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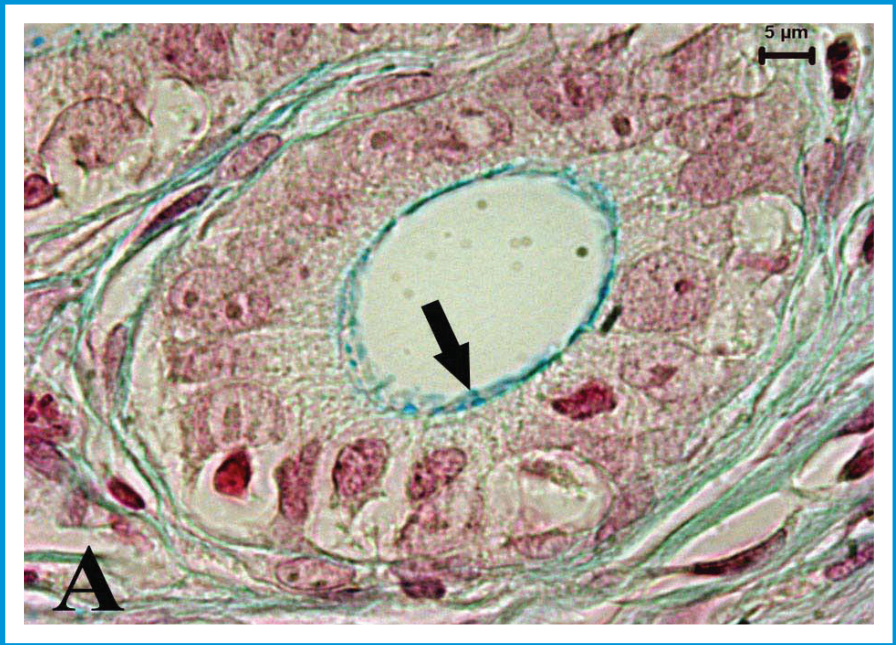

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Original Scientific Article

THE EFFECTS OF TWO WAYS OF STORAGE ON PHYSICOCHEMICAL CHANGES IN PHEASANT MEAT

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Summary: In this study the effects of two ways of storage by chilling and vacuum packaging on the physicochemical changes of hunted pheasant meat were determined during the refrigerate storage. Lactic acid, phosphate and pH value in breast and thigh muscle of chilled (n=10) and vacuum packed pheasants (n=10) were evaluated at 1st, 7th and 14th day after weaning. On day 7 lactic acid concentrations significantly increased in thigh (1.63 ± 0.41 g/100g; $p \leq 0.05$). On day 14, lactic acid in the non-vacuum packed thigh significantly decreased (0.73 ± 0.56g/100g; $p \leq 0.05$) compared to day 7. Significantly higher ($p \leq 0.01$) concentrations of lactic acid were recorded in vacuum packed than non-vacuum packed thigh muscles on day 14. In breast, significant difference ($p \leq 0.01$) in lactic acid between vacuum and non-vacuum packed meat was observed on day 14 (1.88 ± 0.18, respectively 1.15 ± 0.67g/100g). Increased pH value in non-vacuum packed thigh was observed on day 7 (6.49 ± 0.15g/100g), and in vacuum packed on day 14 (6.42 ± 0.13g/100g). Significantly higher concentrations of phosphates in thigh were recorded on day 7 in both groups, vacuum (0.90 ± 0.17; $p \leq 0.05$) and non-vacuum packed meat (0.91 ± 0.10; $p \leq 0.05$), in comparison with day 1. The significant decrease (0.68 ± 0.18; $p \leq 0.01$) of phosphates in thigh were observed on day 14 in non-vacuum packed thigh. In vacuum-packed thigh the phosphates showed significantly increased level (0.89 ± 0.15; $p \leq 0.05$) on day 14 compared with day 1. Our results indicate that the most suitable method of storage of pheasant meat is a combination of chilling and vacuum packing.

Key words: pheasant; meat; lactic acid; pH; vacuum

Introduction

Game birds are hunted mainly for recreational reasons (1) but have also been used as a food source. One of the worlds's most hunted and the most important game bird species in many countries in Europe, Australia, Asia, North and South America and New Zealand is the pheasant *(Phasianus colchicus).* This bird is selected for breeding stock in many countries to produce high nutritive meat (2).

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Pheasant meat ranks among highly appreciated food and mainly breast and thighs of pheasant are highly valued meat portions. Meat is characterized by a high concentration of protein and low content of intramuscular fat and low cholesterol content. Pheasant meat is considered also a good nutritional source of iron (3). These data are valuable for characterizing the nutritional quality of pheasant meat.

However, the other most important traits of quality of pheasant meat are also the pH value, lactic acid and phosphates concentrations. Lactic acid level reflects quantitative transformation

of glycogen and indicates typical or atypical processes of meat ripening (4). The pH value of a muscle is an accepted parameter to identify normal and deviating meat qualities (5). The conversion of muscle to meat is also an energy-demanding process and in the muscle after death, as well as in life, the energy is provided by splitting of ATP to ADP and inorganic phosphorus. In the muscle after death, the ATP is replenished by the conversion of ADP to ATP by the transfer of the higher energy phosphate for creatine phosphate and by degradation of glycogen (6).The maturing processes are running in muscles up to the point of time after death of game, until the supplies of glycogen and energetically valuable phosphates are available.

Physicochemical properties of meat change most intensely during the first hours after the killing of pheasants. This is an interval of most intensive glycolysis and lactic acid increase in the muscle tissues. The speed of this biochemical processes and degree of decreasing of pH values are important for optimal properties of pheasant meat.

One way to maintain the acceptable quality of pheasant meat is chilling. Chilled meat of pheasant should be stored at no more than 4°C in a hygienic manner and this temperature should be maintained throughout the supply chain. Storage of meat at low temperature is a prerequisite for the development of the major eating qualities including tenderness and flavour (7).

The length of refrigeration storage has also a significant impact on the activity of lactate dehydrogenase (LDH) isoenzymes in the blood serum of game after 7 days and the concentrations of LDH isoenzymes - LDH 4 and LDH 5 are reduced (8).

The present study is also focused on the role of vacuum packing of cold-stored pheasant meat. There are many advantages to vacuum packing. The main advantages of vacuum package are that it assists in the preservation of meat, improves tenderness during ageing process as well as shelf life of the meat. Using these modern techniques of packaging we can maintain the microbial and also the sensory quality of the products during the storage (9).

The aim of this study was to compare the effects of vacuum packaging and cold storage of pheasant meat on the physical and biochemical changes (pH value, lactic acid, phosphates concentrations) of meat.

Materials and methods

Pheasants were obtained from regular drive hunts during the hunting season (autumn – winter) in the eastern Slovakia. They had been killed by lead shots. Hunted pheasants were 8 – 9 month-old. Carcass were delivered at 4 °C to the Department of Food Hygiene and Technology in the University of Veterinary Medicine and Pharmacy in Košice (Slovakia). Pheasants were eviscerated 24 hours post mortem and processed. Evisceration (the complete removal all internal organs) is widely recommended method for treatment of feathered game carcasses, to ensure high hygienic quality of the pheasant meat during storage and it is in compliance with the Regulation (EC) of No. 853/2004 (10). The determination of concentrations of lactic acid, phosphates and pH values was carried out 24 hour after death on day 1 of the experiment in all pheasants. Afterwards, samples of muscles from pheasants (20) were divided into 2 groups.

First group consisted of non-vacuum packed pheasant thigh and breast muscle (n=10) . The thigh and breast muscles of the second group of pheasants (n=10), were removed and vacuum packed. The temperature for storing the meat in refrigerator was in both cases 4°C for 14 days.

The concentrations of lactic acid, phosphates and pH values were determined 24 hour after death on day 1 of experiment, as well as on day 7 and on day 14 of the experiment.

Measuring the pH value of meat

Samples of 50g from each group of meat (thigh muscle and breast muscle) were homogenized for 10 minutes. Afterwards, 10g was used for extraction by 100 ml distilled water, and then filtrated**.** The pH values were analysed in a watery meat extract by using a pH meter (InoLab pH720, WTW, Weilheim, Germany) with glass electrode (11).

Capillary electrophoretic analysis

Electrophoretic analyser, Type EA 102 (Villa Labeco, Spišská Nová Ves, Slovak Republic) with a conductive detector was used for measurement of lactic acid and phosphates in meat. The capillary electrophoretic analysis is an appropriate method of determining of lactic acid and phosphates in

meat as an important indicator of quality (12). This method is suitable for analysis of lactic acid changes in the game meat during ageing process (13). The watery extract from the pH measurements was diluted 1:10, and injected into an electrophoretic analyser. The separation analytical system used in analyser consisted of leading electrolyte 10mM HCL, β-alanine and 0.1% methylhydroxyethylcellulose, pH 3.2. Terminator electrolyte consisted of 5mM caproic acid and 5mM hydroxymethyl-aminomethane. The direct currents used in pre-separation and analytical columns were 250 μA and 50 μA. Time of analysis was 10 – 15 min. The results of analysis were evaluated by ITP - Pro 32 software (KasComp. Bratislava, Slovak Republic). The concentrations of lactic acid and phosphates were expressed in $g.100g^{-1}$ of meat.

Statistical evaluation

The results were statistically analysed using GraphPad Prism Software, Version 4.00, San Diego, CA, USA, (2003). One-way analysis of variance (ANOVA) with the post hoc Tukey's multiple comparison test was used to evaluate statistical significance of differences between non-vacuum packed pheasant meat and vacuum packed meat (statistically significant differences are illustrated in the tables by numbers), and between days of the experiment statistically significant differences are illustrated in the tables by letters). The data were presented as mean and standard deviation.

Results and discussion

The course of changes in the concentration of lactic acid and pH during refrigeration storage of pheasant breast muscle is shown in Table 1. The initial mean value of lactic acid concentration measured in the breast muscle within 24 hours after hunting was 1.55 ± 0.69g/100g. On the day 7 of storage, lactic acid concentration increased in both groups. Higher values were measured in the vacuum packed breast muscle (1.92 ± 0.53g/100g), however, there were found no significant differences compared with non-vacuum packed breast muscle (1.84 ± 0.30g/100g). Significantly higher concentrations of lactic acid ($p \leq 0.01$) were observed in vacuum packed than non-vacuum packed breast muscles on day 14 of storage (1.88 ± 0.18, respectively 1.15 ± 0.67g/100g).

On this day of the experiment lactic acid concentration, as a product of lactate dehydrogenase (LDH) activity decreased in both groups, but more significantly in non-vacuum packed breast meat ($p \leq 0.05$).

The initial mean value of phosphates recorded in the breast muscle within 24 hours after hunting (on day 1 of storage) was 0.81 ± 0.22 g/100g (Tab. 1).

Assessment of dynamic phosphates concentrations in vacuum and non-vacuum packed breast meat showed a significant increase (1.01 ± 0.10; $p \leq 0.05$) in non-vacuum packed meat on day 7 of the experiment compared with day 1. An insignificant increase of levels of phosphates was observed also in vacuum packed breast meat on day 7 of experiment.

We observed a significant increase (1.11 ± 0.09; $p \leq 0.01$) of concentrations of phosphates in breast muscle in vacuum packed meat on day 14 of the experiment, as compared with day 7 and day 1.

On the other hand, the significant decrease (0.75 ± 0.14; $p \leq 0.001$) in concentrations of phosphates in breast were observed on day 14 of the experiment in non-vacuum packed meat.

The initial mean value of breast muscle pH was 5.93 ± 0.32. This parameter in both monitored groups did not change significantly during storage at 4 °C. Also, there were observed no significant differences of pH value between breast muscles which were vacuum-packed and those non-vacuum packed (Table 1).

The mean concentration of lactic acid measured in thigh muscles of pheasants within 24 hours after the hunt was 1.14 ± 0.48 g/100g. During refrigeration storage, amount of the lactic acid varied depending on packing method (Table 2). On day 7 of storage, concentration of lactic acid measured in vacuum packed thigh muscles significantly increased (1.63 ± 0.41 g/100g; $p \leq 0.05$). Increase of the quantity of lactic acid was recorded also in non-vacuum packed thigh muscle (1.34 ± 0.28g/100g), but was not statistically significant. The mean value of lactic acid concentration measured in vacuum packed thigh muscle on day 14 of storage was about the same as on day 7 (1.62 ± 0.27g/100g). On the other hand, on day 14 of storage a significant decrease in lactic acid was observed in non-vacuum packed thighs (0.73 ± 0.56g/100g; $p \leq 0.05$) compared to day 7. The most significant difference ($p \leq 0.01$) in

Table 1: Lactic acid and phosphates concentration (g/100g) and pH value monitored in breast muscle of pheasants during storage, V – Vacuum packed, NV – non-vacuum packed

Monitored parameter	Storage method	Day of storage		
		1.	7.	14.
Lactic acid	V	1.55 ± 0.69	1.92 ± 0.53	1.88 ± 0.18 [1]
	NV	1.55 ± 0.69 [ab]	1.84 ± 0.30 [a]	1.15 ± 0.67 [b;2]
Phosphates	V	0.81 ± 0.22 [b]	0.96 ± 0.06 [b]	1.11 ± 0.09 [a,1]
	NV	0.81 ± 0.22 [b]	1.01 ± 0.10 [a]	0.75 ± 0.14 [b;2]
pH	V	5.93 ± 0.32	6.05 ± 0.29	5.91 ± 0.20
	NV	5.93 ± 0.32	5.87 ± 0.26	6.10 ± 0.27

Statistical significant differences between two packing method are illustrated in the lines of tables by numbers and between days of the experiment by letters. Means in the lines with the same superscript (a. b) and means in the columns with the same superscript (1.2) do not differ significantly. Means with different superscripts differ significantly.

Table 2: Lactic acid and phosphates concentration (g/100g) and pH value monitored in thigh muscle of pheasants during storage V – Vacuum, NV – non-vacuum packed

Monitored parameter	Storage method	Day of storage		
		1.	7.	14.
Lactic acid	V	1.14 ± 0.48[b]	1.63 ± 0.41[a]	1.62 ± 0.27[a;1]
	NV	1.14 ± 0.48[ab]	1.34 ± 0.28[a]	0.73 ± 0.56[b;2]
Phosphates	V	0.71 ± 0.15 [b]	0.90 ± 0.17 [a]	0.89 ± 0.15 [a,1]
	NV	0.71 ± 0.15 [b]	0.91 ± 0.10 [a]	0.68 ± 0.18 [b;2]
pH	V	6.38 ± 0.20	6.35 ± 0.18	6.42 ± 0.13
	NV	6.38 ± 0.20[b]	6.49 ± 0.15[ab]	6.62 ± 0.26[a]

Statistical significant differences between two packing method are illustrated in the lines of tables by numbers and between days of the experiment by letters. Means in the lines with the same superscript (a. b) and means in the columns with the same superscript (1.2) do not differ significantly. Means with different superscripts differ significantly.

the concentration of lactic acid measured in the vacuum packed thigh and non-vacuum packed thigh muscle was observed on day 14 of storage, as was ascertained in the breast muscle.

The initial mean concentration of phosphates recorded in the thigh on day 1 was 0.71 ± 0.15 g/100g (Tab. 2). Significantly higher concentrations of phosphates in thigh muscle were recorded on day 7 of the experiment in both groups, vacuum (0.90 ± 0.17; $p \leq 0.05$) and non-vacuum packed meat (0.91 ± 0.10; $p \leq 0.05$), in comparison with day 1.

Similar course of changes in levels of phosphates such as those recorded in breast muscle were recorded in thigh muscle in non-vacuum packed meat. The significant decrease (0.68 ± 0.18; $p \leq 0.01$) in concentrations of phosphates in thigh were observed on day 14 of the experiment.

The concentrations of phosphates in thigh muscle in vacuum packed meat showed significantly increased levels (0.89 ± 0.15; $p \leq 0.05$) on day 14 of the experiment, as compared with day 1.

The pH value measured in the thigh muscle within 24 hours after hunt was 6.38 ± 0.20. During cold storage this value was increasing in both thigh muscle groups. While the increase of pH value in non-vacuum packed thigh was observed already on day 7 (6.49 ± 0.15g/100g), the increase in pH value in vacuum packed thigh was observed on day 14 (6.42 ± 0.13g/100g). As Table 2 shows, more pronounced increase in pH was recorded in thigh muscle which was stored non-vacuum packed, but

statistically significant differences in pH value of vacuum packed thigh and non-vacuum packed thigh muscle were not observed.

From the data presented in tables 1 and 2, pH values measured in the breast muscles were higher throughout the entire storage period, compared to those that were found out in thigh meat. These results are in accordance with studies of other authors (14, 15).

The pH values obtained in our study are closely related with the amount of the lactic acid present in the individual muscles. The concentration of lactic acid was higher in the breast muscle. These differences might result from the different patterns of myofibres. Breast muscles of pheasants are predominantly composed of fast-twitch, glycolytic fibres, whereas thigh muscles have higher percentage of oxidative fibre types (16).

Lactic acid concentration and pH value is influenced by ante-mortem carbohydrate depletion caused by physical activity as a result of chase during hunting, as noted by Paulsen et al. 2008 (17). They recorded, that pH values of hunted non-vacuum packed pheasant muscles were increased during storage, and these values were similar to our results. On the other hand, pH values of vacuum packed meat were almost the same throughout the entire storage period, which is in agreement with the result reported by Pfeifer et al. 2014 (18).

On day 7 of storage the concentration of lactic acid in the vacuum and also in the non-vacuum packed thigh and breast muscles increased. However, pH values measured in those muscles did not decrease; on the contrary, they remained the same as on the first day, or increased. The amount of lactic acid should influence pH value of the muscles, but there are also other factors which have impact on this quality indicator. The decomposition of nitrogenous compounds and formation of alkaline compounds, such as NH_3 causes pH increasing (19). Based on our measured values of pH we can suggest, that the decomposition changes in vacuum packed pheasant meat proceed more slowly compared to non-vacuum packaged meat.

The monitoring of one of indicators of ripening process - the concentrations of lactic acid showed statistically highest differences in vacuum and not-vacuum packed pheasant meat on the day 14 of storage. Lactic acid concentrations measured on day 14 in the vacuum packed breast and thigh meat were almost the same as on the day 7 of storage and were significantly higher ($p \leq 0.01$) compared to values measured in non-vacuum packed meat. The presence of atmospheric oxygen probably affects the degradation of lactic acid, which has impact on sensory properties of meat (20).

These results of dynamic phosphates in non-vacuum packed breast and thigh muscle are in accordance with studies by Januška et al. 2014 (21) who compared physico-chemical changes during maturation of shot and slaughtered pheasants.

In our study we recorded important differences in concentrations of phosphates between vacuum packed and non-vacuum packed meat after 7 day of storage of pheasants meat. The vacuum packed breast meat showed a significantly higher ($p \leq 0.001$) concentrations of phosphates compared with the meat packed in normal atmosphere on day 14 of storage.

Similar, significantly higher ($p \leq 0.01$) concentrations of phosphates were recorded in vacuum packed thigh meat compared with non-vacuum packed meat on day 14 of storage.

Glycolytic pathway plays a key role in skeletal muscle energy metabolism by converting glucose to pyruvate to generate ATP (22). ATP is the essential metabolite for *rigor mortis.* The *rigor* starts when the ATP level decreased less than 85% of the *in vivo* level. The temporal pattern of the post mortem ATP concentration depends on the glycogen and phosphocreatine reserve at the onset of death, as well as the period of anoxia (23).

Inadequate glycogen content in muscle at the moment of death results in little production of lactic acid; therefore meat has a high pH value. The pH value of muscle at 24 h after death is used to determine meat quality and take further processing decisions. Scheffler et al., 2011 (24) reported another explanation for the increased pH decline, instead of lactic acid production, namely the free protons and heat, originating from the ATP hydrolysis.

The rate of post-slaughter degradation of glycogen depends on the *post-mortem* muscle metabolism, is affected by *post-mortem* technologies, such as chilling conditions, which can affect directly the activity of the enzymes that regulate glycogenolysis (25, 26).

During storage, ripening of the pheasant meat occurs, progressively increasing tenderness and developing taste through the proteolytic activity of meat enzymes. Ripening process depends on temperature and can be accelerated by increasing it. However, for hygienic reasons it is recommended, that temperature of 4°C should be

used with a relative humidity of 85–95 %. Like the ageing of meat during cold storage at 4°C, other complementary treatments, maintaining quality of game meat and reducing the risk of microbial spoilage are used.

The reason for the preference of vacuum – packing method is the fact that it can remarkably extend the storage time. In this way stored meat and meat products can be safe and keep quality. For this reason special airtight synthetic films have been developed which can be heat-sealed after removing the air around the packed meat, thus keeping it practically out of contact with the surrounding atmosphere.

The shelf-life of vacuum packed pheasant meat and at the same time stored under 4°C can be remarkably influenced.

Conclusion

In our study we compared the effects of two different methods of treating and storing of feathered game after hunting. These methods impact on the course of physico – chemical changes during ripening process of pheasant meat. It can be concluded that storing pheasant meat by chilling and vacuum packing is the most advantageous combination of the methods that preserves the high hygiene quality of the game meat for a longer period of time. This storage method of pheasant meat enables the final product to become both successful in the wild game meat market and attractive to the consumers.

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UČINKI DVEH NAČINOV SKLADIŠČENJA NA FIZIKALNO-KEMIJSKE SPREMEMBE V MESU FAZANOV

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Povzetek: V raziskavi so bili proučeni učinki dveh načinov shranjevanja fazanjega mesa, in sicer hlajenega v hladilniku ter vakuumsko pakiranega, na fizikalno-kemične spremembe. Koncentracija mlečne kisline in fosfatov ter pH vrednosti prsnih in stegenskih mišic so bile izmerjene pri ohlajenih (n = 10) in vakuumsko pakiranih fazanih (n = 10) 1., 7. in 14. dan po začetku shranjevanja. Sedmi dan so se koncentracije mlečne kisline v stegnu znatno povišale (1,63 ± 0,41 g / 100 g; p ≤ 0,05). Štirinajsti dan so se koncentracije mlečne kisline v nevakuumsko pakiranih stegnih občutno znižale (0,73 ± 0,56 g/100 g; p ≤ 0,05) v primerjavi s 7. dnevom. Značilno višja (p ≤ 0,01) koncentracija mlečne kisline je bila ugotovljena 14. dan v nevakuumsko pakiranih mišicah stegna. Pri prsih je bila značilna razlika (p ≤ 0,01) v koncentraciji mlečne kisline med vakuumsko in nevakuumsko pakiranim mesom opažena 14. dan (1,88 ± 0,18 ter 1,15 ± 0.67 g/100 g). pH vrednost v nevakuumsko pakiranih stegnih je bila 7. dan 6,49 ± 0,15, pri vakuumsko pakiranih pa 14. dan 6,42 ± 0,13 g/100 g). Občutno višje koncentracije fosfatov so bile v stegnih izmerjene 7. dan pri vakuumsko (0,90 ± 0,17; p ≤ 0,05) in nevakuumsko pakiranem mesu (0,91 ± 0,10; p ≤ 0,05) v primerjavi s 1. dnem. Značilno znižanje (0,68 ± 0,18; p ≤ 0,01) fosfatov je bilo opaženo pri nevakuumsko pakiranih stegnih 14. dan. Pri vakuumsko pakiranih stegnih je bila bistveno povišana raven fosfatov (0,89 ± 0,15; p ≤ 0,05) 14. dan v primerjavi s prvim dnem. Rezultati kažejo, da je najprimernejši način shranjevanja fazanjega mesa kombinacija hlajenja in vakuumskega pakiranja.

Ključne besede: fazan; meso; mlečna kislina; pH; vakuum

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Original Scientific Article

USE OF ETHNOVETERINARY MEDICINES BY THE PEOPLE LIVING NEAR PAK-AFGHAN BORDER REGION

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Summary: Ethnoveterinary practices have recently gained importance due to their strong efficacy and fewer side effects on animals system as compared to conventional drugs. The present study was designed to document indigenous knowledge on ethnoveterinary medicines in an unexplored remote Hangu region of Pakistan situated near Pak-Afghan border. Interviews were conducted using semi-structured questionnaires. Data analysis was done using percentage statistics and descriptive statistical indices. Hangu region of Pakistan comprises 24 ethnoveterinary plants belonging to 19 families. Solanaceae, Rhamnaceae, Alliaceae and Euphorbiaceae were found to be the most widely used plant families (2 plant species each) in the studied region. Leaves (13 plant species) were found to be the most frequent plant part used in ethnoveterinary recipes. Total 19 plant species were found to be used against different ailments of cows followed by 12 plant species against buffaloe ailments. Most of plant remedies (9 plant species) were prepared in the form of decoction. The majority of the recipes (71 %) were given to the livestock orally, while 21 % in a topical manner. Gastrointestinal and wound infections were found most frequently in domestic animals and the total of 5 plant species were used against them. Informant consensus results also showed high degree of consensus for gastrointestinal (0.93) and wound healing (0.95) potential of plants. *Withania somnifera* ranked first with FL value (100 %), *Anagallis arvensis* ranked second with FL value (93 %) and *Euphorbia heliscopia* ranked third with FL value (92 %). DMR results showed that *Dalbergia sisso* ranked first, *Morus nigra* and *Melia azedarach* ranked second and *Zizyphus nummularia* ranked third. The present results also showed that these medicinal plants were more often/frequently exploited for medicinal, fuelwood and agricultural purposes. Plants with high Fic and FL value should be subjected to further *in-vitro* phytochemical and pharmacological investigation and protection should be given to multipurpose plant species by providing modern fuel resources and placing restriction on overgrazing.

