČEVALCA ZA BIKOVO SEME (SEXCESS®)

P. Klinc, D. Rath

Povzetek: V raziskavi smo ugotavljali vpliv začasne kemične zaustavitve gibljivosti semenčic na kakovost in oploditveno sposobnost sortiranih in zamrznjenih/odmrznjenih semenčic. Vpliv smo ugotavljali pred in med postopkom ločevanja semenčic po spolu s pomočjo pretočne citometrije. Kakovost in oploditveno sposobnost semenčic, ki so bile začasno imobilizirane s pomočjo natrijevega fluorida (S-AO-NF), smo primerjali z rezultati, pridobljenimi pri semenu, ki smo ga sortirali po standardnem postopku, razvitem v našem laboratoriju (S-AO), ter kontrolnim nesortiranim semenom (C). V poizkus smo vključili seme dveh bikov. Na 197 kmetijah smo opravili 283 prvih osemenitev z 2 milijonoma živih odmrznjenih sortiranih in nesortiranih semenčic. Gibljivost semenčic, ki se je popolnoma zaustavila po dodatku natrijevega fluorida, se je po sortiranju in centrifugiranju vrnila na predhodno stopnjo. Po tajanju in inkubaciji na 37 °C med skupinami ni bilo statistično značilne razlike v gibljivosti semenčic. Po nadaljnji 12- in 24-urni inkubaciji na 37 °C pa je bila gibljivost semenčic statistično značilno višja ($P<0.001$) pri sortiranem semenu v primerjavi s kontrolnim semenom. Pri odmrznjenem semenu je bil odstotek semenčic s poškodovanimi akrosomi statistično značilno višji ($P<0.05$) v skupinah S-AO in C v primerjavi s skupino S-AO-NF. Pri ugotavljanju skupnih morfoloških sprememb smo prav tako ugotovili statistično značilno ($P<0.05$) višji odstotek spremenjenih semenčic v skupinah S-AO in C v primerjavi s skupino S-AO-NF. S pomočjo barvanja FITC-PNA/PI smo ugotavljali odstotek semenčic z reagiranimi akrosomi in odstotek vitalnih semenčic. V obeh skupinah sortiranega semena smo ugotovili statistično značilno nižji ($P<0.001$) odstotek semenčic z reagiranimi akrosomi v primerjavi z ne sortiranim semenom. Odstotek vitalnih semenčic je bil statistično značilno višji ($P<0.05$) v skupini S-AO-NF v primerjavi s skupinama S-AO in C. Po umetni osemenitvi z zamrznjenim/odmrznjenim semenom nismo ugotovili statistično značilne razlike v oploditveni sposobnosti med posameznimi skupinami sortiranega semena in kontrolnim semenom (72.7 % proti 73.3 % proti 79.2 % za skupine S-AO-NF, S-AO in C). Kljub temu da je bil spol potomcev statistično značilno ($P<0.05$) spremenjen v obeh skupinah sortiranega semena, je bila sprememba statistično značilno ($P=0.04$) višja pri skupini S-AO-NF v primerjavi s skupino S-AO.

Ključne besede: seme; semenčice; pretočna citometrija; ločevanje; spol; zamrzovanje / tajanje; umetno osemenjevanje; brejost

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