Key words: traditional practices; livestock ailments; medicinal plants; Pakistan

Introduction

Ethnoveterinary medicines are an important part of the traditional knowledge system of the rural people all over the world. These practices are very useful for keeping the livestock healthy and productive and to treat different ailments of livestock (1). Interests in ethnoveterinary practices have developed recently due to their fewer side effects on the animal system as compared to the western pharmaceuticaly prepared drugs. According to the World health organization total 80 % people of developing world relies on traditional practices for the control of diseases affecting both animals and human (2). The reason of using alternative and complementary medicines is due to no access to veterinary services and high cost of modern veterinary drugs (3).

In developing countries like Pakistan farmers of rural areas mostly have inadequate access to the modern veterinary services and drugs either due to their unavailability or high expenses (4). Majority of the Pakistani farmers are poor and own 5-6 animals per family (5). The farmers of Pakistan

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are greatly dependent on livestock for agricultural purposes and for improving their livelihood. Rural population of Pakistan, especially people of northern areas, have tremendous ethnoveterinary knowledge as majority of medicinal plants are confined to the northern areas due to the presence of Himalayas, Karakoram, Sulaiman, and Hindu Kush mountain ranges (6) that lie in association with Pak-Afghan border. Pakistan and Afghanistan share their boundary of almost 2,500 kilometers called Durand Line separated in 1893 due to synchronization between the Afghan king and British Empire (7). This bordered line demarcates Pashtun ethnic group in the Pak-Afghan border areas. Majority of the population of Afghanistan belongs to Pashtun culture and also have considerable population in Pakistan (8). The majority of the population of northwest region of Pakistan living near border region is rural in nature and relies on their livestock for agricultural purposes and for the future generation.

Tremendous work has been done worldwide on ethnoveterinary practices but very few studies have been conducted in Pakistan so far. The present study was therefore designed to document ethnoveterinary knowledge of an unexplored rural area of Pakistan lying near the proximity of Durand line where locals and farmers are heavily dependent on their livestock and have sound indigenous knowledge regarding their treatment. The present study was designed with the aims i) to identify ethnoveterinary plants of the region, ii) to document ethnoveterinary practices in the region, iii) to provide information on candidate medicinal plants for further *in-vitro* phytochemical and pharmacological investigation, iv) to identify multipurpose ethnoveterinary plants and threats to their extinction and v) to conserve ethnoveterinary knowledge of investigated region before its extinction.

Material and methods

Study area

The present study was carried out in Hangu district located in province Khyber Pakhtunkhwa, Pakistan, near the border region with Afghanistan (Figure 1). Hangu district is located at 33.53 North latitude, 71.06 East longitude, and 858 m above the sea level. Hangu region comprises total area of 1,097 km^2 with total population of 314,529 (9). Hangu region is the area with warm summer season with the mean highest temperature 8.8 °C and the mean lowest temperature 7 °C in December and January (10). Livestock raring and agriculture are the common practices in the region and the major crops are wheat and maize. Due to low literacy rate and financial status local people are heavily dependent on medicinal plants for the treatment of themselves and their livestock as well.

Data collection

Data collection was carried out from March to September 2014. Prior to data collection a detailed meeting was held with the local administrator officers and representatives of the communities in order to tell them about the main theme of the study and to get their consent for data collection and publication. Total 50 informants were selected on the basis of information provided by representatives of the communities. Selected informants were natives of the regions mostly farmers and some were migrants (Afghan refugees). Semi-structured questionnaires were designed for data collection. Informants were interviewed individually in their local language (Pashto). Informants were asked about the type of animals they rear, number of plants they use to treat their livestock, types of livestock ailments they treat, plant parts used, recipe formulation, vehicles used and mode of administration of ethnoveterinary recipes.

Data quality assurance

During data collection each respondent was visited or contacted at least three times for the validity of information provided by them. In case of any deviation of respondent idea from the original information provided, it was rejected and considered irrelevant. Only relevant information was subjected to further analysis process. Further data quality was ensured through proper training of data collectors, pointing out missing information, duplication of material and careful analysis.

Plant collection and preservation

Field visits were made with local informants for identification and collection of documented plants by the informants. Collected medicinal

plants were brought to the laboratory of Kohat University of Science and Technology (KUST), Kohat, Pakistan for further processing. Plant identification was done by the expert taxonomists of Botany Department of KUST. Dried plants were pressed on herbarium sheets and deposited at the Herbarium of Department of Botany KUST, Kohat, Pakistan.

Data organization

All the collected data from informants were organized using Microsoft Word 2007 and Microsoft Excel 2007. All the plants were organized according to their respective families. Growth form of plants were divided into three categories i.e. herbs, shrubs and trees. Plant parts were divided into different categories i.e. leaves, fruits, seeds, stems, roots and whole plants. Livestock ailments were categorized into 9 major disease categories i.e. gastrointestinal, dermatological, antipyretic, wound healing, respiratory, reproductive problems, mastitis, parasitic and rheumatism. Ethnoveterinary recipes were classified into different classes like decoction, powder, paste, infusion, juice, concoction, grinding and extract. Routes of administration of recipes were divided into three categories i.e. oral, topical and both oral and topical.

Data Analysis

Informant consensus (Fic)

Fic is used to recognize widely used medicinal plants for the treatment of species ailments. Prior applying Fic all the animal ailments were classified into 9 major disease categories. Fic value is always high when one or a few plant species are documented by a large number of informants for a specific ailments, while low Fic value means that informants do not agree upon which plant to use. Fic values help to identify plants for further phytochemical and pharmacological investigation (11). Formula used for Fic calculation is as follows:

$$Fic = nur - nt / nur - 1$$

Fic = Informants consensus factor
nur = number of used citation in each category
nt = number of species used

Fidelity level (FL)

FL is helpful for identification of ideal plants use against specific ailment by the informants. Highly favored plants always score high FL values in comparison with those that are less preferred (12).

Formula used to calculate FL value is as follow:

$$FL = Ip / Iu \times 100$$

FL = Fidelity level
Ip = number of respondents who reported the utilization of a medicinal plants for a specific main ailment
Iu = total number of respondents who mentioned the same plant for any ailment

It is understood that plant with high FL value are more likely to be biologically active than those having less FL value (13).

Direct matrix ranking (DMR)

Data on the use of diversity of multipurpose medicinal plants were gathered using DMR practice (14). Total 15 key informants were selected on the basis of their strong traditional knowledge regarding medicinal plants (15). Informants selected for DMR were asked to give values (5 = best, 4 = very good, 3 = good, 2 = less used, 1 = least used, and 0 = not used) to each species for its usage as fodder, fuel or construction timber, in agricultural purposes, or for medicinal recipes. The values (average scores) given to each medicinal plant were summed up and ranked.

Results

In Hangu region of Pakistan the total of 24 plant species belonging to 19 families were used to treat different livestock ailments (Table 1). Solanaceae, Rhamnaceae and Euphorbiaceae were found to be most widely used plant families (2 plants each) in the studied region. Local inhabitants of the region mostly used herbs (50 %) followed by shrubs and trees (25 % each) for the preparation of ethnoveterinary medicines (Table 1). Different plant parts were used for treatment of livestock, however leaves (13 plants species) were found to be the most frequently used plant part followed by whole plant and seeds (6 plants each) (Figure 2). Local people used these ethnomedicines to treat

Figure 1:
Map of the study area

different types of domestic animals such as cows, buffaloes, goats and sheep. Total 19 plant species were found to be used against different ailments of cows followed by 12 plant species against buffalo diseases (Figure 3). Most plant remedies (9 plant species) were prepared in the form of decoction followed by powder (6 plant species) (Figure 4). Traditional people used different types of vehicles in ethnoveterinary medicines, like salt, sugar, milk, water, vegetable oil, etc (Table 1). The majority of the recipes (71 %) were given to the livestock in oral manner while (21 %) in topical manner. Different types of livestock ailments, categorized into 9 major categories, were treated in the region. Gastrointestinal and wound infections were found as the most common in domestic animals and 5 plant species were used against them. Informant consensus results also showed high degree of consensus for gastrointestinal (0.93) and wound healing (0.95) potential of plants (Table 2). Plant species used against dermatological infections, respiratory infections and as antipyretics also scored higher citation (0.97, 0.96 and 0.93 respectively). The present study revealed 9 plant species with highest FL value (Table 3). *Withania somnifera* ranked first with FL value (100%), *Anagallis arvensis* ranked second with FL value (93 %), *Euphorbia heliscopia* ranked third with FL value (92 %) and *Aloe barbadensis* ranked fourth (89 %). DMR exercised on six medicinal plants showed which medicinal plants are more threatened in the study area. According to the results *Dalbergia sisso* ranked first, *Morus nigra* and *Melia azedarach* ranked second and *Zizyphus*

Table 1: Ethnoveterinary medicines used in Hangu region, Pakistan

Families	Plants	Local names	Habit	Part used	Animal	Medicinal use	Recipe	Vehicles	Route
Alliaceae	*Allium cepa* L.	Pyaaz	Herb	Leaves	Buffaloes, cows	External parasites	Poultice		Topical
Amaryllidaceae	*Allium sativum* L.	Ugga	Herb	Leaves	Cows, buffaloes, goats	Mastitis	Crushed	Butter	Oral
Apiaceae	*Foeniculum vulgare* Mill.	Soonphf	Herb	Seeds	Cows, buffaloes, goats	Mastitis	Powder	Vegetable oil	Oral
Asclepiadaceae	*Caralluma tuberculata* N.E.Br.	Pawany	Shrub	Whole plant	Sheep, goats, cows	Removal of placenta	Decoction	Honey	Oral
Arecaceae	*Nannorrhops ritchiana* (Griff.) Aitch.	Mazzari	Shrub	Leaves	Goats, sheep, cows, buffaloes	Gastrointestinal	Decoction	Sugar	Oral
Asteraceae	*Carthamus oxyacantha* M. Bieb.	Speena zgaai	Herb	Seeds oil	Sheep	Wound healing	Powder	Flour	Oral
Cannabaceae	*Cannabis sativa* L.	Bhaang	Herb	Leaves, seeds	Cows	Mastitis	Decoction	Sugar	Oral
Convolvulaceae	*Cuscuta reflexa* Roxb.	Chum bud	Herb	Stem and seeds	Buffaloes and cow	External parasites	Paste		Topical
Euphorbiaceae	*Euphorbia helioscopia* L.	Katta saarai	Herb	Leaves	Buffaloes, cows, sheep	Febrifuge	Concoction	Water	Oral
	Ricinus communis L.	Raanda	Shrub	Seeds, leaves	Cows, buffaloes	Gastrointestinal	Extract	Salt	Oral
Fabaceae	*Dalbergia sisso* Roxb. ex DC.	Shawa	Tree	Whole plant	Cows, goats	Removal of placenta	Crushed	Milk	Oral
Lamiaceae	*Mentha arvensis* L.	Podeena.	Herb	Leaves	Cows and buffaloes	External parasites	Decoction	Sugar	Oral
	Vitex negundo L.	Marmandi	Shrub	Seeds, leaves	Cows, goats, sheep	Wounds, Rheumatism	Infusion, Paste		Topical
Meliaceae	*Melia azedarach* L.	Tora Draka	Tree	Leaves, fruit	Goats, sheep	External parasites, gastrointestinal, fever	Decoction, Juice	Sugar	Oral, Topical
Moraceae	*Ficus carica* L.	Inzeer	Tree	Fruit	Cows	Skin infection	Juice		Topical
	Morus nigra L.	Tor Toot	Tree	Fruit	Cows, buffaloes	Skin infections	Juice	Water	Oral
Myrtaceae	*Eucalyptus lanceolatus* Dehnh.	Lachi	Tree	Whole plant	Goats	Delivery	Decoction	Sugar	Oral
Papaveraceae	*Fumaria indica* Pugsley	Khatee	Herb	Whole plant	Cows, sheep, goats	Antipyretic, wound healing	Powder, decoction, paste	Vegetable oil	Oral, Topical
Primulaceae	*Anagallis arvensis* L.	Dhabbar	Herb	Whole plant	Goats	Chronic cough	Powder	Water	Oral
Rhamnaceae	*Ziziphus mauritiana* Lam.	Bera	Tree	Fruit, leaves	Goats, cows	Fever, wound healing, chronic cough	Powder, crushed	Milk	Oral
	Ziziphus nummularia Aubrév.	Karkata	Shrub	Leaves	Cows, buffaloes, goats	Wounds	Paste		Topical
Solanaceae	*Solanum incanum* L.	Tarkha Mowtngee	Shrub	Roots, leaves	Cows	Mastitis	Powder	Salt	Oral
	Withania somnifera (L.) Dunal	Kapyanga	Herb	Whole plant	Cows, Buffaloes, goats, sheep	Gastrointestinal	Decoction	Honey	Oral
Xanthorrhoeaceae	*Aloe barbadensis* Mill.	Zarpati	Herb	Leaves	Buffaloes	Gastrointestinal	Decoction	Salt	Oral

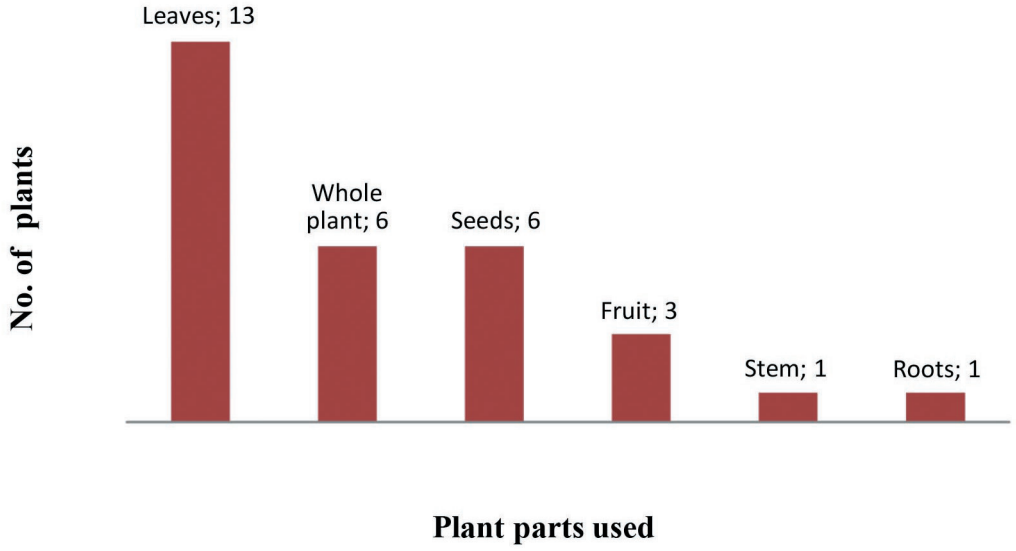

Figure 2: No. of plants part used to treat different livestock ailments

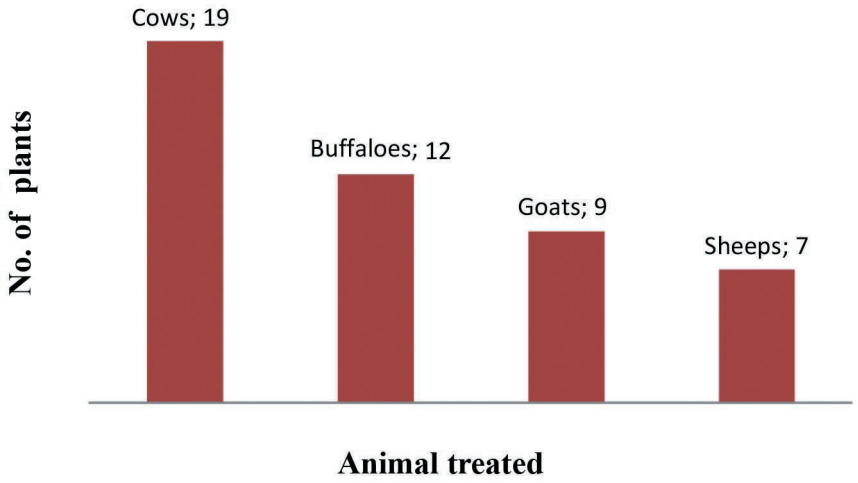

Figure 3: No. of plants species used to treat different types of animal

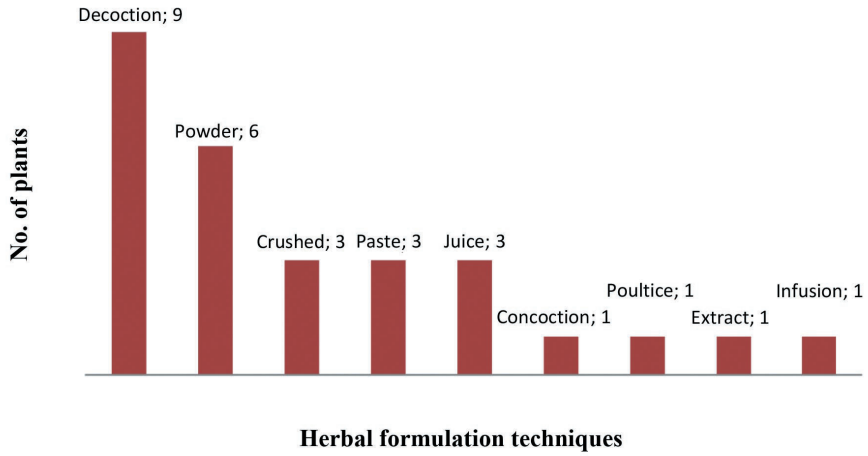

Figure 4: No. of plants species used to prepared different recipes

Table 2: Informant consensus

Disease category	Nur	Nt	Fic
Gastrointestinal	60	05	0.93
Dermatological	42	02	0.97
Respiratory	31	02	0.96
Reproductive problems	18	03	0.88
Parasitic	12	04	0.77
Wound healing	97	05	0.95
Rheumatism	16	01	1.00
Antipyretic	47	04	0.93
Mastitis	09	04	0.62

Table 3: Fidelity level

Number	Plant species	Disease category	Ip	Iu	FL %
01	*Aloe barbadensis*	Gastrointestinal	17	19	89
02	*Caralluma tuberculata*	Reproductive problems	11	14	78
03	*Euphorbia helioscopia*	Antipyretic	24	26	92
04	*Morus nigra* L.	Dermatological	19	25	76
05	*Anagallis arvensis*	Respiratory	14	15	93
06	*Withania somnifera*	Gastrointestinal	23	23	100
07	*Ficus carica*	Dermatological	13	17	76
08	*Carthamus oxycantha*	Antipyretic	20	23	86
09	*Nannorrhops ritchiana*	Gastrointestinal	11	15	73

Figure 4: No. of plants species used to prepared different recipes

Use diversity	*M. nigra*	*D. sisso*	*Z. nummularia*	*Z. mauritiana*	*M. azedarach*	*S. incanum*	Total	Rank
Fodder	4	4	2	2	4	4	20	4
Fuel	5	5	4	3	4	3	24	2
Construction	3	5	3	3	3	2	19	5
Agriculture	4	4	4	4	4	2	22	3
Medicinal	4	5	4	4	5	5	27	1
Total	20	23	17	16	20	16		
Rank	2	1	3	4	2	4		

nummularia ranked third (Table 4). The present results also showed that these medicinal plants were more often exploited for medicinal, fuelwood and agricultural purposes (Table 4).

Discussion

Medicinal plants and their growth form

The present study revealed that locals of the region use 24 medicinal plants for the treatment of livestock ailments. Similar results have also been documented from the other regions of Pakistan (5, 16). Investigate region has rich diversity of medicinal plants (9) and provide conducive habitat for the growth of these medicinal plants as shown by the occurrence of 24 plant species used against livestock treatments. The present area is rural in nature and inhabitants of the region are very much dependent on plant resources for the treatment of their own health as well as their livestock due to their low financial status and literacy rate in the region (9). People of the region mostly used herbs for the preparation of ethnoveterinary medicines. The possible reason behind using higher proportion of herbs might be associated with their ease of finding, their efficacy and easy harvesting. The present findings are in line with the studies conducted in different parts of the world where there is more utilization of herbs for recipes formulation (17, 18).

Plant families used against livestock ailments

Solanaceae, Rhamnaceae and Euphorbiaceae families are highly utilized plant families in the studied region. The highest use of the plants of these families might be due to their higher abundance in the study area or it might be associated with their high bioactivity. The present findings are in contradiction with other studies conducted in different parts of the world (15, 19) which found the use of Asteraceae and Fabaceae families as the highest. These differences might be related to different dominant vegetations of the areas and traditional beliefs of different cultures to utilize specific plants for livestock ailments.

Plant parts used

The local people use different plant parts for ethnoveterinary recipes like leaves, seeds, fruits, stems, roots and whole plants. In comparison the other parts of the plant, leaves are the most frequently used plant part in the Hangu region due to their easy harvesting. Leaves are not only the preferred part in studied region but the majority of the ethnoveterinary and ethnomedicinal studies have proved that leaves are widely used plant parts (4, 20, 21). Leaves are the main sites of photosynthesis and other physiological process that results in the production of different types of secondary metabolites. Wider utilization and high efficacy of leaves might be due to the presence of great accumulation of these secondary metabolites in the leaves as compared to other plant parts. The second most widely used form in the studied region is the whole plant. Collecting whole plants is not a sustainable type of harvesting as compared to leaves. Leaves harvesting does not pose any great damage to the plant life cycle as compared to the whole plant which results in the rapid decline in the population of these species. The present results are in the contradiction with studies conducted elsewhere where roots are widely used plant part for ethnoveterinary medicines (22, 23).

Animals treated in the region

Due to low literacy rate and nature of the region the people are greatly dependent upon livestock for agricultural purposes and for improving their livelihood. Mostly the inhabitants rear cows, buffaloes, goats and sheep. Majority of the plants are used to treat cow's infections followed by buffaloes and goats. Local people do not rear dogs, horses, donkeys or camels which might be due to the fact that these animals do not produce any valuable edible products, while cows, buffaloes, goats and sheep are involved in dairy and meat production which is a part of the monthly income. Similar results have also been conducted by Merwe et al. (24) and Benitez et al. (17).

Types of ailments treated in the region

It was found during research investigation that gastrointestinal infections are more frequent in the studied region. It has also been found that

gastrointestinal infections are more common in lactating animals due to the poor quality of fodder and drinking sources (18). The same number of plant species is also used for wound healing purposes of livestock in the studied area. The reason behind using high number of plant species for wound healing might be associated with the fact that animals usually get injured during food competition or might be due to different parasitic infections. After wound healing and gastrointestinal infections most of the plants are used against fever and mastitis that leads toward increasing the quality of milk production and improving their monthly income. Informant consensus results also showed highest informant citation for gastrointestinal, wound healing, antipyretic, dermatological problems etc. These results give an indication about the bioactivity of medicinal plants used to treat these ailments. According to Heinrich et al. (25), high Fic values are very useful in the selection of specific plants for further search of bioactive compounds. Rheumatism scored 1.00 Fic value because only single species (*Vitex negundo)* was found to be used against arthritis. This indicates that species should be subjected to further in-vitro screening that could lead toward the extraction of some novel compounds against rheumatic problems. Extensively used medicinal plants for specific ailments always score highest fidelity level. Present study determined different plants like *Withania somnifera, Anagallis arvensis*, *Euphorbia helioscopia*, *Aloe barbadensis* etc scored highest fidelity value and could be further search for their *in-vitro* investigation and efficacy.

Ethnoveterinary medicines preparation

Ethnoveterinary medicines formulation techniques vary from individual to individual because same plant can be prepared in different manner by different traditional veterinary healers. In the studied region the most common type of formulation technique is decoction and powdering of plants. It has already been found that powdering and decoction are the most common methods of drug extraction (4). Present results are in line with study conducted in the Malakand valley of Pakistan (5) while contradictory with the study conducted in other parts of the world (2, 26). The majority of the ethnoveterinary recipes in the studied region are prepared by using single species (*Euphorbia helioscopia)*. The recipes were found to be prepared in concoction form and it is generally believed that potency of the drugs can be enhanced when used in concoction form (27). The most preferred route of administration is orally while some of the recipes were applied topically. Oral mode of administration is due to that most of the ailments in the region are internal. These ethnoveterinary medicines are given to the livestock along with different types of vehicles like sugar, salt, milk, honey, water, vegetable oil etc. Similar findings are also reported from the other regions of the world (15, 28). It has already been reported that the use of vehicles is necessary in order to reduce the adstringent effect of herbal formulation and to avoid vomiting. It was noted that there was no uniformity for the dose of ethnoveterinary recipe that might be due to that dose might be increased or decreased depending on the disease severity. Informants reported that the recovery of animals is usually estimated when animals restart their proper feeding and daily activities normally. Similar findings are also reported by other ethnoveterinary studies conducted elsewhere (5, 19).

Multipurpose ethnoveterinary plants and threats to their extinction

DMR results enabled us to recognize highly used medicinal plants in the study area and threats to their extinction. According to the present results *Dalbergia sisso* ranked first, *Morus nigra*, *Melia azedarach* ranked second in the study area. These highly utilized species are trees therefore more exploited in the region for variety of the purposes. After medicinal purposes these species are more harvested for fuel wood, fodder, agriculture and construction purposes. It has already been stated that wood of *Dalbergia sisso* is highly preferred for fuelwood and timber purposes (29). The locals selected for DMR also showed that most of the people of the region are also engaged in exporting timber of *Dalbergia sisso* to the industries located in other regions of Pakistan for generating their income apart from their domestic use as furniture and fuel wood because eighty percent of industrial furniture in Pakistan is being made from *Dalbergia sisso* (30). Other species like *Morus nigra*, *Melia azedarach*, *Zizyphus nummularia* etc are also highly exploited for their fuelwood for different purposes. The high use of fuelwood in the study area is associated with the deficiency of

modern fuel sources in the region. Our findings are also in line with the study by Barkat et al. (31) carried out in district Malakand. He found that in the absence of gas supply and other fuel types in the area, the local people extensively use tree species as fuelwood. Agriculture and livestock raring in the study area are common activities also to support rural livelihood. Therefore grazing is posing another pressure on the flora of the region. The trampling of livestock makes the soil compact resulting in reducing seed germination chances (32). Therefore, there is a dire need to take necessary steps for the conservation of these highly utilized ethnoveterinary medicinal plants before their extinction.

Conclusions

Local farmers and Afghan migrants of the studied region utilize different medicinal plants for the treatment of livestock due to their low income status and high expenses of western drugs. Traditional healers possess tremendous expertise in preparing herbal formulations of medicinal plants. Gastrointestinal and wound infections were most common in the studied region and these disease categories also scored high informant citation. Therefore attention should be given on those plants that are being used against these infection couple with other plants having FL value for further *in-vitro* investigation for their phytochemical analysis and pharmacological activities. Good quality fodder and pure drinking water should be provided to the livestock for decreasing gastrointestinal infections. Multipurpose species especially *Dalbergia sisso* should be given focus from conservation point of view. Modern fuel facilities and control grazing should be promoted in the region in order to conserve these valuable ethnoveterinary plants.

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UPORABA ETNOVETERINARSKIH ZDRAVIL PRI LJUDJEH, ŽIVEČIH V PAKISTANSKO-AFGANISTANSKEM OBMEJNEM OBMOČJU

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Povzetek: Etnoveterinarske prakse so pred kratkim postale pomembnejše zaradi svoje možne učinkovitosti in manjšega števila stranskih učinkov pri živalih v primerjavi s konvencionalnimi zdravili. Študija je bila zasnovana na podlagi dokumentiranja domačega znanja o etnoveterinarskih zdravilih v neraziskani oddaljeni regiji Hangu v Pakistanu, ki leži v bližini pakistansko-afganistanske meje. Opravljeni so bili razgovori s pomočjo polstrukturiranih vprašalnikov. Analiza podatkov je bila opravljena s pomočjo statistike odstotkov in opisom statističnih indeksov. V območju Hangu v Pakistanu uporabljajo 24 etnoveterinarskih rastlin, ki pripadajo 19 družinam. Družine *Solanaceae*, *Rhamnaceae*, *Alliaceae* in *Euphorbiaceae* so najpogosteje uporabljene v obravnavanem območju, saj se uporabljata po dve rastlini iz vsake od naštetih družin. Najpogosteje uporabljani deli rastlin so listi, in sicer od 13 ratlinskih vrst. Največ, 19 rastlinskih vrst, se uporablja pri različnih boleznih goved. Večina rastlinskih sredstev (9 rastlinskih vrst) je bila pripravljena v obliki izvlečka. Kar 71 % jih dajejo živalim oralno, 21 % pa topikalno. Okužbe prebavil in ran so bile najpogostejše težave pri domačih živalih. Skupno 5 rastlinskih vrst je bilo uporabljenih za njihovo zdravljenje. Neformalni rezultati so pokazali visoko stopnjo dopustnega potenciala rastlin za prebavila (0,93) in celjenje ran (0.95). *Withania somnifera* je bila na prvem mestu po vrednosti FL (100 %), *Anagallis* arvensis na drugem mestu z vrednostjo FL (93 %), *Euphorbia heliscopia* pa na tretjem z vrednostjo FL (92 %). Rezultati DMR so pokazali, da se je *Dalbergia sisso* uvrstila na prvo mesto, *Morus nigra* in *Melia azedarach* na drugo, *Zizyphus nummularia* pa na tretje mesto. Dosedanji rezultati so tudi pokazali, da so bile omenjene zdravilne rastline pogosteje uporabljene za zdravila, kurivo in v druge kmetijske namene. Za rastline z visoko vrednostjo FIC in FL bi bilo dobro, da bi jih vključili v nadaljnje fitokemijske in farmakološke raziskave. Te večnamenske potencialno pomembne rastlinske vrste bi bilo potrebno tudi zaščiti z zagotavljanjem sodobnega vira kuriv in omejitvijo pretirane paše.

Ključne besede: tradicionalne prakse; zdravstvene težave živine; zdravilne rastline; Pakistan

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Original Research Article

MICROBIOLOGICAL MONITORING OF MASTITIS PATHOGENS IN THE CONTROL OF UDDER HEALTH IN DAIRY COWS

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Summary: The importance of systematic mastitis control in dairy herds is described through the presentation of data concerning mastitis occurrence and significance in modern milk production. Research was conducted during farm visits and by taking udder quarter samples from all lactating cows at the time of visit. Samples were taken before evening milking. Each sample was tested by the Zagreb mastitis test (ZMT) and examined bacteriologically by inoculation on aesculin blood agar. Identification of grown colonies was carried out using internationally accepted methodology. The obtained results were statistically analysed using the Stata 13.1 statistical package. Udder quarter samples from 385 cows were analysed. ZMT-positive reactions were found in 13.7% of all quarters. Mastitis pathogens were isolated from 175 (13%) of quarter samples. One hundred and forty five of 385 cows (37.3%) included in the study had at least one ZMT positive quarter or permanently lost (dried off) quarter. Mastitis pathogens were isolated in 106 of 363 cows (29.8%) with all four functional quarters. The most frequently isolated pathogens were *Staphylococcus aureus*, *Streptococcus* spp., *Trueperella pyogenes* and *Corynebacterium bovis*. There was no statistical difference in mastitis occurrence between the front and rear mammary quarters. The ZMT results and microbiological examination were moderately correlated (Kappa index = 0.4662).

Key words: cow; mastitis; mastitis pathogens; Croatia

Introduction

The occurrence of mastitis in a dairy herd above the tolerable limit causes multiple losses. One is lower milk prices or even confiscation of milk. Another, often overlooked, is lower milk production. Taking into account the costs of mastitis such as treatment, withdrawal of milk from the market and additional labour, it can be concluded that mastitis is a greater threat to the cow owner than to the animal's health.

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Among the economic losses due to mastitis, the dominant is reduced milk production, at 60% of total losses, followed by additional labour (16%), confiscated milk (9%), higher replacement rate (7%), lower market value of milk (4%), drugs (3%) and veterinary costs (1%) (1). Mastitis is an inflammation of the mammary gland of varying aetiology (2). However, mastitis is most commonly caused by numerous bacterial species. Hence, milk from mastitic glands is a possible threat to human health since it contains harmful bacteria and their toxins. Mastitis is the result of continuous competition between the pathogen and host defence mechanisms. Many

environmental factors favour bacterial success for the invasion of the mammary gland, such as: inadequate milking procedures, vacuum level of milking machine outside the recommended values, improper hygiene of milking machines, inadequate hygiene level of stalls and animals, poor microclimate conditions in stalls, insufficient feeding, etc. (1,3,4).

Milking technique is one of the key factors which are able to initiate infection and subsequent inflammation of the mammary gland. Either too short or too long milking time can be an initial point for mammary gland infections (5,6). Improper udder hygiene, milking of dirty or inadequately sanitized udders can also lead to mastitis. Furthermore, wounds on the udder and/or teat skin and anatomical failures of teat ends and sphincter are predisposing factors for mastitis. Supernumerary teats and fistulae serve as atria for the entry of bacteria into the mammary gland (7).

No other infectious disease can compare to mastitis in terms of the number of possible causative agents. The literature has recorded 150 or 200 different microbial species isolated from mastitis cases, predominantly bacteria, but also fungi or even monocellular achlorophylic algae. Despite the multitude of therapeutic agents and preventive measures, mastitis is still a predominant cause of losses in dairy production (8,9,10).

Mastitis appears in two forms: (i) subclinical with no clinical symptoms and (ii) clinical with one or more clinical signs such as: redness, oedema, pain, elevated local temperature and organoleptic changes in the milk (11). Regarding clinical appearance, mastitis occurs with a wide spectrum of symptoms, from no visible signs to extremely difficult symptoms which may lead to death of the infected animal. The clinical approach differentiates several types of mastitis regarding the intensity of infection, such as latent infection, catarrhal inflammation, acute phlegmonous or parenchymatous and oozing mastitis (12,13). Infective mastitis can be contagious, caused by a few of bacterial species such as *S. aureus, Streptococcus agalactiae* and *Corynebacterium bovis,* or environmental, caused by bacteria from the environment such as other streptococci, coliform bacteria, enterococci, etc. (14).

Despite many diagnostic methods available today, routine mastitis diagnosis is still mostly based on microbiological laboratory examination of properly taken udder quarter samples (15). Among different methods designed for screening in field conditions, mastitis testing using a reagent is inexpensive and accurate, and is easy to perform and interpret (16). Udder quarters giving a positive reaction should be submitted for microbiological examination (17).

This study emphasizes the importance of mammary gland health control in a systematic manner, showing the occurrence of mastitis in dairy herds, and the frequencies of isolated mastitis pathogens. Furthermore, it provides statistical evidence for the influence of quarter position (front/rear) on mastitis occurrence. Finally, it provides statistical evidence on the accordance between microbiological examination and field tests carried out using the Zagreb mastitis reagent.

Materials and methods

Animals

A total of 385 dairy cows from 15 farms were included in the study. Farms were located in Zagreb County, Sisak-Moslavina County and Karlovac County. Herd size ranged from 10 to 70 cows. Cows belonged to Simmental breed, Holstein-Friesian breed and their crosses.

Cows were kept in closed stalls, in smaller herds or free in larger farms. At all visited farms, cows were milked twice daily. Larger farms with a free stall system had stationary milking parlours, while at smaller farms, milking was performed at standing places either using a milking machine or by hand. The floor was either bedded with straw or sawdust in smaller herds or covered with rubber material in larger farms with a free stall system. Udder preparation for milking was performed by washing with warm water and drying with clean paper towels or individual cloths.

Sampling

Samples for examination were taken before the evening milking. After washing and drying, teat ends were disinfected with cotton swabs soaked in 70% ethanol. The first few streams were discarded. Approximately 10 mL of milk from each udder quarter was taken into sterile tubes. Samples were

transported to the laboratory on ice and stored at 4 °C until laboratory examination which was performed within 12 hours from sampling.

Each sample was examined using the Zagreb mastitis test (ZMT, Croatian Veterinary Institute, Zagreb, Croatia). ZMT is a field test intended for the identification of cows and quarters with abnormal udder secretions. It contains alkyl-aryl sulphonate which destroys cell membranes and induces DNA polymerisation. Hence, the intensity of the reaction in the mixture of equal aliquots of milk and reagent, i.e. consistency of the mixture, serves as an approximation for somatic cell number in the milk sample. Depending on the number of cells, the visible change in the mixture varies from no reaction (up to 3×10^5 cells per mL) to the formation of a gel with the consistency of egg white. Reactions in the mixture of ZMT and tested milk samples in this study were graded according to the manufacturer's recommendations as mild (visible threads in mixture), moderate (visible gel formation, but still liquid mixture consistency), and strong (formation of a gel consistency resembling egg white). Microbiological examination (MBE) was carried out according to the method recommended by the NMC (1999). Primary isolation was carried out by inoculation of samples onto nutrient agar (Merck, Germany) with 5% ovine blood and 0.1% aesculin and incubation at 37 °C. Inoculated plates were checked at 24-hour intervals. Grown colonies were stained according to Gramm (Merck, Germany), checked for catalase and oxidase production and further subcultured onto differential or selective media. Presumptive staphylococci colonies were subcultured onto Baird-Parker agar. Coagulase production in grown colonies was verified using 0.5 mL rabbit plasma (Merck, Germany). CAMP test was carried out to identify *Streptococcus agalactiae* among the presumptive streptococci. Gram-negative bacteria were subcultured onto MacConkey agar and Triple sugar iron agar (Merck, Germany). Pathogens were finally identified by biochemical profiling using Micronaut identification systems (Merlin Diagnostika, Germany) for gram-positive bacteria and gram-negative fermentative bacteria.

Statistical analyses were performed using the Stata 13.1 statistical package (Stata Corp, USA). Observed differences among udder quarters were examined using the chi-square test. Accordance between different diagnostic tests was tested by the Kappa statistics test.

Results

Results of ZMT for single quarters

Testing using ZMT encompassed 1540 udder quarters, of which 84.53% samples included somatic cells below the detectable level for mastitis reagents (< 3×10^5/mL). Among the samples with observed positive reaction, most were assigned to the mildest gradation (marked + or 1), which corresponds to a number of somatic cells from 3–5 × 10^5/mL. In the clinical examination performed during sampling, it was established that 29 udder quarters (1.88%) were permanently dysfunctional or lost for further milk production (Table 1).

Table 1: Data obtained by the ZMT in dairy cows

Reaction in ZMT	Dairy cows	
	n	%
Negative (-)	1300	84.53
Mild (+)	103	6.70
Moderate (++)	89	5.79
Strong (+++)	16	1.04
Pus	3	0.20
Nonfunctional quarter	29	1.88
Total	1540	100

Results of the MBE

In the MBE, 86.88% of all examined quarters were negative, *i.e.* none of the causative agents of mastitis were isolated. Among the isolated causative agents, the most frequent was *Staphylococcus aureus* in 69 (4.48%) of examined samples (Figure 1). The frequency of members of genus *Streptococcus* isolated from 37 samples (2.39%) was lower than that of *S. aureus* (Figure 2). Among other causative agents, a rather significant number of the isolated species was *Trueperella pyogenes* (previously: *Arcanobacterium*) from 26 (1.69%), *Corynebacterium* spp. from 25 (1.62%) and *Streptococcus uberis* from 18 (1.17%) of samples, whereas other causative agents were isolated only sporadically (Table 2; Figure 3).

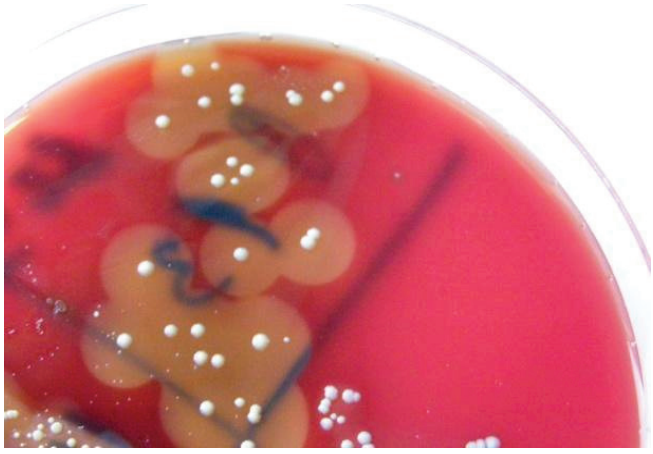

Figure 1: Colonies of *Staphylococcus aureus* on blood agar

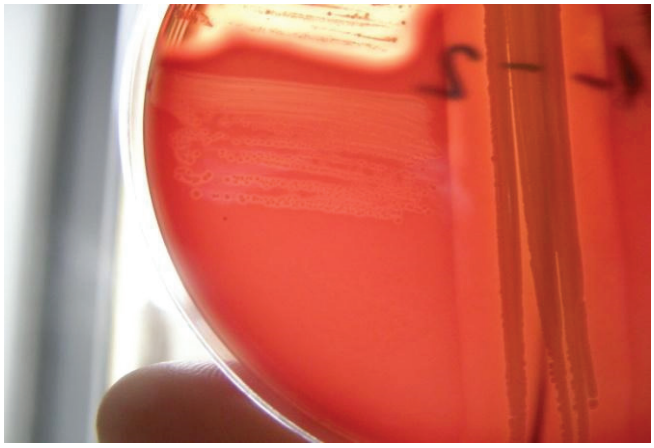

Figure 2: Colonies of *Streptococcus dysgalactiae* on blood agar

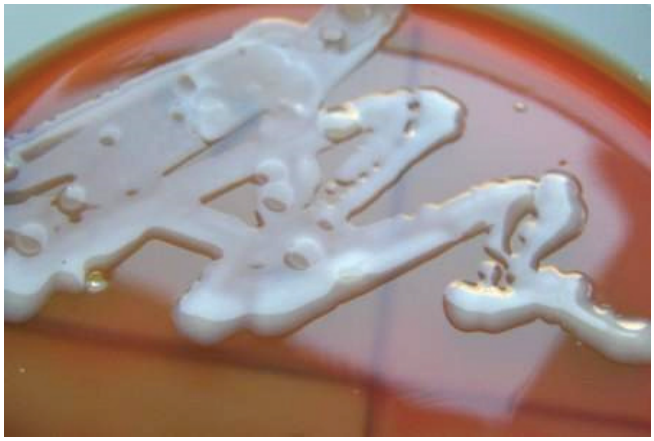

Figure 3: Colonies of *E. coli* on blood agar

Table 2: Microbes isolated by the MBE from dairy cows with mastitis

Isolated organisms by MBE	Dairy cows	
	n	%
Staphylococcus aureus	69	4.48
Trueperella pyogenes	26	1.69
Corynebacterium spp.	25	1.62
Streptococcus uberis	18	1.17
Streptococcus spp.	11	0.71
Enterococcus spp.	5	0.32
Streptococcus dysgalactiae	5	0.32
Staphylococcus spp.(CNS)	4	0.26
E. coli	3	0.19
Streptococcus agalactiae	3	0.19
Klebsiella spp.	1	0.06
Pasteurella spp.	1	0.06
Proteus sp.	1	0.06
Enterobacter sp.	1	0.06
Pseudomonas sp.	1	0.06
Yeasts	1	0.06
Non-functional quarter	27	1.75
No growth	1338	86.88

Results of examinations according to number of dairy cows

Of total 385 cows examined, no clinical changes were observed in 240 (62.34%) cows. Clinical changes in one quarter were recorded in 17.66% of examined cows, and in two quarters in about 8% of cows. In 26 cows (6.75%), quarters were found to have chronic damage, resulting in a total loss of function. Of the 363 cows with functional all udder quarters, 257 (70.8%) had no quarters infected. Among the microbiologically positive cows, the highest proportion had one infected quarter (61 cows or 16.8%) (Figure 4).

The proportion of quarters that showed no reaction to the ZMT, *i.e.* in which the number of cellular elements was below the level of detection by ZMT reagent, was between 82.6% and 86.16%, depending on the anatomical position of the quarter. The observed difference in frequency of a positive reaction between front and rear quarters was not statistically significant ($P>0.05$) (Table 3).

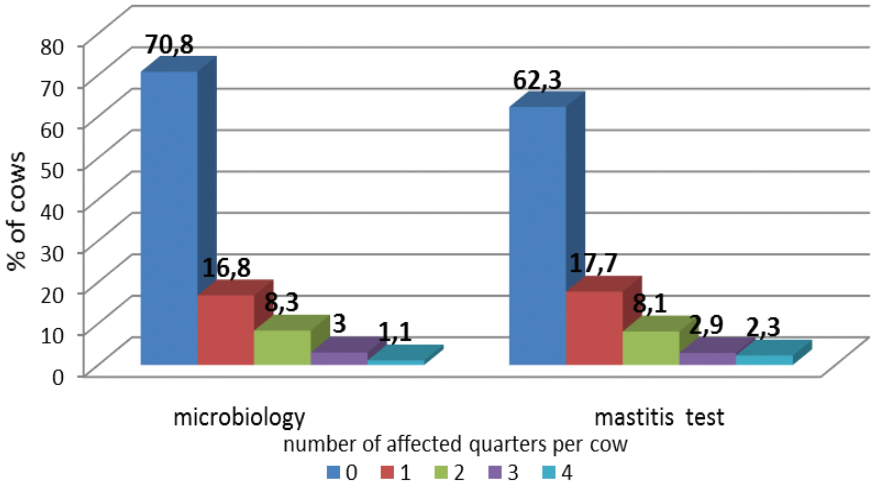

Figure 4: Comparison of the results obtained by MBE and ZMT per cow

Table 3: Intensity of reaction in ZMT per single quarters of dairy cows

Reaction in ZMT	I quarter		II quarter		III quarter		IV quarter		Total		*P*-value
	n	%	n	%	n	%	n	%	n	%	
Negative (-)	330	86.2	328	85.2	318	82.7	324	84.2	1300	84.4	0.32
Mild (+)	26	6.8	21	5.4	28	7.3	28	7.3	104	6.7	
Moderate (++)	16	4.2	20	5.2	32	8.3	21	5.4	89	5.7	
Strong (+++)	4	1.0	4	1.0	2	0.5	6	1.6	16	1.0	
Pus	1	0.3	1	0.3	-		1	0.3	3	0.2	
Nonfunctional quarter	8	1.6	11	2.9	5	1.3	5	1.3	27	1.8	

Table 4: Data obtained by MBE per quarter of dairy cows

Udder quarter	None of agents isolated		Isolated causative agent		*P*-value
	n	%	n	%	
I	337	88.9	42	11.1	0.58
II	337	90.1	37	9.9	
III	332	87.4	48	12.6	
IV	332	87.3	48	12.6	
Total	1338	88.4	175	11.5	

Table 5: Congruence between ZMT and MBE results

ZMT result	MBE result		Total
	Negative	Positive	
Negative	1228	72	1300
Positive	108	103	211
Kappa index	0.4661		

The proportion of udder quarters from which a causative agent of mastitis was isolated ranged from 9.9% (in the II quarter) to 12.6% (in III and IV quarters). The observed differences in the frequency of infections between different quarters were not statistically significant (Table 4). Accordance between the data obtained by ZMT and MBE was found to be moderate, with Kappa index of 0.466 (Table 5).

Discussion

Though modern milk production includes a spectre of preventive measures for the prophylaxis of mastitis, this disease is still the cause of the highest economic losses in dairy cattle breeding. There are many reasons why the causative agents of mastitis are successful in infecting the udder. Very few of infectious diseases are comparable to mastitis in terms of potential numbers of causative agents. According to the literature, more than 150 different microbial species have been indicated as causative agents of mastitis. These belong to a wide range of taxonomic categories. The most frequent are: bacteria, fungi and even monocellular aclorophylic algae [18,19].

Most other infections occur by the galactogenic route, *i.e.* by the entry of the causative agents into the udder through the teat canal. The teat canal is open during milking and stays partially open for a short time after milking. This period could be prolonged if the milking machine is not technically functional. The mucosae of the teat canal may even prolapse in cases when the vacuum pressure for milking is higher than recommended. Hence, mastitis is very frequently a consequence of the technical malfunction of milking machines. Furthermore, many cows share same the milking unit. When the disinfection procedure is not performed after the milking of each cow, the possibility of infection transmission increases even if a single cow in the milking order is infected. In other words, it is known that many causative agents are present in the milking environment and their transmission from infected to non-infected animal during milking is possible if farmers do not follow the standard hygienic procedures. Furthermore, cows in one phase of lactation produce a high milk yield, which demands increased feed intake and thus increased energy and entering into a negative energy balance (NEB). In such cases, cows use their own body reserves in order to produce milk yield in accordance to their genetic potential. During the period of NEB followed by increased metabolic demand, cows are more susceptible not only to mastitis, but also to other, particularly metabolic disorders such as ketosis (20,21,22).

In the present study, more than 15% of the examined udder quarters showed signs of disturbance in secretion in testing with ZMT. In comparison with a similar studies, the proportion of quarters with a positive reaction was extremely decreased. Namely, in the studies conducted in 1997 and 2004, the proportion of positive reactions was 34% and 28%, respectively (23). These studies were performed using similar methodology but in other parts of the country and at the time of enforcement of new legislation regarding the calculation of raw milk prices. Accordingly, it can be assumed that the decreased number of quarters with positive reaction to ZMT are the result of the implementation of preventive measures directed towards udder health and thus to the hygienic quality of milk.

By MBE, the causative agents of mastitis were isolated in 175 (13%) of tested samples. The most common was *S. aureus* (4.5%), as previously reported in Croatia and worldwide (24,25). However, the total number of infected quarters by this agent was less than half in comparison to the previous studies (11%). This finding supports the assumption of improved overall hygienic standards in primary milk production. Namely, at the farms visited, it was observed that the teat was routinely dipped into disinfectant following milking. This procedure was applied without exception at all farms with a milking parlour. It should be emphasized that teat dipping following milking is considered to be an effective measure to control and reduce contagious mastitis (26).

The second typical contagious agent, *Streptococcus agalactiae*, was isolated from less than 1% of tested samples. In all performed tests, the frequency of this agent was less than 3%. However, isolation of this agent, even in low numbers, indicates possible errors in mastitis prevention at dry-off. Namely, this agent survives almost exclusively in the udder, while it is very rapidly destroyed in the environment; therefore, this is one of the rare causative agents which could be totally eradicated in the herd. In thise regard, the use of antibiotics in dry-off with other preventive

measures is effective in dairy cattle herds (27).

More often than in previous studies, bacteria from the genus *Corynebacterium* and *Trueperella pyogenes* were isolated. *Corynebacterium bovis* is a mild pathogen, but it colonizes the teat canal and occasionally induces a small increase of somatic cell number and lowers milk yield. In this regard, infections caused by these bacteria are mild and more difficult to detect (15).

With respect to previous studies, more cases of *T. pyogenes* were recorded in the present study. This causative agent very often causes purulent inflammations, and in addition to mastitis, it also induces changes in other organs and tissues. It is related to summer mastitis, which is a more significant health problem in the northern Europe. It is assumed that flies play an important role in the spread of this pathogen from infected to non-infected animals. Since the present study was performed in late winter and early spring when flies are not active, and we have observed an increased frequency of this agent in comparison to previous studies, it would be advisable to enhance monitoring in dairy cow herds during spring and summer, in order to avoid the possible spread of this agent by the increased activities of flies (28).

Significant numbers of *Streptococcus uberis* were isolated. This pathogen most frequently infects cows from the environment, but with a higher frequency in the early dry-off period than during other phases of lactation. In this regard, in order to prevent mastitis caused by this agent, it is crucial to ensure a clean and dry environment, particularly in the dry-off period. The frequency of other causative agents was relatively low and sporadic and the obtained results were similar to data reported previously. Some of these agents may infect the udder relatively frequently with a lethal outcome, such as in the case of *E. coli* infection. The reason for low prevalence in such systematic studies is that infections are of a short duration. The chance for isolation of *E. coli* from an affected udder is greater when the sample for examination is taken immediately after the appearance of the first mastitis symptoms. It is assumed that live bacteria are no longer present in udder secretions after 5 to 6 hours from the first appearance of symptoms. Clinical disorders appear due to endotoxin, which is released from dead bacterial cells (29).

When considering the results of ZMT and MBE per animal, it is evident that a relatively high proportion of the dairy cows were affected by the causative agents. More than one-third of animals showed a positive reaction to ZMT in at least one udder quarter. According to some authors, up to 50% of cows are affected by mastitis. The proportion of cows with the presence of pathogens within at least one udder quarter was almost 30% in this study. Almost half of the cows, from which the pathogens were isolated, had more than one udder quarters infected. When mastitis is considered from this aspect, the status of its spread, significance for the modern milk industry and importance of systematic control of mastitis in dairy cow herds (2) becomes clearer.

Regarding the intensity of reaction observed in ZMT testing, the highest proportion of reactions were of the lowest degree that could be detected by this method. This fact supports the statement that the mastitis prevention is at a relatively high level in the examined herds. Farmers promptly react to changes in secretion and thus very few cases end with a chronic form of mastitis with extreme reactions (30). The detection of mastitis is performed by the mastitis test, and in some farms with milking parlours, the milking machine measures the electric conductivity of the milk. Namely, in inflamed quarters, at the beginning of the milking process, the milk's electric conductivity is altered due to an increased concentration of chlorides, which are characteristic of the early phases of the inflammatory process (31).

Some studies have reported differences in mastitis frequencies between the front and rear udder quarters. It is assumed that the reason for more frequent infection of rear quarters than front quarters is in their anatomical position and higher exposure to trauma. In the present study, differences were found among quarters and also in the intensity of reaction between quarters, although they were not statistically significant ($P>0.05$). Similarly, the differences in the frequency of isolation of the causative agent between the quarters were also not statistically significant ($P>0.05$) (25).

MBE is often used as the gold standard for validation of reliability of other diagnostic tests. In this study, we compared the results of the ZMT with those of the MBE by means of the Kappa test. Accordance between these two methods was found to be moderate (0.4661). Although the result of the ZMT is a moderate indicator of inflammation, it should be pointed out that an increased number of cellular elements in milk

might be a consequence of trauma or other causes. A reliable scientific approach for more accurate determination of inflammation status of the udder quarters requires more frequent milk sampling during the same milking or more sampling at regular time intervals.

When considering the importance of the mastitis diagnosis, its public health and economic significance should be taken into account. The number and variety of possible causative agents, differences in their spread and survival in the environment are reasons for the use of the MBE as an essential method. Some of the causative agents may pose risk for human health. Furthermore, bacterial resistance to antibiotics should not be overlooked. In other words, bacteria are capable of acquiring and transferring antibiotic resistance to other, taxonomically related and unrelated bacteria. Since the susceptibility test is an essential part of the MBE, the importance of this method in the diagnostics of mastitis and in monitoring trends in the appearance and spreading of resistance to antibiotics is evident (32).

Among mastitis cases, subclinical infections are dominant and the only sign of infection is an elevated somatic cell count. Hence, problematic herds are often revealed during routine controls of somatic cell counts at the purchase of raw milk. Milk with a high somatic cell count is unacceptable for further processing and sale, which increases the total costs of mastitis. However, lower milk yield in cows with infected udders is the predominant cause of economic loss due to mastitis. Picinni et al. (33) calculated that the individual loss per cow in the herd due to mastitis is between 55 and 113 euros.

Conclusions

More than one-third of cows in the present study had changes in at least one udder quarter. The degree of change ranged from the mildest degree that could be detected by the ZMT, to permanently dry-off and loss of quarters for further milk production. About 30% of examined cows in this study had at least one udder quarter infected. The most frequently isolated causative agents of mastitis were *S. aureus* and streptococci, although in a lower degree than in similar previous studies. Concordance of the data obtained by the methods applied in this study was moderate. The ZMT proved to be a rapid, simple and low cost means of mastitis detection in dairy cows. However, it should be supported with data obtained from the MBE in order to select the most effective therapy and to prevent the occurrence of antibiotic resistance.

Conflict of interest

Authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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MIKROBIOLOŠKO SPREMLJANJE POVZROČITELJEV MASTITISA OB NADZORU ZDRAVJA VIMENA PRI KRAVAH MOLZNICAH

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Povzetek: Pomen sistematičnega nadzora mastitisa pri čredah krav je opisan s predstavitvijo podatkov o pojavnosti mastitisa in njegovemu pomenu za sodobno proizvodnjo mleka. Raziskava je bila opravljena v obdobju obiskov kmetij ob odvzemu vzorcev iz vsake vimenske četrti vsem kravam v laktaciji med obiski. Vzorci so bili odvzeti pred večerno molžo. Vsak je bil testiran z zagrebškim »mastitis testom« (ZMT) in za bakteriološki pregled nasajen na krvni agar z eskulinom. Identifikacija zraslih kolonij je bila izvedena s pomočjo mednarodno sprejete metodologije. Dobljeni rezultati so bili statistično obdelani s pomočjo statističnega paketa Stat 13.1. Analizirani so bili vzorci vimenskih četrti 385 krav. ZMT-pozitivne reakcije so bile ugotovljene v 13,7 % vimenskih četrtih. Povzročitelji mastitisa so bili ugotovljeni v 175 vzorcih (13 %) četrti. Od 385 krav, vključenih v raziskavo, jih je 145 (37,3 %) imelo vsaj eno ZMT pozitivno vimensko četrt ali trajno osušeno vimensko četrt. Povzročitelji mastitisa so bili izolirani pri 106 od 363 krav (29,8 %), iz vseh štirih funkcionalnih četrti. Najpogosteje izolirani povzročitelji so bili *Staphylococcus aureus*, *Streptococcus spp.*, *Trueperella pyogenes* in *Corynebacterium bovis*. Statistično značilnih razlik v pojavljanju mastitisa med sprednjo in zadnjo vimensko četrtjo ni bilo. Rezultati ZMT in mikrobiološki pregled sta v zmerni korelaciji (indeks Kappa = 0,4662).

Ključne besede: krava; mastitis; povzročitelji mastitisa; Hrvaška

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Original Research Article

OESTROGENS AND PROLACTIN REGULATE MAMMARY GLAND EPITHELIAL CELL GROWTH BY MODULATION OF THE WNT SIGNAL PATHWAY

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Summary: Oestrogens and prolactin can regulate mammary gland development and epithelial cell growth as well as lactation. Meanwhile, the Wnt-signalling pathway and subsequent upregulation of ß-catenin driven by the downstream target of cyclinD1 also play a role in development of the mammary gland. This study aimed to assess the possible involvement of oestrogens and prolactin in the regulation of cell growth in the mammary gland. Bovine mammary gland epithelial cells (MECs) were treated with ß-estradiol (E2) and prolactin (5 μg mL^{-1}) for 48 h and then measured for cell viability. mRNA and the protein expression level of genes (E-cadherin, CyclinD1 and ß-catenin) related to the Wnt pathway were measured by qRT-PCR and Western blot, respectively, while sub-cellular localizations of the proteins in MECs were further monitored by immunofluorescence. The expressions of E-cadherin and CyclinD1 were highest at 36 h ($P<0.05$) whereas ß-catenin was lowest at 36 h after treatment with E2 and prolactin. The protein level of the E-cadherin and cyclinD1, which are the targets of the Wnt signal pathway, were unregulated. ß-catenin protein level decreased in both hormone groups. In conclusion, prolactin and E2 could efficiently affect the cell growth of MECs and increase the expression of E-cadherin and cyclinD1 at both the mRNA and protein levels. Immunofluorescence suggested that prolactin and E2 impact the nuclear expression of ß-catenin protein. The current study indicated that the proliferative efficacy of prolactin and E2 on MECs was modulated through the Wnt-signalling pathway.

Key words: mammary gland development; hormone; Wnt pathway

Introduction

The mammary gland is an organ in female mammals for milk production and contains a dynamic tissue structure derived from the epidermis that achieves full maturity in the adult (1). Embryonic-branching morphogenesis and lactation mirror the changes within the endocrine environment. Resolving the hormone interactions that modulate mammary gland growth and morphogenesis is essential for understanding the physiological basis of a successful lactation and development (2). Oestrogens and prolactin function to regulate mammary gland development, cell growth, and lactation (1), while the Wnt-signalling pathway also plays a role in the development of the mammary gland (3). For example, E-cadherin is a calcium-dependent cell-cell adhesion glycoprotein and is composed of five extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail (2). E-cadherin is a key molecule in the cell-cell adhesion and in maintaining the morphogenesis of

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a variety of organs, including mammary epithelial cells (4). Furthermore, β-catenin is a dual function protein, regulating the coordination of cell-cell adhesion and gene transcription (5). β-catenin directly interacts with the cytoplasmic domain of E-cadherin to form an adhesion molecule complex (6) and also acts as the key mediator in the canonical Wnt-signalling pathway (7). Increased expression of β-catenin protein promoted the transcription of genes important in regulating the cell cycle (8). β-catenin is stable in the cytoplasm, and during activation, it will translocate into the nucleus (9,10) and, in turn, interact with the T-cell factor (TCF)/Lef transcription of target genes, including CD1, c-Myc, and metalloproteases that promote cell proliferation, differentiation, and tissue development (11,3).

Multiple stages of mammary developments are regulated by the Wnt-signalling pathway. Compelling evidence indicates that the Wnt-signalling pathway was activated in a subset of epithelial cells accompanying initial branch formation of the mammary gland (12) and mammary bud development, and thus, plays a major role in embryonic development, cell proliferation, and differentiation or even oncogenesis (13). Prolactin has an important biological function in promoting survival of the cells (14). Thus, oestrogens and prolactin exert their effects in the mammary gland primarily via paracrine interactions. Oestrogens are significant mediator of the development of the mammary epithelium during alveolar proliferation, ductal morphogenesis, and functional differentiation, accompanied by progesterone peptide hormones in combination with stromal signals (15). Oestrogen is a key determinant of ductal elongation in the mammary glands of mice (16) in various hormone replacement and genetic knockout studies. The principal role of prolactin in the mammary glands of mice during pregnancy is to promote alveolar development and lactation (17).

The molecular mechanisms underlying the effects of prolactin and oestrogens on E-cadherin, β-catenin, and cyclinD1 in the Wnt-signalling pathway in the mammary gland of cows have not been fully elucidated. Therefore, in this study, we hypothesized that prolactin and oestrogens may impact the Wnt/β-catenin pathway to regulate mammary gland development and cell proliferation. We then investigated this specific transduction pathway along with E-cadherin, β-catenin, and its main downstream protein, cyclinD1. These data could provide an insight into the link between oestrogens/prolactin and the Wnt/β-catenin pathway for mammary gland development and cell proliferation.

Materials and methods

Primary cell culture and treatment

Mammary gland epithelial cells from bovine (MECs) were obtained from the Key Laboratory of Dairy Science, Northeast Agricultural University, China and cultured in a cell culture medium of mixed Dulbecco's modified Eagle's medium (DMEM) with F12 (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% foetal bovine serum (Invitrogen), 5 μg mL^{-1} Hydrocortisone (Sigma-Aldrich, Bangalore, India), 5 μg mL^{-1} insulin (Sigma–Aldrich). 100 IU mL^{-1} Penicillin and 100 IU mL^{-1} Streptomycin (Invitrogen) in a humidified incubator with 5% CO_2 at 37 °C. Prolactin and 17β-estradiol (E2) were purchased from Sigma–Aldrich. The D-Hanks' medium was composed of 8.00 g L^{-1} NaCl, 0.40 g L^{-1} KCl, 0.086 g L^{-1} Na_2HPO4, 0.060 g L^{-1} KH_2PO_4, and 0.35 g L^{-1} $NaHCO_3$. For experiments, the logarithmic growth phase of MECs was inoculated at a concentration of 3×10^4 cells cm^{-2} into 6-well culture plates and grown, and the medium was refreshed after 24 h. The cells were then stimulated by adding prolactin (5 μg mL^{-1}) according to a previous study (18) for 0, 6, 12, 24, 36 or 48 h and harvested. For E2 (5 μg mL^{-1}), it was the same concentration as prolactin. The experiments were in triplicate, and the control group was replaced with fresh medium and cultured to 48 h.

Cell viability assay

Cells were harvested by trypsinization and suspended at a final concentration of 2×10^4 cells mL^{-1} in fresh DMEM. Aliquots of 100 μL/well cell suspension were plated in 96-well tissue culture plates, and cells were treated with prolactin and E2 at 5 μg mL^{-1} of each for 0, 6, 12, 24, 36, and 48 h, respectively. Cells without prolactin and E2 were used as negative controls. At the end of each experiment, 10 μL/well cell proliferation Reagent WST-1 (Roche, Basel, Switzerland) was added to the cell culture plates and further incubated for 4 h at 37 °C in a humidified atmosphere containing

5% CO_2; the cells were then mixed thoroughly for 1 min on a shaker and the absorbance of the cells against a background control was measured using a microplate reader (Thermo Fisher Scientific, United States) at 450 nm. Each experiment was repeated three times.

RNA isolation and qRT-PCR

Prolactin- and E2-treated cells were subjected to RNA isolation using the Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol and dissolution in RNAse-free water and quantified using a spectrophotometer and qualified using Agilent (Palo Alto, CA, USA) bioanalysis. The RNA sample (1 μg) was reversely transcribed into cDNA using the PrimeScript® RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions and then subjected to qPCR amplification using SYBR® Premix Ex Taq™ (TaKaRa) on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers were designed using Premier 5.0 (see Table 1), and all qRT-PCR amplifications were performed in duplicate and repeated three times. The relative expression value of each target gene was normalized to GAPDH expression.

Protein extraction and Western blotting

MECs were treated with either prolactin or E2 at 5 μg mL^{-1} in six plates for 36 h, and untreated cells were used as a negative control. Cells were lysed in a RIPE buffer (Beyotime, Shanghai, China) (100 μL mL^{-1} to the plate plus 2 μL mL^{-1} protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined with Bradford reagent (Sigma-Aldrich), and the same amount of protein samples (20 μg) was separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membranes (Amersham, Freiburg, Germany). After blocking in 5% non-fat milk in Tris-buffered saline (TBS, 0.1 mol L^{-1}, pH 7.4) for 90 min, the membranes were incubated with E-cadherin, β-catenin, cyclinD1, or GAPDH antibody at 4 ºC overnight. Antibody against E-cadherin (#sc-1500), β-catenin (#sc-53483), cyclinD1 (#sc-718) or GAPDH (#sc-25778) and a goat anti-mouse IgG-HRP (#sc-2005) and goat anti-rabbit IgG-HRP (#sc-2004), donkey anti-goat IgG-HRP (#sc-2020) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After being washed with TBST, the membranes were further incubated with a secondary antibody (an HRP-conjugated goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP or donkey anti-goat IgG-HRP antibody diluted at an appropriate dilution in 5% non-fat milk in TBS) for 2 h at room temperature. Protein bands were then detected by incubation with enhanced ECL (Amersham Pharmacia Biotech) on X-RAY film (Kodak, NY, USA). The protein abundance of GAPDH served as an internal control. Each experiment was repeated three times.

Immunofluorescence

An immunofluorescence (IF) assay was performed to assess the sub-cellular localizations of proteins related to the Wnt pathway in MECs. Briefly, cells were grown on coverslips at a density of 3×10^4 cells cm^{-2} and treated with prolactin and E2 at 5 μg mL^{-1} for 36 h, and cells without prolactin and E2 treatment were a negative control. Thereafter, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 min and then washed thoroughly with Tris-buffered saline with 1‰ $Triton_X$-100 (TBSTx, pH 7.4) three times for 5 min each and incubated with 5% bovine serum albumin (BSA) for 1 h and further incubated with a

Table 1: Primers used during real-time PCR

gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
E-cadherin	NM_001002763	CGTATCGGATTTGGAGGGAC	CATCATCGAGGAACAAGAGCAG
β-catenin	NM_001076141	CCAAGTGGGTGGCATAGAGG	GGCTGGTCAGATGACGAAGG
CycinD1	NM_001046273	GGACCGCTTCCTGTCGCT	GCCAGGTTCCACTTGAGTTTGT
GAPDH	AB098934	TGCTGGTGCTGAGTATGTGGT	AGTCTTCTGGGTGGCAGTGAT

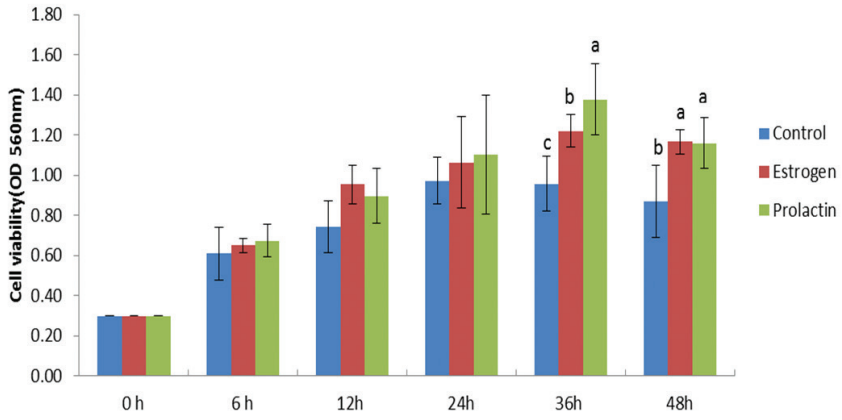
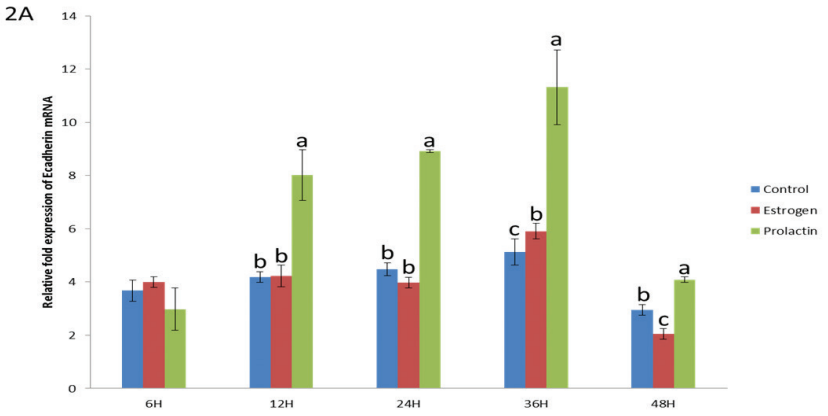
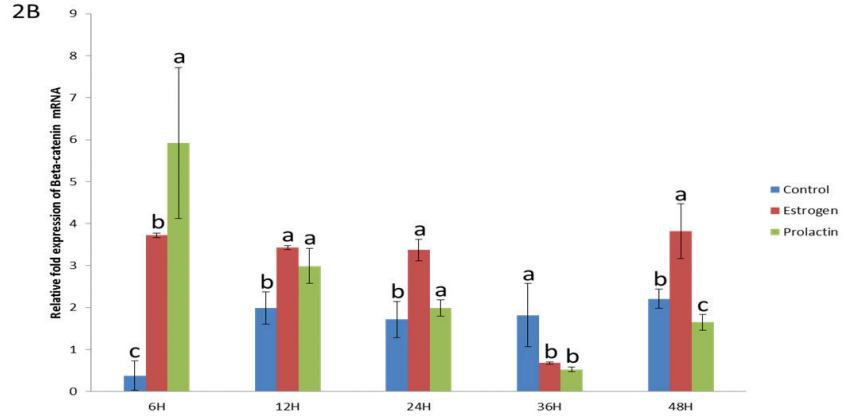


Figure 1: MECs Growth curve *in vitro* by prolactin and E2 (5 μg mL^{-1}) from 0 h to 48 h. The data were represented as mean ± SEM. Different letters indicated a significant level of P<0.05 in comparison to the control





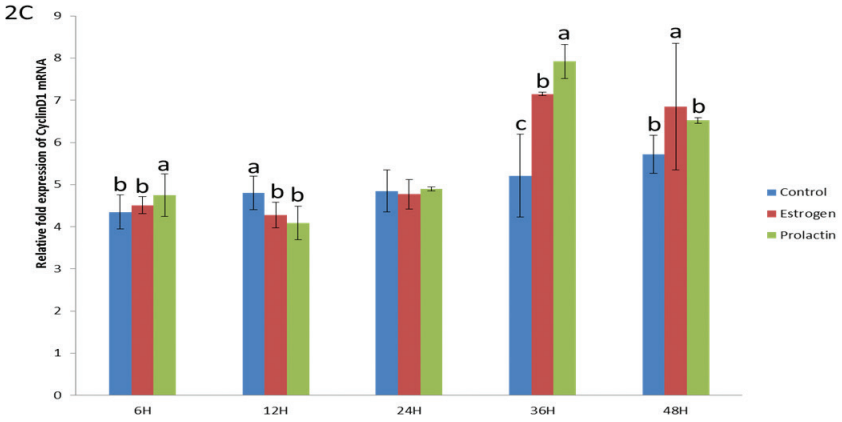


Figure 2: mRNA expression levels of E-cadherin (2A), β-catenin (2B) and cyclinD1 (2C) with treatment of prolactin or E2 for 0, 6, 12, 24, 36 and 48 h. GAPDH was used for normalization. Different letters indicate a significant level of P<0.05 in comparison to the control

primary antibody against E-cadherin and β-catenin at 4 °C overnight. On the next day, cells were washed with TBSTx thrice and then incubated with the rabbit anti-goat FITC conjugated (#sc-2777), and goat anti-mouse TRITC (#sc-2092) antibodies (Santa Cruz Biotechnology (Santa Cruz, CA, USA)) secondary antibody in a 1:200 dilution in TBSTx for 1 h. The nuclei of MECs were stained with DAPI for 15 min at room temperature. After that, the coverslips were mounted with a fluorescence-quenching mounting medium (Beyotime, Shanghai, China) and reviewed under a confocal microscope; the images of cells were captured by the Z-stacking function for serial confocal sectioning at 2 μm intervals (Leica TCS SP2, Buffalo Grove, IL, USA) and then analysed with Leica software. The experiments were performed in triplicate and repeated at least once.

Statistical analysis

Comparison of differences between treatment and control was statistically analysed by using SPSS 16.0 software (SPSS, Chicago, IL, USA). Association among the expression of E-cadherin, β-catenin, and cycinD1 before and after treatment was analysed using the ANOVA two-way measures. Cell proliferation in different time points (0, 6, 12, 24, 36 and 48 h) after prolactin and E2 treatment was evaluated using the analysis of ANOVA repeated measures. For protein intensity, the data were analysed with one-way ANOVA repeated measures with the bandscan 5.0 software and the data were summarized as mean ± SEM. A p-value ≤ 0.05 was considered to be statistically significant.

Results

Effects of prolactin and E2 on regulation of cell proliferation

MECs growth curve revealed that the largest cell population was reached at 36 h after treatment with 5 μg mL-1 prolactin and E2. In comparsion to the controls, an increase of cell population in both prolactin and E2 groups was observed. Notably, numbers of cells at 36 h were significantly higher than those of other time points in prolactin and E2 treatment (Figure 1). Therefore, the optimal treatment time of prolactin and E2 was determined to be 36 h.

Effects of Prolactin and E2 treatment on expression of E-cadherin, ß-catenin and cyclinD1 in MECs

The effects of prolactin and E2 treatment on the expression of E-cadherin, β-catenin and cyclinD1 in MECs were then examined. The relative mRNA expression of E-cadherin and cyclinD1 was slightly increased in comparison to the control group, during the period from 12 h to 48 h after the treatment (Figure 2AC). Notably, during treatment with either prolactin or E2, mRNA expression levels of β-catenin were increased in comparison to the controls between 6 and 24 h, but remarkably reduced at 36 h after the treatment (Figure 2B). However, there is no significant difference between the prolactin and E2 groups in the effect of the treatment.

The protein expression of E-cadherin, β-catenin and cyclinD1 were observed in MECs after treatment of 5 μg mL^{-1} prolactin and E2 at 36 h. Western blot and the desitometric analysis results are depicted in Figure 3. Desitometric analysis indicated a decrease in β-catenin protein during either prolactin or E2 treatment in comparison with control cells ($P<0.05$). Moreover, E-cadherin and cyclin D1 protein quantities were greater than in the control cells ($P<0.05$). However, the protein amount of E-cadherin, β-catenin and cyclinD1 did not differ between the prolactin and E2 groups ($P>0.12$).

Immunofluorescence

Using laser-scanning confocal microscopy, we explored whether prolactins and E2 could modulate the expression of Wnt pathway components. Our results showed that within 36 h of treatment of either prolactin or E2, the fluorescence of E-cadherin protein was strong (green fluorescence) in perinuclear of MECs (Figure 4B, 4C). However, red signals were the tetramethylrhodamine isothiocyanate (TRITC)-labeled β-catenin, which was expressed in the nuclear compartment. It was suggested that the expression of β-catenin be decreased receiving either prolactin or E2 treatment (Figure 4B, 4C). Therefore, these results revealed that the expression of E-cadherin in MECs treated with prolactin and E2 was upregulated, however, β-catenin expression was lower than in the control cells.

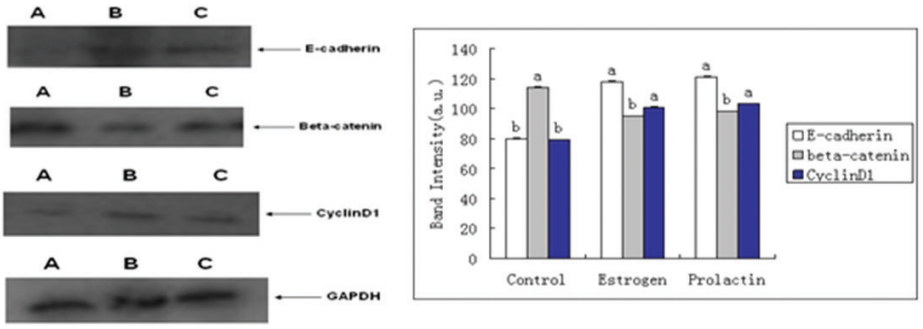


Figure 3: Western blot of E-cadherin, β-catenin and cyclinD1 in response to prolactin or E2 treatment (36 h). A: Control group; B: E2 treatment for 36 h; C: prolactin treatment for 36 h. GAPDH was used for normalization. Values are represented as mean ± SEM. Different letters indicate a significant level of $P<0.05$ in comparison to the control

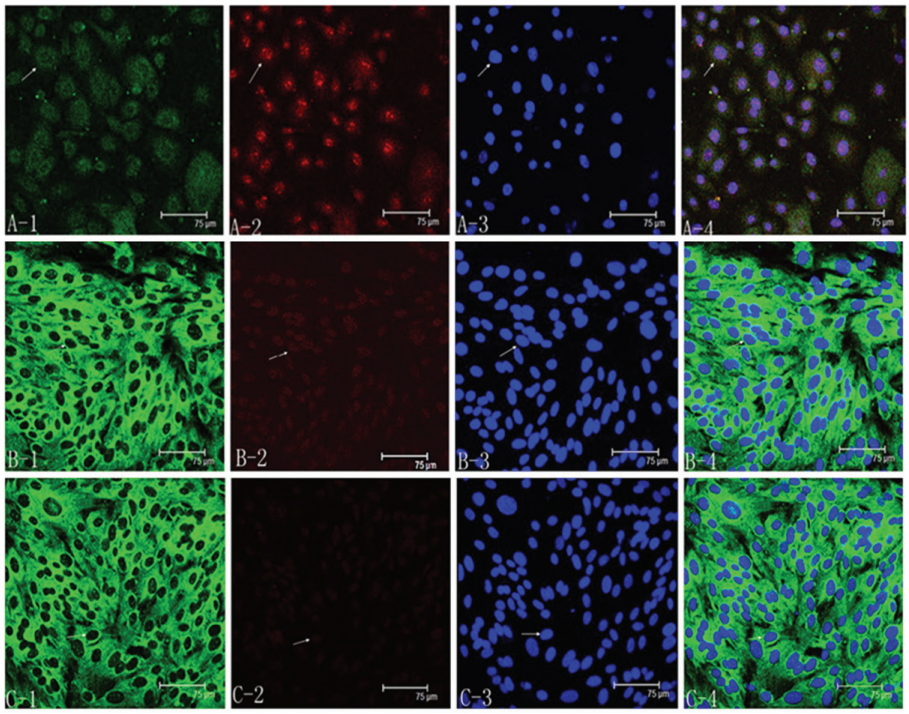


Figure 4: Immunofluorescence analysis revealed sub-cellular localizations of the proteins involved in Wnt-signalling in MECs with the treatment of prolactin and E2 for 36 h respectively. (A) Control group without prolactin and E2 treatment; (B) E2 treatment group; (C) prolactin treatment group (A-C original magnification 200×). Green, red, and blue fluorescent signals represent E-cadherin, β-catenin and DAPI-dyed nuclei, respectively

Discussion

Oestrogens and prolactin function to regulate mammary gland development and cell growth and lactation, while the Wnt-signalling pathway also plays a role in the development of the mammary gland. Overall, the Wnt/ β-catenin-signalling pathway is a known regulator of cellular functions including embryonic development and cell proliferation, differentiation, survival and adhesion (19,20). E-cadherin and β-catenin as a complex of the Wnt/β-catenin signalling pathway are specifically involved in regulating cell adhesion, and they have a close relationship with the development of the mammary gland. The current study explored the E2 and prolactin effects on MEC proliferation and the underlying molecular event *in vitro*, which could provide insightful information regarding the potential molecular actions of the mammary gland of cows. In the current study, we found that E2 and prolactin at a concentration of 5 μg/mL of each impacted MEC proliferation and the expression of E-cadherin, β-catenin, and cyclinD1. Future studies will investigate whether knockdown of the Wnt/β-catenin signalling pathway gene expression could alter the effects of oestrogens and prolactin on mammary gland development and cell growth.

Oestrogens and prolactin are required for the growth and morphogenesis of bovine mammary glands via the essential regulation of mammary epithelial cell proliferation and differentiation. Our current data has demonstrated that 5 μg/mL treatments with prolactin or E2 markedly in-

creased MEC viability and also significantly impacted the expression of E-cadherin, β-catenin, and CyclinD1, which could be crucial for exploiting oestrogens and prolactin-related mammary gland development. Specifically, postnatal mammary gland development is under the control of various hormones, including oestrogens, progesterone, growth hormone (GH), prolactin, and cell fate-determining signalling pathways (21). Our results were in accordance with previous studies showing that prolactin, by regulation of the PRL-mediated Jak-Stat signalling pathway-mediated expression of the milk proteins and oestrogens, promoted ductal extension and lobular alveolar morphogenesis (22). It has been indeed well-characterized that the bovine mammary gland-secreted oestrogens during the last week of pregnancy affects some functions of bovine neutrophils (23), which could account for our present observation that oestrogens increased cell proliferation. We found that prolactin and E2 can promote cell proliferation and survival, as well as increase cell motility, which is in accordance with Tworoger (24) who showed that that estradiol increased cell proliferation 1.5 times compared to control cells in a breast cancer cell line. Our data further revealed that the expression of cyclinD1 was affected by these two hormones, which is consistent with the observed cyclinD1 as the Wnt-signalling target gene (19). Indeed, cyclinD1 has been considered to be one of the most important factors in the regulation of cell cycle progression (25). Thus, the hormones activated β-catenin by translocation of the cytoplasmic β-catenin into the nucleus and in turn regulated gene expression, including cyclinD1, for the promotion of cell proliferation, differentiation, and organ development (26). In line with this, MEC proliferation was observed in the present study (Fig. 1). Interestingly, down-expression or destruction of E-cadherin and β-catenin is one of the changes that characterize an invasive phenotype. These genes are also considered to be invasion/tumour suppressor genes (17). Our data demonstrated that E2 and prolactin increase the expression level of E-cadherin and CyclinD1 and arrest β-catenin (Fig. 2), suggesting that prolactin and E2 enhance the Wnt-signalling activity. These results are consistent with a previous study that the proportion of the mammary epithelial cells after E2 treatment was increased and imply that Wnt signalling can substitute for oestrogens to drive total population growth (27). In addition, prolactin and E2-treated MECs showed that E-cadherin expression was markedly increased. It is possible that the proliferative effects of prolactin and E2 are mediated by the promotion of cyclinD1 expression in MECs, which further supported a previous study showing that oestrogens significantly increased expression of both c-Myc and cyclinD1 proteins and then contributed to cell cycle progression (28). Our current data showed that prolactin and E2 markedly increased the expression levels of cyclinD1 in a time-dependent manner in MECs.

Furthermore, a previous study (29) showed that soy isoflavone genistein increased E-cadherin expression through an ERβ-mediated pathway, upregulated E-cadherin–β-catenin cell adhesion complex formation, but decreased Wnt-induced cytosolic and nuclear β-catenin accumulation, and the transcription of proliferation-associated cyclinD1 and c-Myc genes. Therefore, prolactin and E2 are effective for MEC proliferation. The Wnt/β-catenin signalling is necessary for for growth survival and differentiation of the mammary gland, and it can lead to cell proliferation. Moreover, E-cadherin can sequester β-catenin away from the nucleus, acting as a tumour suppressor (30). The cadherin-catenin complex is a group of membrane proteins to regulate cell-cell adhesion. Disruption of this cell-cell adhesion in malignant, transformed cells could contribute to enhanced cell migration and proliferation. This can be confirmed by our current data that β-catenin was affected by prolactin and oestrogens after 36 h treatment, indicating that the Wnt/β-catenin pathway was activated and the downstream target gene, cyclinD1 was increased (17). Thus, further study of the Wnt/β-catenin pathway could lead to better understanding of mammary gland development, milk production, and carcinogenesis.

In summary, our current data demonstrated that the effects of prolactin and E2 on the proliferation of mammary epithelial cells and on modulation of the key components of the Wnt signalling. E2 could increase the mammary gland epithelial cells validity and the expression of a cell-cycle key gene- cyclinD1. We also emphasized the interactions between hormone-activated pathways as the principal determinants of the mammary gland development. Our results highlighted the importance of the Wnt components E-cadherin, β-catenin, and cyclinD1, which may serve as future targets for regulating the cell development and lactation of mammary glands.

Acknowledgements

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The authors declare that they have no conflict of interest.

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ESTROGENI IN PROLAKTIN URAVNAVAJO RAST EPITELIJSKIH CELIC MLEČNE ŽLEZE S SPREMEMBO SPOROČILNE POTI WNT

J. Tong, H. Zhang, Y. Wu, Y. Wang, Q. Li, Y. Liu

Povzetek: Estrogeni in prolaktin lahko uravnavajo razvoj mlečne žleze ter razvoj in rast epitelijskih celic, pa tudi laktacijo. Poleg tega ima vlogo pri razvoju mlečne žleze znotrajcelična pot sporočanja preko dejavnikov Wnt ter povečanja izraženosti ß-katenina, povzročeno preko molekule ciklina D1. Ta študija je bila namenjena oceni morebitne udeležbe estrogenov in prolaktina pri uravnavanju rasti celic v mlečni žlezi. Epitelijske celice mlečne žleze goveda (MECS) so bile 48 ur tretirane z ß-estradiolom (E2) in prolaktinom (5 μg mL^{-1}), nato pa je bila pri njih izmerjena sposobnost njihovega preživetja. mRNK in beljakovinska raven izražanja genov (E-kadherina, ciklina D1 in ß-katenina) v povezavi s potjo Wnt sta bili izmerjeni s qRT-PCR in metodo Western blot, medtem ko je bila subcelična lokalizacija proteinov v MECS opazovana s pomočjo imunofluorescence. Izražanje E-kadherina in ciklina D1 je bilo najvišje po 36 urah ($p < 0,05$), medtem ko je bila raven izražanja ß-catenina najnižja 36 ura po dodajanju E2 in prolaktina. Raven beljakovin E-kadherina in ciklina D1, ki sta tarči znotrajceličnega signaliziranja Wnt, je bila neuravnana. Beljakovinski nivo ß-katenina se je zmanjšal v obeh hormonskih skupinah. Zaključimo lahko, da prolaktin in E2 učinkovito vplivata na rast celic MECS ter povečanje izražanja E-kadherina in ciklina D1 na obeh stopnjah - stopnji mRNK in beljakovinski stopnji. Rezultati imunofluorescence kažejo na to, da prolaktin in E2 vplivata na izražanje beljakovine ß-catenin v jedru. Sedanja študija je pokazala, da je bila proliferacijska sposobnost prolaktina in E2 na MECS uravnana preko NT-signalne poti.

Ključne besede: razvoj mlečne žleze; estrogeni; progesteron; pot Wnt

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Original Research Article

CHANGES IN THE UTERUS AND VAGINA OF RATS WITH EXPERIMENTALLY INDUCED DIABETES AND THE EFFECT OF LYCOPENE ON THE CHANGES

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Summary: The aims of this study were to identify the changes that occurred in the uterus and vagina of rats with experimental diabetes, and the effects of orally administered lycopene on these changes. For this research, 42 four-month-old female Wistar Albino rats were used. Experimental diabetes was induced using a single dose of 50 mg/kg streptozotocin (STZ). The control and diabetic rats were randomly separated into four groups as follows: control+corn oil, control+lycopene, diabetes+corn oil, and diabetes+lycopene. Crossman's triple staining and the Periodic Acid Schiff (PAS) methods were applied to the uterine and vaginal slides. Additionally, the uterus was stained with Alcian Blue (AB) pH: 2.5. While the number of glands, the thickness of the endometrium, and the PAS-positive reaction in the glandular epithelium were lower, the AB positive reaction in the glandular epithelium was higher in the diabetes+corn oil group in comparison with the control+corn oil group in the uterus. In contrast, while the height of the lamina epithelialis and the thickness of the tunica muscularis were smaller in the diabetes+corn oil group, in comparison with the control+corn oil group, the thickness of the tunica muscularis was larger in the diabetes+lycopene group, in comparison with the vagina of the diabetes+corn oil group. The changes caused by diabetes in the uterus and vagina, as well as the existence of the protective qualities of lycopene were revealed for the first time, to the best of our knowledge.

Key words: experimental diabetes; lycopene; rat; uterus; vagina

Introduction

Diabetes mellitus is an endocrine disease with serious complications (1). In the uterus, the thicknesses of the endometrium (2), myometrium (3), and the height of the epithelium decrease with the effects of diabetes. The endometrial stroma is also disrupted (4). Furthermore, the immune and vascular defects can occur in gestational endometrium (5). In addition, it was stated that the rate of luminal epithelial cell division was depressed in the uterus with the effect of diabetes (6). In contrast, the mRNA expressions of insulin-like growth factors and their receptors change during the preimplantation periods in the uterus of diabetic animals and thus, may result in diabetic embryopathy (7). In the vagina, the thickness of the epithelial layer decreases (2), and the amount of collagen and apoptosis rate increase with diabetes (8). A narrowing in the muscularis area and truncation in the elastic fibres are observed (9) while the submucosal vasculature decreases and the risk of fibrosis increases (10). Moreover, it was emphasized that the oestrogen, progesterone, and androgen receptor expressions significantly

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were reduced with the effect of diabetes in the vagina and thus, the vaginal functions may be affected negatively (11). Conversely, increased free radicals play a major role in the pathogenesis of the complications of diabetes (12). While the glutathione (GSH), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels decrease, the malondialdehyde (MDA) level increases with the effects of diabetes in the uterus (13). However, diabetes increases the reactive oxygen species but reduces the SOD levels in the vagina (8).

Lycopene is a member of the carotenoid family and has antioxidant properties (14). It causes a decrease in the glucose, hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substance (TBARS) levels, and it increases the catalase (CAT), SOD, GPx antioxidant enzyme activity, and insulin concentration (15). In addition, it attenuates diabetic neuropathic pain (16) as well as endothelial (17) and erectile dysfunction by reducing oxidative stress (18). Moreover, it was stated that it had a cardioprotective effect (19) and may be useful in the prevention of atherosclerosis (20). However, it was indicated that lycopene inhibited tumour growth (21) and decreased the metastatic capacity (22).

The aim of the present study was to examine the histological, histometrical, and histochemical changes that occur in the uterus and vagina of rats induced with diabetes, and the effects of orally administered lycopene on these changes.

Materials and methods

Animals

In this study, 42 adult female Wistar Albino rats (average body weight 160 g, 4 months old) were used. The rats were obtained from the Department of Laboratory Animals, Ege University in Izmir, Turkey. The rats were housed in a room with a temperature of 24±1°C. The animals were kept under conventional conditions, with a 12-h light/dark cycle, while they were provided with food and water ad libitum. All procedures were approved by the Ethics Committee of Adnan Menderes University, Turkey.

Induction and assessment of diabetes

Experimental diabetes was induced in 30 rats using a single dose of 50 mg/kg of streptozotocin (BioShop STR 201.1), in a 0.01 M citrate buffer (pH 4.5), administered intraperitoneally. After three days, the fasting blood glucose levels were measured, using a glucometer (Contour TS Bayer), in the blood samples taken from the tails of the rats (15). The rats with fasting blood glucose levels of 250 mg/dl or more were included in this study (16). An equal amount of 0.01 M citrate buffer (pH 4.5) was applied to the rats in the control groups, intraperitoneally. Experimental diabetes did not develop in five animals. Therefore, they were removed from the study. Seven animals died in the experiment term in diabetes groups. Therefore, the study was carried out with 30 animals.

Experimental design

Three weeks after diabetes was induced, the control and diabetic rats were randomly separated into four groups as follows: control+corn oil (n=6), control+lycopene (n=6), diabetes+corn oil (n=8), and diabetes+lycopene (n=10). The control+lycopene and diabetes+lycopene groups of rats were fed 4 mg/kg of lycopene (10% FS; Redivivo TM; Code 7803; DSM Inc., Istanbul, Turkey) dissolved in 4 ml/kg of corn oil via gavage for four weeks. The control+corn oil and diabetes+corn oil rats were fed 4 ml/kg of corn oil every day.

Sample collection and preparation

At the end of the experiment term, all animals in all groups were checked for oestrous cycles using the vaginal smear method. When the animals were in the diestrus period, they were sacrificed by cervical dislocation under ether anaesthesia. Samples were taken from the right horn of the uterus and the vagina; then, the tissues were fixed for 24 hours, at +4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH: 7.4). After routine histological processing, the tissues were embedded in Paraplast, and 5 µm-thick cross-sections were taken.

Histological, histometrical and histochemical analyses

All histological and histometrical evaluations of the tissue sections were performed using Crossman's triple-staining method. In addition, the Periodic Acid Schiff (PAS) staining method was

used in the uterus and vagina, as was Alcian Blue (AB) (pH: 2.5) staining method was applied to the uterus for histochemical examinations. PAS and AB pH 2.5 histochemical staining techniques were employed in order to demonstrate the neutral and acidic mucosubstances, respectively. Changes in the amount of carbohydrate components in the uterus and vagina of the animals in the different groups were determined, and the effect of lycopene with these dyes was identified.

The changes that occurred (in comparison with the normal histological appearance of the tissues), numerical values, and histometrical measurements were determined in the two sections that belonged to each animal. The thickness of the endometrium and the height of the surface epithelium were identified by the measurements taken from five different areas. The height of the glandular epithelium and gland area were measured from five different randomly selected glands in the endometrium, and the glands of the uterus were counted in the sections of each animal. The height of the lamina epithelialis and the thickness of the tunica muscularis were determined using the measurements taken from five different areas in the vagina. The relative PAS and AB pH: 2.5 positive reaction intensities in the surface and glandular epithelial cells of the uterus, as well as the PAS-positive reaction intensity in the lamina epithelialis of the vagina, were evaluated by one person subjectively, and given a score as follows: 0: no reaction (-); 1: weak reaction (+); 2: moderate reaction (++); and 3: strong reaction (+++) (23). PAS-positive cell counts were obtained from five different 28224 μm^2 unit areas. Finally, a light microscope (Leica DMLB) equipped with an image analysing system (Leica Q Win Standard) was used for measuring, counting and scoring the procedures; photographs were also taken.

Statistical analysis

The SPSS statistical package (version 17.00) software was used for all analyses, and the data were analysed using the one-way analysis of variance (ANOVA). If there was a difference in the ANOVA, Duncan's test was used as a post hoc test. The values are presented as the means ± standard errors, and values of $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***) were considered to be statistically significant.

Results

Histological and histometrical evaluations

The heights of the surface and glandular epithelia, gland area, number of glands, and thickness of the endometrium were examined in the uterus. The number of glands in the endometrium and the thickness of the endometrium were lower in the diabetes+corn oil group, in comparison to the control+corn oil group. Similarly, it was observed that the gland area was smaller in the diabetes+lycopene group, in comparison to the control+lycopene group ($P<0.01$, $P<0.001$) (Figures 1, 2 and 7).

The height of the lamina epithelialis and thickness of the tunica muscularis were measured in the vagina, where they were smaller in the diabetes+corn oil group, in comparison to the control+corn oil group. However, they were larger in the control+lycopene group, in comparison to the control+corn oil group. Similarly, the thickness of the tunica muscularis was larger in the diabetes+lycopene group, in comparison to the diabetes+corn oil group ($P<0.001$) (Figures 3 and 8). Furthermore, vacuoles were observed in the lamina epithelialis of the vagina in the diabetes groups (Figure 9).

Histochemical evaluations

A positive PAS reaction was seen in the apical parts of the surface and glandular epithelia in the uterus. PAS-positive cells were also observed in the endometrial connective tissue. The intensity of the PAS-positive reaction in the glandular epithelium was lower in the diabetes+corn oil group, in comparison to the control+corn oil group ($P<0.001$) (Figure 10). However, a positive PAS reaction was observed in the superficial cell layers of the lamina epithelialis in the vagina. It was seen that the intensity of the PAS-positive reaction was higher in the diabetes+lycopene, in comparison to the control+lycopene group ($P<0.05$) (Figure 11).

A positive AB pH: 2.5 reaction was observed in the apical parts of the surface and glandular epithelia in the uterus. It was determined that the intensity of the AB pH: 2.5 positive reaction was higher in the glandular epithelium in the diabetes+corn oil group, in comparison to the control+corn oil group ($P<0.001$) (Figure 12). Figures 4–6: include units in the figures.

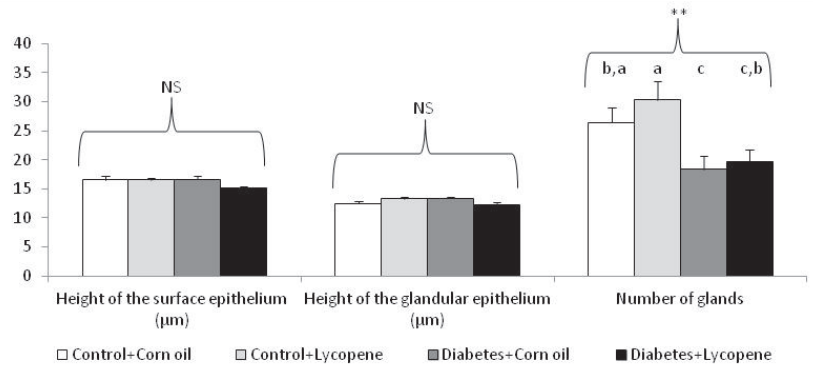

Figure 1: Heights of the surface and the glandular epithelium, and the number of glands in the uterus of the control and diabetes groups a,b,c: Different superscripts in the bars indicate a significant difference. NS: Non-significant. **: P<0.01

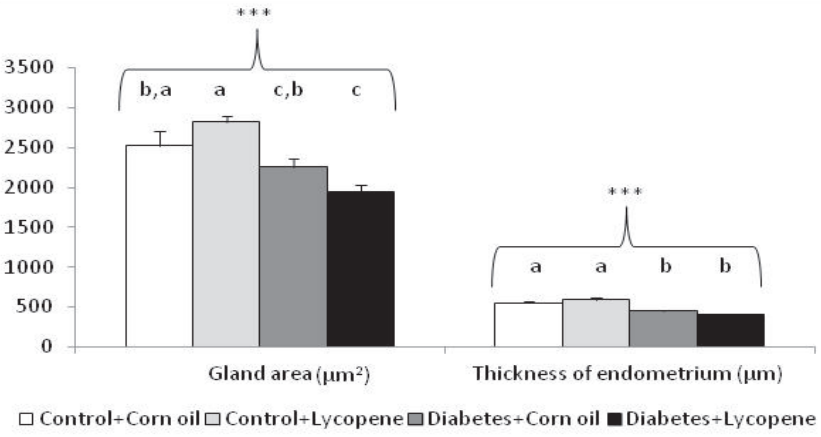

Figure 2: Values of gland area and thickness of endometrium in the uterus of the control and diabetes groups. a,b,c: Different superscripts in the bars indicate a significant difference. ***: P<0.001

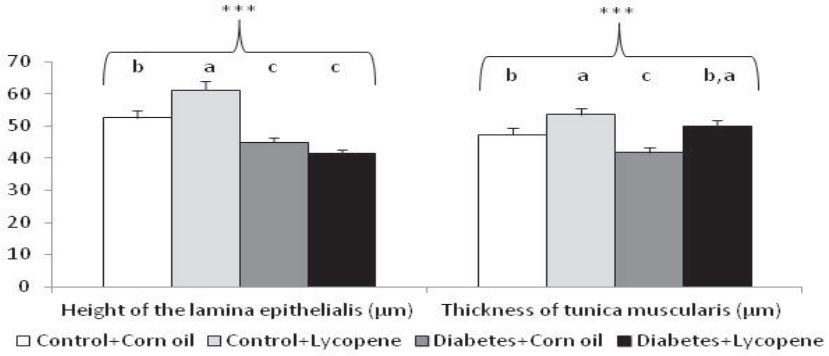

Figure 3: Histometrical measurement values in the vagina of the control and diabetes groups. a,b,c: Different superscripts in the bars indicate a significant difference. ***: P<0.001

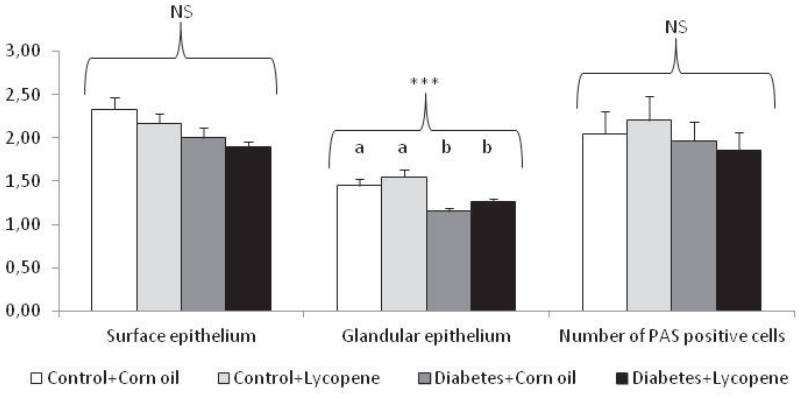

Figure 4: The intensity values of the PAS-positive reactions in the surface and glandular epithelia of the uterus, and the number of PAS-positive cells in the unit area in the endometrial connective tissue of the control and diabetes groups. a,b: Different superscripts in the bars indicate a significant difference. NS: Non-significant, ***: P<0,001

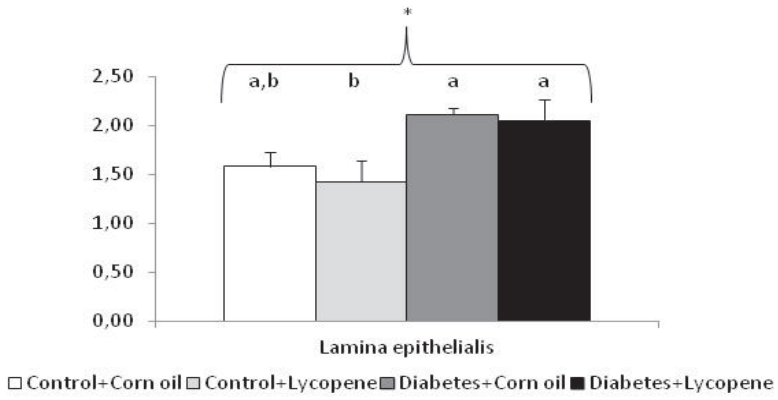

Figure 5: The intensity values of the PAS-positive reaction in the lamina epithelialis of the vagina in the control and diabetes groups. a,b: Different superscripts in the bars indicate a significant difference. *: P<0,05

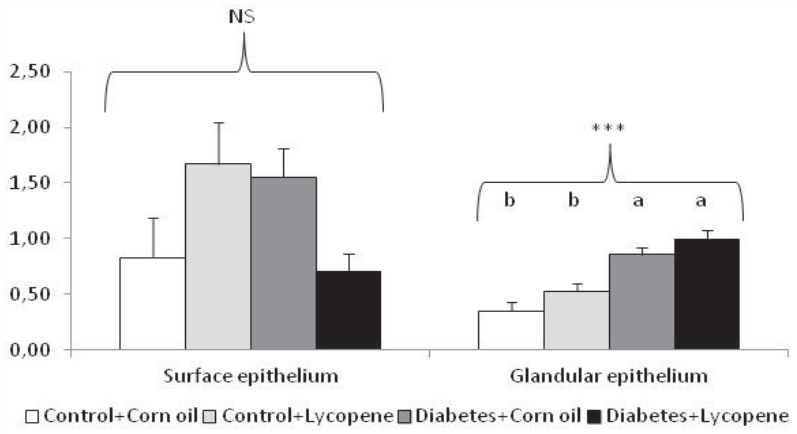

Figure 6: The intensity values of the AB pH: 2.5 positive reactions in the uterus of the control and diabetes groups. a,b: Different superscripts in the bars indicate a significant difference. NS: Non-significant, ***: P<0,001

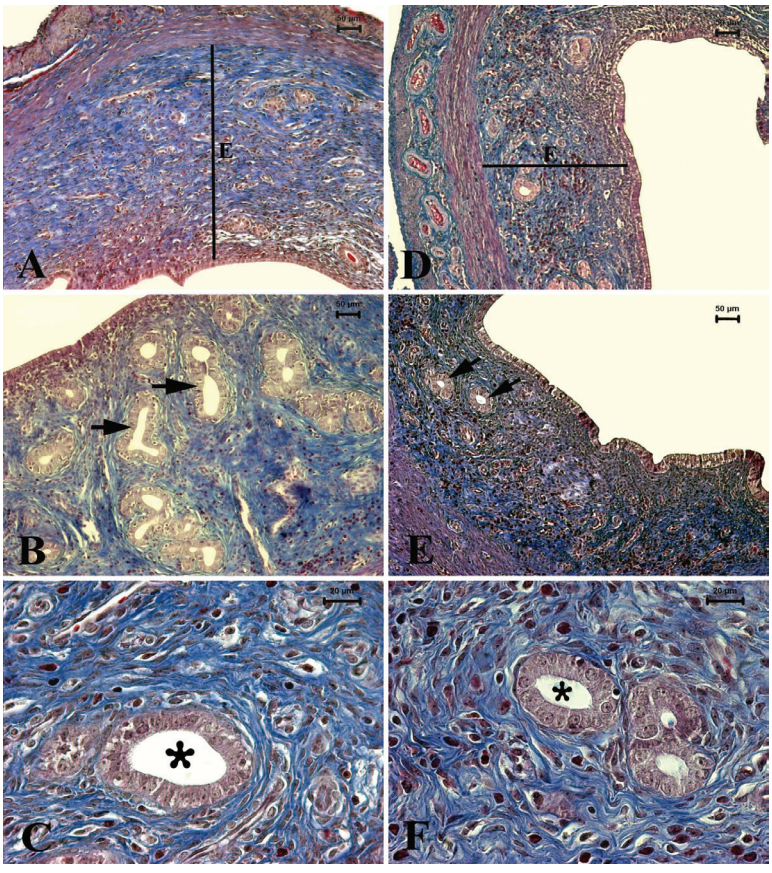

Figure 7: Uterine sections in the control and diabetes groups. It can be seen that the thickness of the endometrium (A, D) (black lines, E) and the number of glands (B, E) (arrows) were lower in the diabetes+corn oil group (D, E), in comparison to the control+corn oil group (A, B). It was also determined that the gland area (asterisks) was smaller in the diabetes+lycopene group (F), in comparison with the control+lycopene group (C). Triple staining method. Bar: 50 µm (A, B, D, E); 20 µm (C, F)

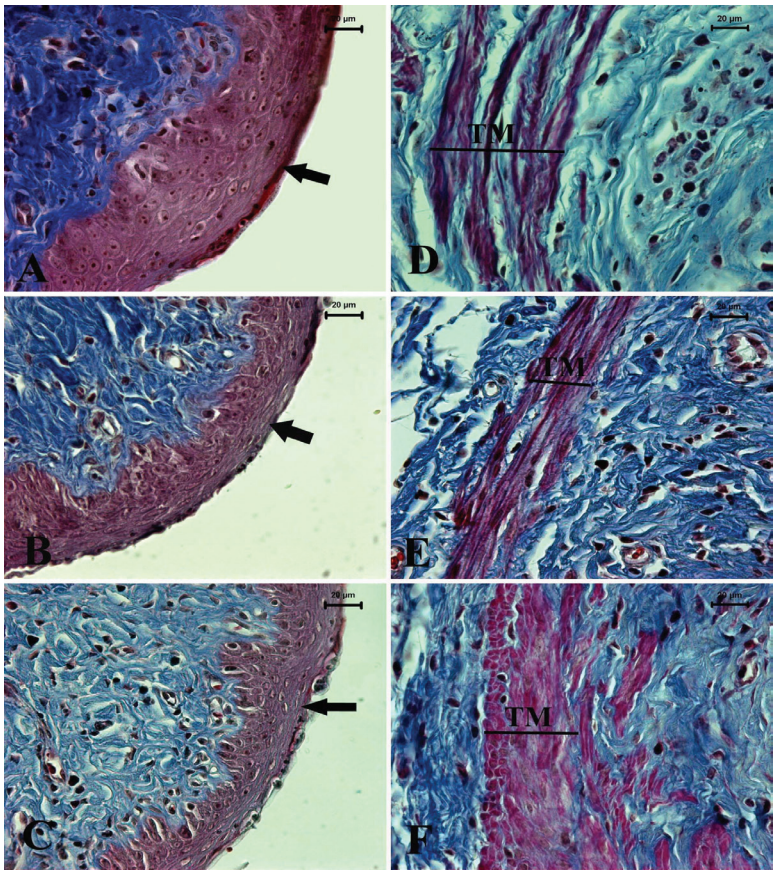

Figure 8: Vaginal sections in the control and diabetes groups. It can be seen that the height of the lamina epithelialis (A-C, arrows) was smaller in the diabetes+corn oil (B) and diabetes+lycopene (C) groups, in comparison to the control+corn oil group (A). Similarly, it was determined that the thickness of the tunica muscularis (D-F) (black lines, TM) was smaller in the diabetes+corn oil group (E), in comparison to the control+corn oil (D). However, it was larger in the diabetes+lycopene group (F), in comparison to the diabetes+corn oil group (E). Triple staining method. Bar: 20 µm

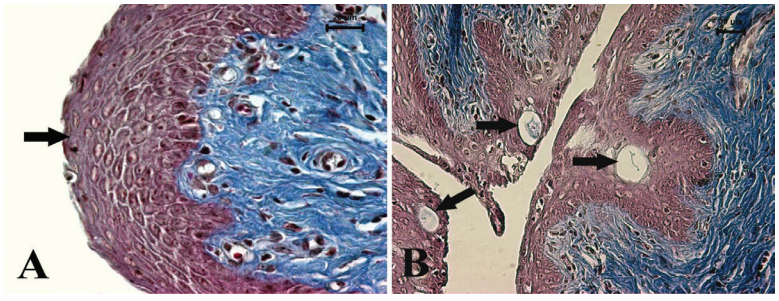

Figure 9: Lamina epithelialis of the vagina in the control+corn oil group (A) (arrow). Vacuoles in the lamina epithelialis of the vagina in the diabetes+corn oil group (arrows). Triple staining method. Bar: 30 µm

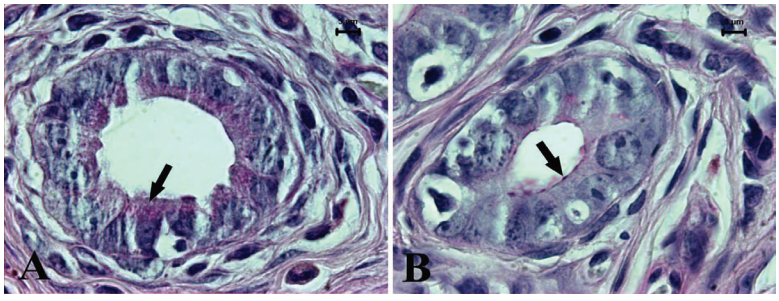

Figure 10: PAS-positive reaction in the glandular epithelium of the uterus in the control+corn oil (A) and diabetes+corn oil (B) groups. It can be seen that the intensity of the PAS-positive reaction was lower in the glandular epithelium of the uterus in the diabetes+corn oil group, in comparison to the control+corn oil group (arrows). PAS method. Bar: 5 µm

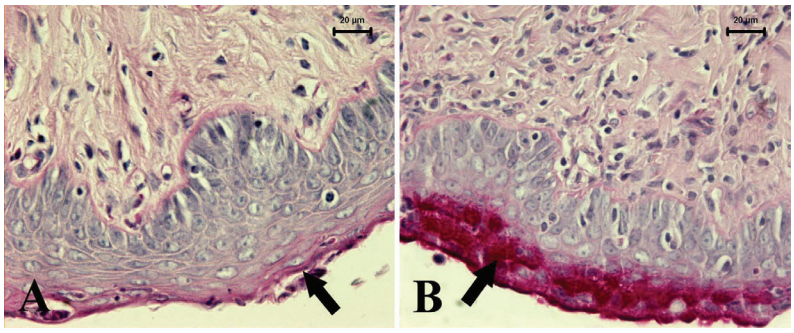

Figure 11: Vaginal sections in the control+lycopene (A) and diabetes+lycopene (B) groups. It can be seen that the intensity of the PAS-positive reaction was higher in the lamina epithelialis of the vagina in the diabetes+lycopene group, in comparison to the control+lycopene group (arrows). PAS method. Bar: 20 µm

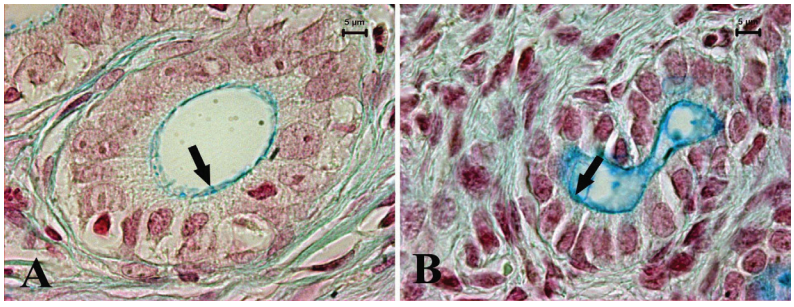

Figure 12: AB pH: 2.5 positive reaction in the glandular epithelium of the uterus in the control+corn oil (A) and diabetes+corn oil (B) groups. It can be seen that the intensity of the AB positive reaction was higher in the glandular epithelium of the uterus in the diabetes+corn oil group, in comparison to the control+corn oil group (arrows). AB pH: 2.5 staining method. Bar: 5 µm

Discussion

Diabetes mellitus causes various complications in the uterus and vagina. Garris and Smith (4) determined that the endometrial stroma and secretory activity of the epithelium of the uterus were disrupted by the effects of diabetes. In addition, Tatewaki *et al.* (24) showed that the lipid deposits increased in the uterine epithelium of diabetic animals. In our study, it was seen that the number of glands in the endometrium was lower in the diabetes+corn oil group, in comparison to the control+corn oil group. Based on these results, it can be seen that the secretory activity might change in the uterus with the effects of diabetes. It has been reported that the submucosal vasculature (10) and α-smooth muscle actin decreased in the vagina, but the amount of collagen and the apoptosis rate increased with the effects of diabetes (8). In this study, vacuoles were observed in the lamina epithelialis of the vagina in the diabetes groups; therefore, it was confirmed that diabetes mellitus led to structural disruptions in the vagina.

The effects of diabetes on the histometrical measurements of the uterus and vagina have been reported in various studies. In the uterus, it has been reported that diabetes decreased the height of the uterine epithelium (4) and the thickness of the myometrium (24). Similarly, Kim *et al.* (25) and Cushman *et al.* (9) determined that diabetes resulted in the thinning of the epithelium and a decrease in the muscularis area in the vagina. Park *et al.* (10) showed that the fibrosis risk increased in the vagina, with diabetes. In this study, it was observed that the thickness of the endometrium in the uterus, the height of the lamina epithelialis and thickness of the tunica muscularis in the vagina were smaller in the diabetes+corn oil group, in comparison to the control+corn oil group. Therefore, the secretory activity may decrease in the uterus, and structural atrophy may occur, with the effects of diabetes in the uterus and vagina. Moreover, the gland area in the uterus was smaller in the diabetes+lycopene group, in comparison to the control+lycopene group in the study. Based on these results, it may be expressed that this result is produced in diabetic animals only. However, the height of the lamina epithelialis and thickness of the tunica muscularis were larger in the control+lycopene group, in comparison to the control+corn oil group in the study. Therefore, it may be considered that lycopene might be beneficial in healthy individuals.

Lycopene is a powerful antioxidant with some protective effects. Zhou *et al.* (26) indicated that lycopene partially alleviated pulmonary fibrosis. In addition, Atessahin *et al.* (27) stated that lycopene ameliorated pathological changes, including tubular necrosis and degeneration. It was also found that lycopene reduced myocyte (28) and myocardial

damage (29), as well as having pharmacological potential in protecting against cardiomyocyte apoptosis (30). In our study, it was determined that the thickness of the tunica muscularis was larger in the diabetes+lycopene group, in comparison to the diabetes+corn oil group in the vagina. It may be suggested that lycopene might decrease the rates of fibrosis, apoptosis, and degeneration; therefore, it might prevent atrophy in the vagina.

A positive PAS reaction has been identified in the uterus and vagina. For example, Larsen (31) reported that PAS was observed in the uterine epithelium, staining the luminal cytoplasm of the non-ciliated cells. In addition, Pluta *et al.* (32) determined that the neutral mucin content was affected by the epithelial location and that it was greater in the basal than in the apical areas of the cervix. Noci *et al.* (33) found that an accumulation of glycogen was present at the cell base of the glandular epithelium in the endometrium; however, PAS-reactive cytoplasmic granules were seen in the natural killer (NK) cells in the uterus (34, 35). In addition, Wick and Kress (36) stated that macrophages showed PAS-positive reactions in the endometrial stroma in most cases. In this study, the PAS-positive reactions were seen in the apical parts of the surface and glandular epithelia in the uterus. Furthermore, the intensity of the PAS-positive reaction in the glandular epithelium was lower in the diabetes+corn oil group, in comparison to the control+corn oil group. For this reason, it can be indicated that the neutral mucin synthesis changed with the effects of diabetes in the uterine glandular epithelium. PAS-positive cells were observed in the endometrial connective tissue; based on the literature, these could be NK cells or macrophages (34–36).

Flamini *et al.* (37) reported that the epithelium in the vagina had two to three cellular layers with PAS-positive superficial cells, which is consistent with our results. Additionally, Groot *et al.* (38) determined that the PAS-positive granules showed an increase in the superficial layer of the vaginal epithelium due to androgenic influences. In our study, a PAS-positive reaction was observed in the superficial cell layers of the lamina epithelia in the vagina, and the intensity of the PAS-positive reaction was higher in the diabetes+lycopene group, in comparison to the control+lycopene group. This shows that the neutral mucins may be higher with the effect of diabetes in the vagina, and this result is only produced in diabetic animals.

It has been reported that the non-ciliated cells of the luminal epithelium in the uterus along the apical membrane stained (positive) with AB (36). In this study, an AB pH: 2.5 positive reaction was observed in the apical parts of the surface and glandular epithelia in the uterus. In addition, it has been found that the intensity of the AB pH: 2.5 positive reaction was higher in the glandular epithelium in the diabetes+corn oil group in comparison to the control+corn oil group. Therefore, it could be said that the acid mucosubstance may be higher with the effect of diabetes in the glandular epithelium of the uterus.

In conclusion, the changes in the uterus and vagina caused by diabetes, as well as the existence of the protective qualities of lycopene (in some parameters) have been revealed for the first time. We believe that further studies increasing the lycopene dosage and the implementation duration for diabetic animals will show that lycopene has a pronounced antioxidant effect on the uterus and vagina.

Acknowledgements

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SPREMEMBE V STENI MATERNICE IN NOŽNICE PODGAN Z EKSPERIMENTALNO POVZROČENO SLADKORNO BOLEZNIJO IN VPLIV LIKOPENA NA TE SPREMEMBE

M. Yildiz, M. Sandikci

Povzetek: Namen raziskave je bil ugotoviti spremembe, ki so se zgodile v steni maternice in nožnice podgan z eksperimentalno povzročeno sladkorno boleznijo ter učinke peroralno apliciranega likopena na povzročene spremembe. Raziskava je zajela 42 štirimesečnih podganjih samic seva Wistar. Eksperimentalni diabetes je bil povzročen z enojnim odmerkom 50 mg/kg streptozotocina (STZ). Podgane iz kontrolne skupine in skupine s sladkorno boleznijo so bile naključno razdeljene v štiri skupine: kontrolna skupina podgan + koruzno olje, kontrolna skupina podgan + likopen, podgane s sladkorno boleznijo + koruzno olje in podgane s sladkorno boleznijo + likopen. Trojno barvanje po Crossmanu in metoda barvanja PAS (iz angl. Periodic Acid Schiff) sta bili uporabljeni pri histoloških rezinah maternice in nožnice. Dodatno so bile rezine maternic obarvane z barvilom alcian modro (AB). Število žlez, debelina endometrija in PAS-pozitivna reakcija žleznega epitelija v maternici so bili nižji pri podganah s sladkorno boleznijo, pozitivna reakcija AB v žleznem epiteliju pa je bila višja pri skupini podgan s sladkorno boleznijo v primerjavi s kontrolno skupino. Višina epitelija ter debelina mišične plasti stene maternice sta bili nižji pri podganah s sladkorno boleznijo, zanimivo pa je, da je likopen pozitivno vplival na debelino mišične plasti, saj je bila ta pri podganah s sladkorno boleznijo, ki so dobivale likopen, višja kot pri podganah, ki likopena niso dobivale.

Ključne besede: eksperimentalna sladkorna bolezen; likopen; podgana; maternica; nožnica

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Original Research Article

DYNAMICS OF SOME VAGINAL PARAMETERS IN NON-PREGNANT BITCHES AFTER MID-LUTEAL AGLEPRISTONE TREATMENT

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Summary: The aim of our study was to investigate the effects of the antiprogestin aglepristone after its application in bitches with high serum progesterone concentrations on some specific vaginal parameters. Twelve non-pregnant female dogs from different breeds and ages were included in the study. The bitches were divided into two groups. Group I (n = 6) received aglepristone (10 mg/kg, subcutaneously) injected twice, 24 h apart, on days 29 and 30 after the estimated day of ovulation, which was determined by progesterone assays. Group II (n = 6) served as a control group and received a placebo. The electrical resistance of vaginal mucus, vaginal pH and serum P_4 levels were determined on days 29, 30, 33, 36, 39, 42 and 45 after ovulation. Additionally, vaginal smears were performed to evaluate the changes in vaginal cells. Partial luteolysis was detected at day 32.5 ± 2.26 (mean ± SD) and 44 ± 1.73 in treated and control bitches, respectively ($p < 0.001$). Complete luteolysis ($P_4 < 2$ ng/ml) was observed on day 41.5 ± 2.26 in treated bitches. Beginning on the day after the first treatment, a decrease in electrical resistance of vaginal mucus was measured in the experimental group. A significant reduction ($p < 0.05$) occurred on day 41.5±2.26 compared with day 29. The pH of vaginal secretions of all bitches in Group I increased during the period starting from the day after the first treatment. A significant difference ($p < 0.05$) was found on day 32.5 ± 2.26. No changes were detected for either parameter in the control group. The cell populations in vaginal smears of all animals were similar. In conclusion, aglepristone administration to dogs during the mid-luteal stage influenced vaginal pH and the electrical resistance of vaginal mucus.

Key words: vagina; bitch; aglepristone; electrical resistance; pH

Introduction

The vagina and the vestibulum are important structures for the reproductive health of female dogs. Determination of specific vaginal parameters such as electrical resistance, pH and type of cell populations could provide some information about the local or general health of the animal.

Over the past decade, the use of antiprogestins has increased. They are competitive progesterone antagonists, which bind to specific receptors, causing their structural transformation, preventing hormonal biological effects (1).

The progesterone antagonist aglepristone is commonly used for induction of abortion or parturition in bitches, as well as for treatment of various gynaecological disorders associated with high serum progesterone levels. There are many studies on the use of aglepristone, involving induction of abortion (2), conservative treatment of pyometra (2-6), cystic endometrial hyperplasia (7), acromegaly, insulin resistance (8) and mammary GH-induced IGF-1 secretion (9). Although the

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effect of antiprogestogens on the uterus, ovarian function and mammary gland in the bitch has been investigated, little is known about their effect on external genitalia, especially on the vagina.

The aim of the present study was to investigate the effects of the antiprogestogen aglepristone on some specific vaginal parameters after its administration to bitches with high serum progesterone concentrations.

Materials and methods

Twelve non-mated bitches from different breeds (American Cocker Spaniel-2, English Cocker Spaniel-2, Samoyed-2, Golden retriever-1, German Hunting Terrier-1, mixed breeds-4) and ages (3-6 years, mean ± SD 4.08 ± 1.16) in diestrus without any reproductive problems were included in the study. All animals were clinically and gynaecologically healthy. The study has started 29 days after ovulation day, which was indicated by serum progesterone (P4) levels between 4-10 ng/ml during the last estrus.

The animals were divided into treatment (Group I) or control (Group II) groups. On day 29 and 30 after ovulation, Group I (n = 6) received 10 mg/kg body weight aglepristone (Alizine®, Virbac Laboratories, Carros, France) subcutaneously, while Group II (n = 6) received saline solution (0.3 ml/kg body weight, subcutaneously).

Blood samples were collected from each bitch on days 29, 30, 33, 36, 39, 42 and 45 after ovulation by venipuncture of the cephalic vein. Blood vials without anticoagulant were used and centrifuged (3000 X g for 15 min). The sera were stored at -20°C until assayed for serum progesterone levels. Serum progesterone levels were measured by an enzyme immunoassay (EIA) using a progesterone kit (Human, PROG ELISA, GmbH, Germany). The analytical sensitivity of the progesterone ELISA test was 0.03-0.07 ng/ml (range: 0 - 40 ng/ml) with an intra-inter assay coefficient of variation < 10%.

Vaginal electrical resistance and pH levels were determined and vaginal smears were performed on the same days, immediately following the blood collection.

Vaginal electrical resistance was measured using the "Draminski Dog Ovulation Detector" (Draminski®, Poland). The vulva was cleaned with a dry paper towel and the probe was inserted into the vagina. The button was then pressed three times with full rotation (360˚), so that the electrodes came into full contact with the vaginal mucus. The result was recorded on a checklist. Vaginal pH levels were determined using two types of strips (4.0-7.0 and 6.5-10.0) (Merck KGaA). A speculum was inserted and indicator strips were placed on the vaginal wall for at least 3 seconds. The changes in the colour of the indicator were compared with the reference table for both strips.

Vaginal smears were collected with sterile cotton swabs (size Ø2.5x170) to evaluate the changes in vaginal cells, and stained with Haemacolor® (Merck KGaA). A minimum of 10 fields of view were observed with a light microscope at magnifications of 160 to 400 X. Cells from the vaginal wall were differentiated as basal, parabasal, intermediate, superficial or keratinized (10).

Partial luteolysis was defined on the basis of a 50% decrease in serum P_4 concentrations from the mean pre-treatment value, while complete luteolysis was defined at P_4 concentrations below 2 ng/ml (11).

The results were expressed as mean ± SD and analysed using ANOVA for repeated measures. $P \leq 0.05$ was considered significant.

Results

The results for aglepristone treatment effects on P_4 levels, vaginal electrical resistance and vaginal pH are presented on Figures 1, 2 and 3, respectively.

Before treatment, the mean serum progesterone concentrations in treated (Group I, n = 6) and control (Group II, n = 6) bitches were 60.33±27.39 and 57.29±24.21 ng/ml, respectively. Partial luteolysis was observed at day 32.5±2.26 and 44±1.73 in the treated and control groups, respectively ($p<0.001$). Complete luteolysis (P4<2 ng/ml) was observed at day 41.5±2.26 only in Group I.

The electrical resistance of vaginal mucus of all animals in Group I decreased, starting on the day after the first treatment and continued to decrease after the second administration; a statistically significant difference ($p<0.05$) was observed in all Group I bitches on day 42 after ovulation, i. e. after the complete luteolysis (41.5±2.26). In Group II bitches, vaginal electrical resistance was not reduced.

Vaginal pH values increased in all Group I bitches, beginning on the day after the first and

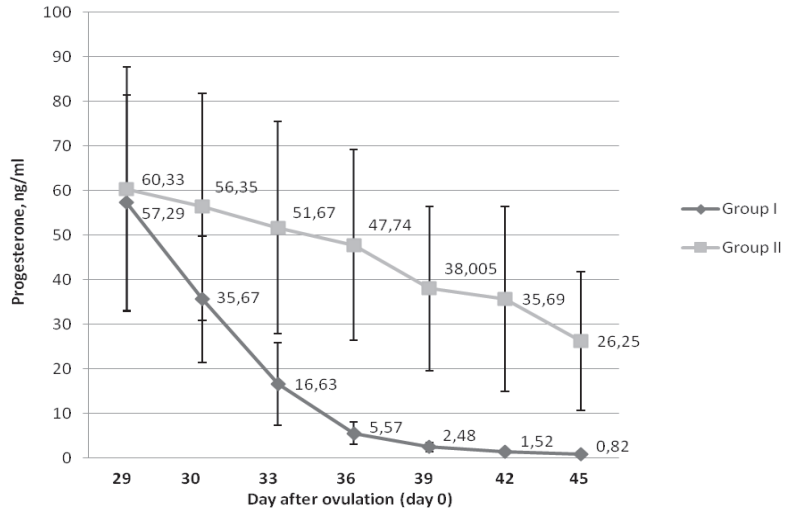

Figure 1: Serum P_4 levels in treated (Group I, n = 6) and control (Group II, n = 6) bitches

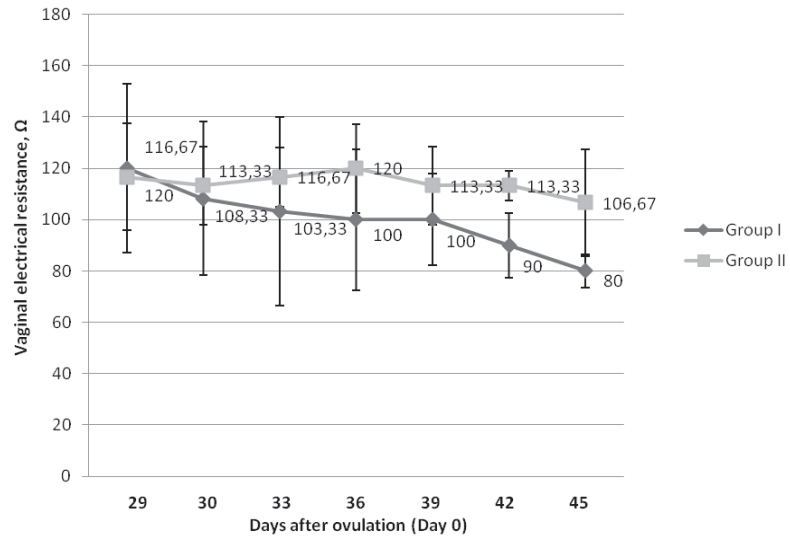

Figure 2: Vaginal electrical resistance in treated (Group I, n = 6) and control (Group II, n = 6) bitches

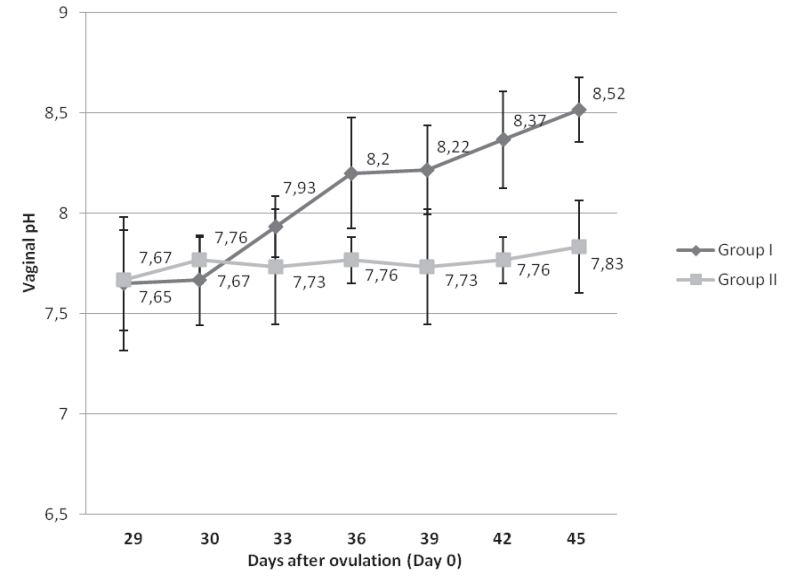

Figure 3: Vaginal pH in treated (Group I, n = 6) and control (Group II, n = 6) bitches

kept on increasing after the second treatment. A statistically significant difference ($p<0.05$) was observed in all bitches on day 33 post ovulation, i.e. after partial luteolysis (32.5±2.26) compared with day 29. This decrease persisted in the subsequent days of measurement. In Group II, vaginal pH also increased, but the difference was not significant until day 45.

The cell populations in vaginal smears of all animals in Groups I and II were similar and consisted of parabasal cells, small intermediate cells and neutrophil leukocytes.

Discussion

According to previous studies (12, 13), antiprogestogens do not have direct or indirect luteolytic effects in female dogs (in contrast to humans). Aglepristone administered to bitches during the early luteal stage decreased the interestrous interval without effect on the duration of the luteal stage (14). Injected in the mid-luteal stage, the same antiprogestogen induced early luteal regression and a decrease in serum progesterone levels in treated bitches (11). In our study, we also treated the animals during the mid-luteal stage (days 29 and 30 after ovulation) and found similar results about the luteal function.

Our study is the first investigation of the dynamics of specific vaginal changes in bitches after the termination of the biological activity of progesterone. The results confirm that these vaginal parameters depend on progesterone concentration, especially the changes in vaginal electrical resistance. Until now, most studies have only demonstrated the influence of progesterone levels and dynamic changes of this parameter depending on the LH surge and estrogen levels in females (15).

There are two key aspects in the interpretation of the results. The first is related to the time of statistically significant changes in the values of the parameters after aglepristone administration.

The changes in vaginal impedance after aglepristone treatment occurred only after a statistically significant decrease in serum progesterone levels. Substantial changes in vaginal fluid pH were found even earlier, on the 4th day after the first aglepristone administration, when serum progesterone levels were still relatively high. It is well known that the environmental pH influences the development of microorganisms and the effect of some therapeutic agents. The increase in pH resulting from the aglepristone treatment should be taken into consideration in decision-making for optimal therapy for diseases of the genital organs.

According to other reports (15-20) as well as previous works of our group (21, 22), vaginal electrical resistance and pH during proestrus and estrus are extremely dynamic parameters that vary over a very short interval of time: within 1-2 days. It is believed that this is a response to constantly changing levels of progesterone and estrogen and to the LH peak (15). Measurement of vaginal resistance and pH during estrus helps determining the optimal time for insemination. Changes in these parameters following aglepristone administration during the diestrus depend on the period of time after progesterone receptors are blocked, which is ultimately followed by a drop in progesterone levels. Similarly, the occurrence of abortion is 6-7 days after antiprogestin administration (23). It is well known that progesterone is required to maintain pregnancy.

The second aspect involves the direction of the changes in studied parameters following the administration of aglepristone. It was detected that the vaginal resistance decreases and in the meantime the pH increases. Similar changes occur during estrus, after ovulation, when the levels of progesterone are constantly increasing (16, 18, 21, 22). Future studies should address the exact mechanisms of these changes in vaginal resistance and pH.

The absence of changes in the vaginal cell populations after the use of antiprogestins during diestrus confirm the dominant role of estrogens in the changes of vaginal wall's cell populations.

In conclusion, the present study demonstrates that the treatment of bitches with the antiprogestogen aglepristone during diestrus results in statistically significant decrease in vaginal electrical resistance on the 14th day after the 1st injection, when the serum progesterone levels were lower than 2 ng/ml, as well as a significant increase in the pH of vaginal fluids on the 4th day after the 1st injection, when partial luteolysis was detected. A change in the vaginal cell populations was not detected.

Acknowledgements

We thank all the breeders who have accepted to participate in our study.

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DINAMIKA NEKATERIH PARAMETROV NOŽNICE PRI NEBREJIH PSICAH PO TRETIRANJU Z AGLEPRISTONOM PO SREDNJI LUTEALNI FAZI

A. Antonov, P. Georgiev, J. Dineva, T. Conze, R. Dimitrova, A. Wehrend

Povzetek: Namen naše raziskave je bil proučiti učinke aplikacije antiprogestina aglepristona psicam z visoko koncentracijo progesterona v serumu, na nekatere specifične nožnične parametre. V raziskavo je bilo vključenih dvanajst nebrejih psic različnih pasem in starosti. Psice so bile razdeljene v dve skupini. Skupina I (n = 6) je prejela aglepristone (10 mg/kg subkutano), injicirano dvakrat v 24 urah, 29. in 30. dan po predvideni ovulaciji, ki je bila določena z merjenjem progesterona. Skupina II (n = 6) je služila kot kontrolna skupina in je prejemala placebo. Električna upornost nožnične sluzi, nožnični pH in serumski nivo P4 so bili izmerjeni 29, 30, 33, 36, 39, 42 in 45 dni po ovulaciji. Poleg tega so bili odvzeti nožnični brisi za vrednotenje sprememb v celicah nožnice. Delna luteoliza je bila odkrita 32,5. ± 2,26 dan (povprečje ± SD) pri skupini zdravljenih psic in 44. ± 1,73 dan pri skupini kontrolnih psic ($p < 0,001$). Popolna luteoliza ($P_4 < 2$ ng/ml) je bila opažena 41,5. ± 2,26 dan pri zdravljenih psicah. Na dan po prvem tretiranju je bilo izmerjeno zmanjšanje električnega upora vaginalne sluzi, izmerjene v eksperimentalni skupini psic. Značilno zmanjšanje ($p < 0,05$) je bilo zaznano 41.5. ± 2,26 dan v primerjavi z 29. dnem. pH nožničnih izločkov vseh psic v skupini I se je povečal v obdobju od dneva po prvem tretiranju. Značilne razlike ($p < 0,05$) so bile vidne 32,5. ± 2,26 dan. V kontrolni skupini niso zaznali sprememb pri vseh parametrih. Populacije celic v vaginalnih brisih vseh živali so bile podobne. Iz opisane raziskave lahko zaključimo, da dodajanje aglepristona psicam v srednji lutealni fazi vpliva na nožnični pH in na električno upornost nožnične sluzi.

Ključne besede: vagina; psica; aglepristone; električna upornost; pH

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Original Research Article

BLINDED PLACEBO STUDY OF BILATERAL OSTEOARTHRITIS TREATMENT USING ADIPOSE DERIVED MESENCHYMAL STEM CELLS

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Summary: Mesenchymal stem cell therapies attract a lot of attention and also controversy in veterinary medicine. In the present study, mesenchymal stem cell therapy, using autologous cells from adipose tissue was evaluated for the treatment of osteoarthritis in the knee. Ten dogs with bilateral osteoarthritis in the knees were included in the study. After collection of adipose tissue and expansion of cells, 2 to 3 millions of mesenchymal stem cells were injected into one knee while sterile phosphate buffer (same as used for resuspension of cells) was injected into the other knee as a placebo. Dog owners were blind in regard which knee received cells and which placebo. Dogs were clinically evaluated before treatment and 3, 6 and 12 months after the treatment, and synovial fluid was collected and evaluated before treatment and after 12 months. Radiographs of both knees were taken before treatment and 6 and 12 months after the treatment. In 9 out of 10 dogs enrolled in the study, there was a significant improvement of lameness after 1 year. Radiographs did not show improvement in the cartilage condition 1 year after the treatment. However, in 7 out of 10 treated joints the osteoarthritis did not progress while in all 10 of placebo treated joints there was a significant worsening of the osteoarthritis. Analysis of the synovial fluid before and 1 year after the treatment showed no statistically significant differences between mesenchymal stem cells treated and placebo treated knees. Results of this study suggest beneficial effects of mesenchymal stem cells treatment in osteoarthritis in dogs and confirm that mesenchymal stem cells treatment is a viable option for managing this debilitating disease in dogs.

Keywords: dog; osteoarthritis; knee; mesenchymal stem cells

Introduction

Osteoarthritis is a common disease in dogs that affects dogs of all ages. Although large joints such as hips, elbows and knees are most often diagnosed with osteoarthritis, this disease could affect many other joints including vertebral facet joints, carpal and tarsal joints, and also metacarpal and metatarsal joints (1, 2). The incidence of osteoarthritis in general population of dogs is about 20 %, while in aging (older than 8 years) dogs, it can reach up to 80 % (3). Clinical signs of osteoarthritis are very variable, but most common signs are pain and reduced physical functioning of dogs (1, 2). Currently, osteoarthritis cannot be cured and dog owners are usually offered pain management. With proper pain management the quality of life of dogs could be improved, although the disease cannot be cured and regular pain management therapy is both financial and life-style burden for dogs and their owners, and could have significant side effects (1, 2).

In recent years, stem cells have attracted a lot of attention as potential source of biological agents for regenerative treatments. Basic studies in laboratory rodents are slowly translated into clinical settings.

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Stem cells are generally divided into three categories: (i) embryonic stem cells that are obtained from very early embryos, (ii) adult stem cells that can be found in different adult tissues, and (iii) induced pluripotent stem cells which are adult differentiated cells turned into stem cells by activation of several genes in these cells (4-7). Adult stem cells are most often derived from adipose tissue or bone marrow and are usually called mesenchymal stem cells (8). These are currently being exploited in numerous basic studies and clinical trials. Adult mesenchymal stem cells have several important advantages for potential clinical use as they can be easily obtained from different tissues, and they are suitable for autologous treatments as they can be obtained from diseased patients (8).

Several basic and clinical studies have suggested beneficial effect of adult mesenchymal stem cells in the treatment of osteoarthritis in different species from laboratory animals to dogs and horses (9-14), although there is still an urgent need for more clinical trials to confirm the benefit of stem cell treatments in dog osteoarthritis.

In the present study, we have therefore evaluated the efficacy of mesenchymal stem cell treatment for knee osteoarthritis in adult, clinical patients, dogs.

Material and methods

Patients

Ten dogs with bilateral osteoarthritis were included in the current study. All dogs were clinical patients at the Clinic for small animals at Veterinary faculty, University of Ljubljana. Five of them were German boxer and five were other breeds. Five were male and five were female. Average age of dogs was 6.2 years. Dog owners voluntarily participated in the study after being informed about all potential risks and benefits of such trial treatments and have signed an informed consent (in Slovenian language). All animal procedures were performed according to the best practice of Veterinary care. As study was done on a client owned clinical patients, no approval from ethical committee was needed according to the Slovenian legislation.

Before treatment all dogs were clinically, neurologically and radiographically examined and the osteoarthritis was confirmed in both knee joints. All dogs were negative to tick diseases and had no sign of any infection.

On day one all dogs were lame. Lameness was divided into five groups according to Brunnberg (15) from 0 to IV. O = normal; I = hardly noticeable lameness; II = noticeable, leg is still loaded; III = noticeable, leg is occasionally not loaded; IV = leg is not loaded.

On radiographs all dogs had severe signs of osteoarthritis and were also divided in five groups according to Brunnberg (28) 0 = normal; I = minimal osteophyte formation; II = obvious osteophyte formation; III = multiple moderate osteophyte formation; IV = large osteophyte formation and deformity of bone ends.

Tissue collection

Dogs were premedicated with morphine (Morphini chl., Alkaloid, Skopje Macedonia) 0.3 mg/kg s/c, and anesthesia was induced with midazolam (Midazolam Torrex, Chiesi Pharmaceuticals, Manchaster, UK) 0.1 mg/kg iv and propofol (Norofol, Norbrook Laboratories, Corby, UK) 3 - 4 mg/kg iv and maintained with sevoflurane (Sevorane, Abbott, Maidenhead, UK) in oxygen. Dogs were administered Lactated ringer's solution (Hartmann solution, B Braun, Melsungen, Germany) during anesthesia (5 ml/kg/h i/v). Adipose tissue was collected through small incision on the back between scapulae. Tissue was immediately placed in the transport media and transported into the cell culture laboratory.

Cell preparation

Tissue was dissected into small pieces and incubated with 1 mg/ml collagenase (Sigma, Taufkirchen, Germany) overnight. Following day, digested tissue was centrifuged at 3000 g for 5 minutes. Supernatant was discarded and pellet of cells was resuspended in cell culture media containing DMEM and 10 % fetal bovine serum. Cells were plated into 6 well plates and grown in standard conditions at 37 ^{o}C and 5 % CO2. After reaching confluency, cells were trypsinyzed and transferred into larger, 25 cm^2 dish, where they were grown again until confluency. Confluent cells from 25 cm^2 dish were trypsinized, centrifuged at 3000 g for 5 minutes, washed with 1 ml of PBS, centrifuged again and finally resuspended in 1 ml of sterile PBS.

Cell injection

Two to three millions of cells were placed in the syringe and transported to the orthopaedic veterinarian. Dogs were again anaesthetized. Cells were injected directly into one of the ostheoarthritic knees, while sterile PBS was injected as placebo into the other knee. Prior to application, synovial fluid was collected from both knees, and examined for appearance, total protein, albumins, total cell count, alkaline phosphatase (ALP) and alanine transaminase (ALT).

Clinical evaluation

All dogs were examined 3, 6 and 12 months after stem cell application. During clinical examination, differences in lameness, joint pain, signs of inflammation or infection, neurologic deficit and any other diseases were observed. At the same time the owners were asked about their observations.

Radiography

Radiographs of all dogs were taken 6 and 12 months after the treatment and examined for any signs of improvement or progression of osteoarthritis and any signs of infection or other pathologic bone conditions.

Synovial fluid analyses

Synovial fluid was collected at the time of stem cell injection and 1 year after the treatment and also examined for any signs of osteomalacia, infection, inflammation, bone destruction.

Results

Clinical evaluations

All dogs were examined 3, 6 and 12 months after treatment. Before treatment, 7 dogs showed signs of limping scored by 2 according to Brunnberg scale, and 3 dogs showed limping scored by 3. One year after treatment, limping was improved in all but one dog and results for all individual dogs are presented in figure 1.

Radiographic evaluations

Dogs were subjected to radiography before treatment and at 6 and 12 months after the treatment (Figure 2). Although there was no significant improvements in the size of osteophytes in the joints observed on autoradiographs after 12 months, in all (10 out of 10) joints that received placebo treatment there was worsening of the joint status, while in 7 out of 10 joints treated with mesenchymal stem cells the radiographic score in the joints remained the same, suggesting that stem cell treatment did stall the progress of osteoarthritis. The difference was statistically significant using chi-square test with $p < 0.001$ (Table 1).

Synovial fluid examination

Concentration of total protein content, albumins, alkaline phosphatase and alanine aminotransferase did not differ significantly between treated and untreated joints, and before and after the treatment (Fig 3a and 3 b), suggesting that stem cell application did not cause any long

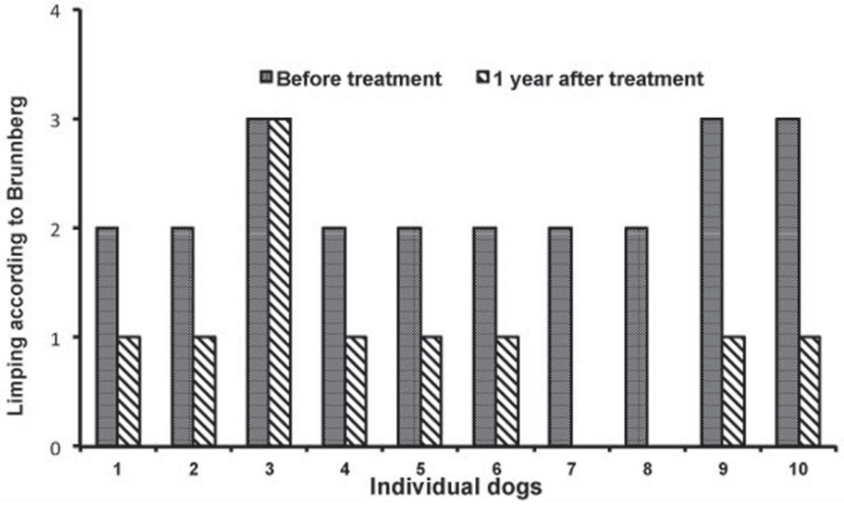

Figure 1: Limping in dogs before treatment and one year after treatment scored according to Brunnberg scale. In all but one dog significant improvement in limping was observed

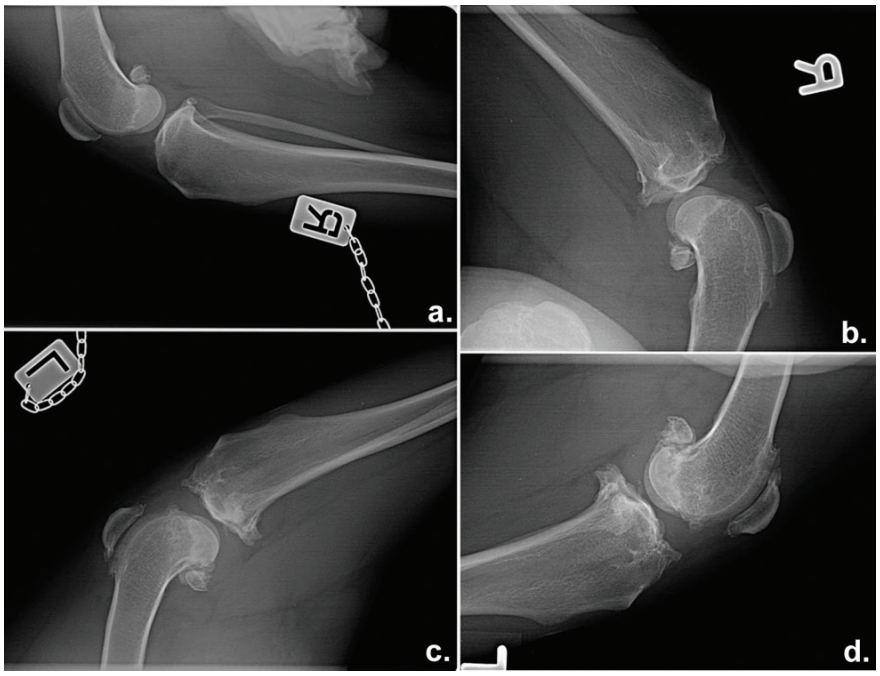

Figure 2: Representative X-ray photographs of both knees from same dog before treatment (a, c) and 12 months after treatment (b, d). In the placebo treated knee, osteoarthritis worsened during one year significantly (a - before treatment, b - 12 months after treatment) while in MSC treated knee, the osteoarthritis did not progress (c – before treatment, d – 12 months after treatment). Similar situation was observed in seven out of 10 dogs

Table 1: Autoradiographic evaluation revealed that size of osteophytes increased in all placebo treated joint while in 7 out of 10 joints treated with MSC the osteoarthritis did not progress (*Chi^2 $p < 0.001$)

Radiographic evaluation before and 12 months after treatment	MSC treated joint	Placebo
Same	7*	0
Worse	3*	10

lasting inflammation of the joints. Similarly, total number of cells per ml was similar between treated and untreated joints and before and 1 year after treatment (Fig. 3c). All values were within the physiological levels for synovial fluid.

Discussion

Ostheoarthritis is debilitating disease that affects many dogs and could affect different joints (1, 2). Currently, there is no treatment for this disease. In the present study, we have therefore evaluated clinical and autoradiographic outcomes of mesenchymal stem cell treatment in dogs, suffering from osteoarthritis in both knees.

Osteoarthritis is very prevalent in older dogs, where it can affect up to 80 % of dogs over 8 years old, but it could also affect much younger dogs (3). Although some of the symptoms of osteoarthritis can be eased with pain relief therapy, the disease is currently incurable and presents an important financial and psychological burden for dog owners. Current methods for managing osteoarthritis include classical pain relief and nonsteroidal antiinflamatory drugs, often combined with functional foods, physical therapy and alternative therapies such as acupuncture. In severe cases, surgery and joint replacement could be also performed, but this is very costly procedure and therefore not suitable for average dog owner (2).

Stem cells from different sources have attracted a lot of attention in recent years due to their potential use in regenerative medicine. Several studies, both preclinical and clinical in dogs and also in humans have provided some evidence that mesenchymal stem cells might have beneficial effects in the treatment of osteoarthritis (9-11, 14). However, there are still very few clinical studies that would confirm positive effects of mesenchymal stem cells in clinical patients. In the present study mesenchymal stem cells obtained from adipose tissue of dogs, affected by severe

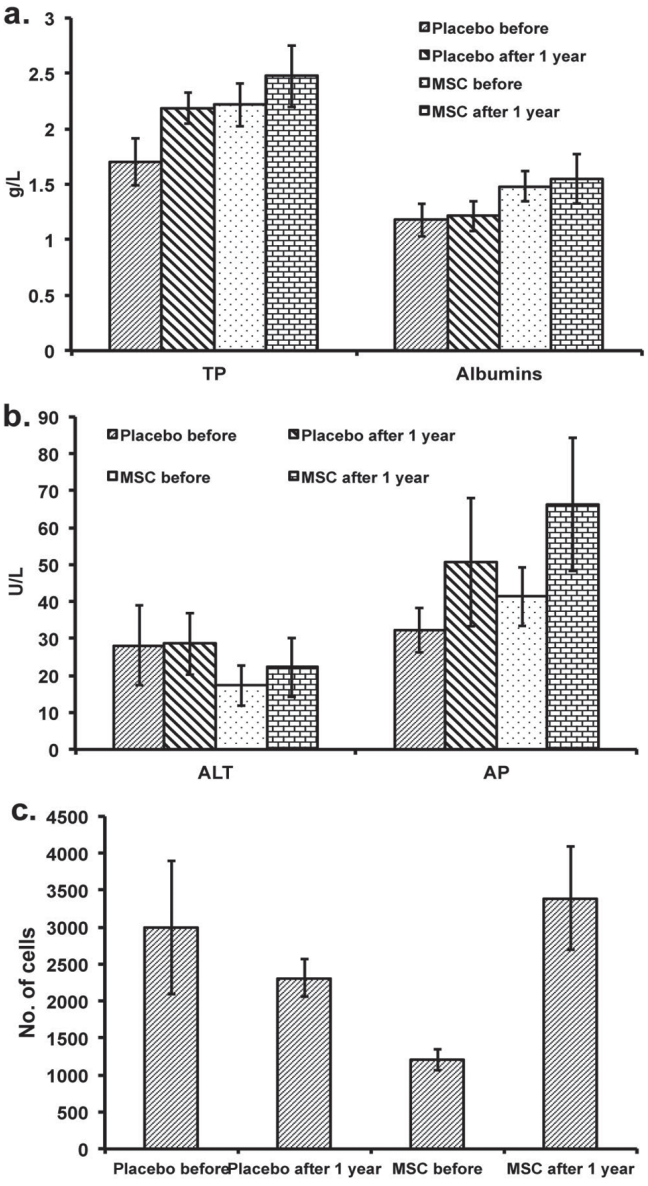

Figure 3: Total protein content (TP) and albumins (a), alanine aminotransferase (ALT) and alkaline phosphatase activity (AP; b) and number of cells (c) were similar in MSC treated and placebo joints before and after the treatment, suggesting that MSC application did not cause any long lasting inflammation of the joints. All values were within the physiological levels for synovial fluid

ostheoarthritis, were used for the treatment of dogs with bilateral osteoarthritis in the knee. Adipose tissue derived stem cells were chosen as they are easily obtainable in large quantities from adipose tissue of dogs, affected by osteoarthritis.

Clinical evaluations of our patients revealed significant improvement in limping as assessed by Brunneberg scale (15). Interestingly, the first clinical signs of improvement were noticeable only 2 to 3 months after treatment, and were very obvious one year after treatment. Although some owners reported improvements much earlier, this was not confirmed during clinical examination and is presumably placebo effect on dogs' owners. Interestingly, although there was a marked clinical improvement, autoradiographic evaluations 12 months after the treatment did not reveal improvements in the joint structure in any of the treated knees. However, in comparison to untreated joints, the stem cell therapy seemed to slow down or even stopped degenerative processes, as in 7 out of 10 treated knees, the autoradiographic evaluation did not show any progression of disease while in all 10 knee joints receiving placebo treatment, osteoarthritis have significantly progressed. Interestingly, 4 out of 10 dogs came to the clinic again 18 months after the treatment and underwent x-ray imaging again. In all four dogs, there was an improvement of joint surface (significant reduction of osteophytes) in treated joints only.

Although several studies have shown beneficial effects of mesenchymal stem cell treatments in osteoarthritis in different species, it is not known how is this beneficial effect achieved. Several studies, mostly in rodents, have tried to track mesenchymal stem cells after injection into affected joints and most studies could not find any evidence of stem cells engrafting into the cartilage or bone tissue (16-20), although some engrafting cells were detected in synovial membranes and menisci in rabbits and goats (16, 19). Dessando et al. (16) have shown that in rabbits, joints treated with MSC had reduced expression of tumour necrosis factor alpha, an inflammatory cytokine, and reduced expression of matrix metaloproteainase 1. Similarly, studies in mice (21), goats (19) and horses (22) have shown anti-inflammatory effects of MSC in osteoarthritic joints. Therefore, it is currently thought that mesenchymal stem cells effect in osteoarthritis might be more due to their anti-inflammatory effects, rather then regenerative capabilities. This is supported by our study as we did detect significant clinical improvement in dogs, but that was not, at least one year after the treatment, accompanied by the improvement in the structure of the joints.

As described before, results of our analysis of the inflammatory response following the application of MSC suggest that the application of stem cells did not cause any long-lasting inflammation in the joint. Nevertheless we have only used synovial fluid as a sample for the analysis due to the non-invasiveness of the collection method, as our study was done on owned dog patients and any more invasive procedures would be unethical. Therefore, the possible effect of stem cells on the local inflammation and concentrations of the immune system mediators in the synovial membranes was not examined. However, there is a substantial body of evidence showing that the secretom of mesenchymal stem cells does reduce the local inflammation in the synovial membrane and in the extracellular matrix of the cartilage. The mediators involved in this process are thought to be Indolamin-2,3-dioxigenase-1, Interleukin 6, Tumor factor alpha and Tumor factor beta (23-25). These mediators would serve as good markers of local inflammatory response for further investigation.

Some studies have reported only short to medium term beneficial effects of MSC treatment in osteoarthritis (26, 27). However, in our study, we have found that beneficial effect of MSC treatment was prolonged and was evident by clinical examination at least a year after the treatment, and even more interestingly, the improvement of the cartilage appeared in some dogs only 18 months after the treatment. We were not able to trace the cells in the dogs, as all dogs were clinical patients and not laboratory dogs, and therefore we could not used labeled cells. Therefore, we do not know if there was an engraftment of MSC in our study, although prolonged effect of MSC does suggest that stem cells must have engrafted into some tissues. However, this does not mean that stem cells had to engraft into the cartilage and differentiate into cartilage tissue, perhaps, as suggested by previous studies, MSC engraft only in soft tissues but nevertheless secret anti-inflammatory substances and trophic factors that contribute to the healing of the joint from these tissues.

Presented blind placebo study of bilateral knee osteoarthritis shows that MSC treatment had beneficial effect in almost all treated dogs with the improvement of clinical signs. Although there was no evident improvement in radiographs 1 year after the treatment, it appears that stem cell treatment did stall the progress of osteoarthritis as in all joints receiving placebo, severity of osteoarthritis progressed in one year while in majority of MSC treated joints there was no progression of osteoarthritis. This study therefore suggests a positive effect of MSC treatment for managing osteoarthritis in dogs.

Acknowledgment

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SLEPA PLACEBO RAZISKAVA ZDRAVLJENJA VNETJA KOLENSKEGA SKLEPA Z MEZENHIMSKIMI MATIČNIMI CELICAMI

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Povzetek: Zdravljenje z mezenhimskimi matičnimi celicami v zadnjih letih priteguje veliko pozornosti in tudi razprav v veterinarski medicini. V predstavljeni raziskavi smo preverjali učinkovitost zdravljenja z matičnimi celicami pri ventju kolenskih sklepov pri psih. V raziskavo je bilo vključenih 10 psov z vnetjem obeh kolenskih sklepov. Psom je bil odvzet košček maščobnega tkiva, iz katerega smo osamili in pripravili mezenhimske matične celice. Dva do tri milijone celic v sterilnem fosfatnem pufru smo nato injicirali v eno obolelo koleno, medtem ko smo v drugo obolelo koleno injicirali samo fosfatni pufer kot placebo. Lastniki psov niso vedeli, v katero koleno so psi prejeli celice in v katero koleno placebo. Psi so bili klinično pregledani 3, 6 in 12 mesecev po zdravljenju. Sinovialno tekočino smo iz sklepa odvzeli ob vnosu matičnih celic ter 12 mesecev po zdravljenju. Ob začetku zdravljenja ter 6 in 12 mesecev po zdravljenju smo naredili tudi rentgenske posnetke obeh kolenskih sklepov. Od 10 zdravljenih psov smo po enem letu pri devetih opazili značilno izboljšanje šepanja po enem letu. Rentgenske slike po enem letu sicer niso pokazale izboljšanja sklepne površine, vendar pa pri 7 od 10 zdravljenih sklepov ni prišlo do napredovanja bolezni, medtem ko je pri vseh 10 nezdravljenih sklepih prišlo do značilnega poslabšanja sklepne površine (večje število oziroma velikost izrastkov). Analiza sinovialne tekočine ob začetku zdravljenja ter eno leto po zdravljenju ni pokazala statistično značilnih razlik v preiskovanih parametrih, kar kaže, da v sklepih ni prišlo do vnetnih sprememb kot posledica zdravljenja z mezenhimskimi matičnimi celicami. Rezultati te raziskave kažejo pomembne učinke mezenhimskih matičnih celic pri zdravljenju vnetja sklepov pri psih in potrjujejo, da je takšno zdravljenje primeren način zdravljenja te nevarne kronične bolezni psov.

Ključne besede: pes; vnetje sklepov; koleno; mezenhimske matične celice

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Case Report

Brevundimonas vesicularis SEPTICAEMIA IN A KID WITH CONGENITAL GOITRE

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Summary: In this article a case of septicaemia, caused by *Brevundimonas vesicularis* in a newborn pygmy goat with congenital goitre, is described. *B. vesicularis* is an aerobic non-sporulating non-fermenting motile Gram-negative bacillus, ubiquitous in the environment. It is susceptible to aminoglycosides and anti-pseudomonal penicillins and resistant to ampicillin and cephalosporins. To date, *B. vesicularis* has been isolated from water, soil, plants and several human clinical specimens, mostly in immunosuppressed patients causing endocarditis, arthritis, keratitis and septicaemia. It has never been reported to cause any problems in animals.

A newborn pygmy goat kid died 18 hours after a normal parturition. Prior to death, no clinical abnormalities were observed, the kid was normally developed, in good condition and had a normal suckling reflex. At necropsy, a severely enlarged thyroid gland, an acute embolic pneumonia, acute catarrhal enteritis and a moderate splenomegaly were noticed. Microscopically, severe thyroid gland hyperplasia, embolic pneumonia and catarrhal enteritis were confirmed. In the liver and myocardium small multifocal necroses, surrounded by single neutrophils, were noticed. A severe diffuse lymphoreticular hyperplasia was diagnosed in the spleen and a diffuse parenchymatous degeneration was noticed in the kidneys. Numerous small colonies of Gram-negative bacteria were observed in the liver and lungs. Intensive growth of smooth, thick, convex, yellowish colonies on blood agar and green colonies on Drigalski agar was obtained from the liver, spleen and lung samples. The bacteria were identified as *B. vesicularis* by a commercial kit. The bacterium resulted sensitive to broad-spectrum antimicrobial agents, including ampicillin, cefotaxime, ceftriaxone and ceftazidime-clavulanate.

To the best of our knowledge, this is the first report of *B. vesicularis* septicaemia in animals. Weak immunodeficient goitrous kids are predisposed to many bacterial infections, including the ubiquitous and opportunistic *B. vesicularis*, which can cause fulminant septicaemia and death within a few hours after birth.

Key words: *Brevundimonas vesicularis*; congenital goitre; newborn kid; pneumonia; septicaemia

Introduction

Brevundimonas vesicularis is an aerobic non-sporulating, non-fermenting, motile Gram-negative bacillus, ubiquitous in the environment (1,2,3). It was first isolated from a leech in 1954 and classified as a member of group IV of the genus *Pseudomonas* and named *Pseudomonas vesicularis* (1). In 1994 it was reclassified in the genus Brevundimonas (4). In humans, *B. vesicularis* has been isolated from blood, cerebrospinal fluid, urine, eye, wound and vaginal cultures (1), but was only occasionally implicated in human infections, mostly in immunocompromised patients (1,5-8), making it an opportunistic bacterium. It has never been reported to cause any problems in animals. To the best of our knowledge, this is the first report of septicaemia caused by *B. vesicularis* in animals.

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Materials and methods

The pygmy goat kid was dissected at the Instutute of Pathology, Wild animals, Fish and

Bees of the Veterinary Faculty, University of Ljubljana immediately after death.

Representative specimens of the thyroid gland, spleen, liver, small intestine, lungs, heart and kidneys were fixed in 10% neutral buffered formalin for 24 hours, routinely embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin (HE) and Gram. Samples of the spleen, liver, small intestine, lungs, heart and kidneys were taken for bacteriological examination. The standard bacteriological procedure for isolation of aerobic bacteria was used. Briefly, the samples were plated on blood and Drigalski agar plates and incubated for 24 h at 37°C under aerobic conditions. The isolate was tested for antimicrobial susceptibility using the disk diffusion method on Muller Hinton agar (Oxoid). Incubation time was 24 h at 37°C. The following antimicrobial agents were tested, using antibiotic discs (Becton Dickinson): amikacin (30 µg), piperacillin (30 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Ceftazidime - calavulanate (30 µg), Gentamicin (15 µg), Imipenem (5 µg), Aztreonam (15 µg), Ciprofloxacin (30 µg), Polymyxin B (30 µg), Colistin (30 µg), Sulfamethoxazole-trimetoprime (30 µg), Amoxicillin-clavulanate (30 µg), Erythromycin (30 µg), Azithromycin (30 µg), and Tetracycline (30 µg). The strain was classified as resistant, intermediate or susceptible to antimicrobials tested according to NLSI breakpoints used for *Pseudomonas* sp., as proposed by Karadag et al. (9).

Results

History

A newborn, apparently healthy female pygmy goat kid from the Ljubljana Zoo, of normal birth weight (1.3 kg) died suddenly 18 hours after a normal parturition of twins. At the time of death a prominent enlargement in the throat region was observed. The gestation was normal and both doe and the other kid were healthy.

Necropsy

The kid was normally developed and in good condition. No alterations were noticed in the umbilicus, and the umbilical vessels were normally obliterated. Abomasum was filled with 0.5 dl of partially clotted milk and in the initial part of the small intestine there was a moderate amount of dense milky fluid. The thyroid gland was severely enlarged with each lobe measuring 4 cm x 2.5 cm x 1.5 cm (Figure 1). An acute embolic pneumonia with multifocal sub-pleural petechial haemorrhages, diffuse acute catarrhal enteritis, a moderate splenomegaly and mild liver, heart and kidney congestion were also observed. No changes were observed in other organs and tissues.

Histopathology

Microscopically, a severe hyperplasia of the thyroid gland was diagnosed: the tissue consisted of numerous solid clusters and enlarged follicles filled with pale colloid and lined by flat or prismatic epithelium; multifocally, papillary processes protruded into the follicular lumina. The pulmonary alveoli were multifocally densely infiltrated with neutrophils and some alveolar macrophages (Figure 2). Small, scattered colonies of Gram-negative bacteria were observed in the alveoli. In the liver, random multifocal necroses with numerous colonies of Gram-negative bacteria in their centre were observed. At the periphery of the necrotic area, single neutrophils were noticed. Large bacterial colonies were also seen in the hepatic blood vessels. Small multifocal necroses with small groups of neutrophils and macrophages were observed in the myocardium. Acute diffuse catarrhal desquamative enteritis, severe diffuse lymphoreticular hyperplasia with severe hyperaemia of the spleen and diffuse parenchymatous degeneration of the kidneys were also observed.

Bacteriological culture

Intensive growth of smooth, thick, convex, yellowish colonies on blood agar and green colonies on Drigalski agar was obtained from the liver, spleen and lung samples. Gram staining revealed Gram-negative bacilli. Rapid oxidase test was positive and rapid indole test was negative. The bacteria were identified as *Brevundimonas vesicularis* by commercial kit (BBL Crystal Enteric/Nonfermenter ID Kit, Becton Dickinson). *Enterobacter cloacae* was detected on small intestine plates. Kidney sample plates remained sterile.

B. vesicularis resulted susceptible to all the tested antibiotics.

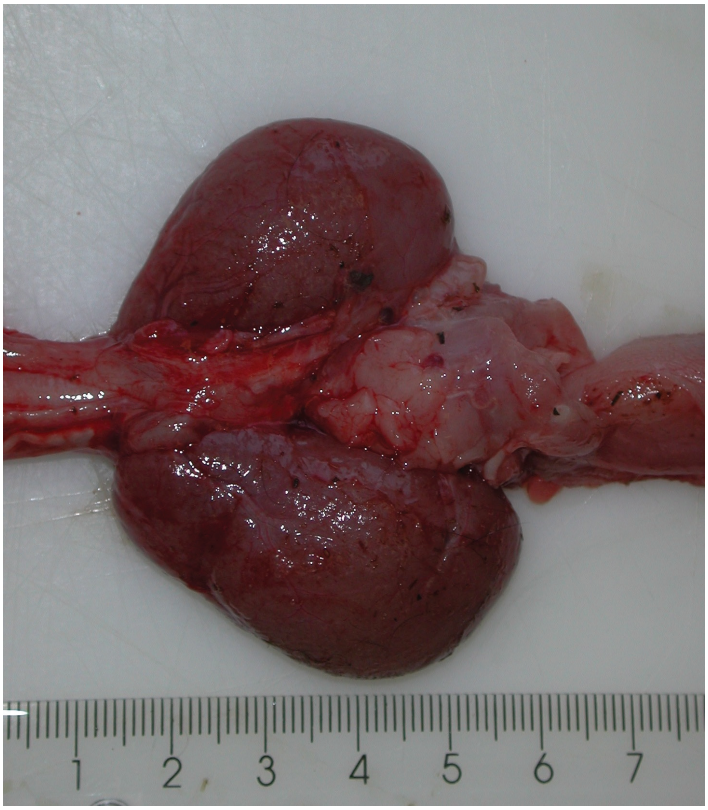

Figure 1: Necropsy finding. Severely enlarged thyroid gland

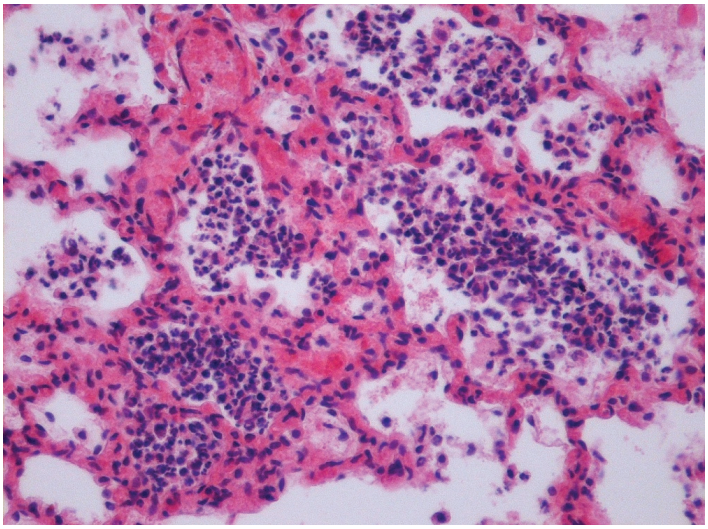

Figure 2: Embolic pneumonia. Note the pulmonary alveoli densely infiltrated with neutrophils and some alveolar macrophages. Hematoxylin and eosin, x 200

Discussion

In the first few hours after birth a kid is susceptible to a number of specific infective pathogens and other infectious agents, normally considered to have low virulence, but can also cause disease if the immunological status of the neonate is not at an optimal level (10). Goitrous kids are usually stillborn, or are born weak (11) and usually die within a few hours after birth (11,12) due to respiratory problems or increased susceptibility to stress and infection, mainly caused by *Escherichia coli*, *Clostridium perfringens* type B, *Staphylococcus aureus*, *Streptococcus* spp., *Corynebacterium* spp. or *Salmonella*, and often resulting in septicaemia (12,13). The increased susceptibility to infection is

linked to altered status of thyroid hormones, which affect natural killer cell activity and cell-mediated immune response, making goitrous patients immunocompromised (14). Albeit gestation of goitrous kids is significantly prolonged and dystocia and retained foetal placenta are often reported (11), this was not the case in our kid. The doe and the other twin were (and still are) clinically healthy. The kid later diagnosed with congenital goitre also showed no clinical disorders, but we believe it was immunocompromised and had died due to *B. vesicularis* septicaemia - *B. vesicularis* was isolated from the lungs, liver and spleen. Small colonies of Gram-negative bacteria were microscopically observed in inflamed pulmonary alveoli, hepatic blood vessels and in hepatic necroses.

B. vesicularis is a ubiquitous environmental microorganism that has been isolated from water, soil, plants and several human clinical specimens (1,6). Infection has so far only been described in humans, mostly in immunosuppressed patients suffering from leukaemia (1), sickle cell anaemia (8), biliary pancreatitis (5) or after surgical procedures (7,15). *B. vesicularis* was the cause of endocarditis (3), arthritis (2), keratitis (15), urinary tract infection (16), liver abscess (17) and bacteraemia/ septicaemia (1,5-8,18). Most of the infected patients recovered completely after adequate antibiotic therapy (2,3,5-7,15-17). *B. vesicularis* is uniformly susceptible to aminoglycosides and anti-pseudomonal penicillins and resistant to ampicillin and cephalosporins (7). In our case, the bacterium was sensitive to broad-spectrum antimicrobial agents, including ampicillin, cefotaxime, ceftriaxone and ceftazidime-clavulanate.

To the best of our knowledge, this is the first report of *B. vesicularis* septicaemia with microscopically described changes in organs in a kid with congenital goitre. Weak immunodeficient goitrous kids are predisposed to many bacterial infections, including the ubiquitous and opportunistic *B. vesicularis*, which can cause fulminant septicaemia and death within few hours after birth.

In conclusion, *B. vesicularis* may be an emerging pathogen in immunosuppressed animals and also in neonatal infections in animals.

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Brevundimonas vesicularis SEPSA PRI KOZLIČKU S PRIROJENO GOLŠAVOSTJO

M. Gombač, I. Gruntar, P. Kvapil, T. Švara

Povzetek: V prispevku smo opisali sepso, ki jo je pri novorojenem pritlikavem kozličku z golšavostjo povzročila bakterija *Brevundimonas vesicularis*. *B. vesicularis* je aerobna, nesporogena, gibljiva, gramsko negativna ubikvitarna bakterija, ki je občutljiva na aminoglikozide in nekatere peniciline. Do sedaj so jo izolirali iz vode, zemlje, rastlin in ljudi, predvsem imunosupresivnih bolnikov, pri katerih je povzročila endokarditis, artritis, keratitis in sepso. V literaturi ni zabeleženo, da bi ta bakterija pri živalih povzročila katere koli bolezenske spremembe.

Pritlikavi kozliček je poginil 18 ur po normalnem porodu. Pred poginom ni kazal bolezenskih znakov, bil je normalno razvit in v dobri telesni kondiciji, normalno je sesal. Med raztelesbo smo ugotovili obojestransko močno povečano ščitnico, akutno embolično pljučnico, akutni kataralni enteritis in zmerno splenomegalijo. S patohistološko preiskavo smo potrdili močno hiperplazijo ščitnice, embolično pljučnico in kataralni enteritis, v jetrih in miokardu smo opazili majhne multifokalne nekroze, obdane s posameznimi nevtrofilci, vranica je bila močno hiperplastična, v ledvicah pa smo ugotovili močno parenhimsko degeneracijo tubulocitov. V pljučih in jetrih smo opazili številne majhne kolonije gramsko negativnih bakterij. Na krvnem agarju so iz pljuč, jeter in vranice v čisti kulturi zrasle gladke, debele, izbočene, rumenkaste bakterijske kolonije, na agarju po Drigalskem pa zelene bakterijske kolonije. Izolirane bakterije smo determinirali kot *B. vesicularis*. Bakterije so bile občutljive na širok spekter antibiotikov, vključujoč ampicilin, cefotaksim, ceftriakson in ceftazidim-klavulanat.

Predstavljeni primer je prvi opis z B. vesicularis povzročene sepse pri živali. Imunosupresivni kozlički so zaradi golšavosti dovzetnejši za številne bakterijske okužbe, med katere gotovo lahko prištevamo tudi okužbo z *B. vesicularis*, ki lahko povzroči sepso in pogin v nekaj urah.

Ključne besede: *Brevundimonas vesicularis;* kongenitalna golšavost; novorojeni kozlički; pljučnica; sepsa

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