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RABBIT ADIPOSE-DERIVED STEM CELLS MAINTAIN THEIR CHROMOSOMAL COUNTS DURING PASSAGING: SHORT COMMUNICATION

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ABSTRACT

Monitoring of stem cells genetic stability is one of the most important safety points, because the number of chromosomes can change throughout the culture and *in vitro* manipulation of these cells. In our study the stem cells metaphases were analysed using the G-staining method. At least 60 metaphases in three samples of rabbit adipose-derived stem cells were assessed in three subsequent passages. Results of our study showed that at least 70 % of cells in each passage can maintain their stable karyotype. The highest proportion of aneuploidy (30 %) was recorded in the third passage. Even though we observed a slight increase of aneuploidy during passaging, statistical analysis did not show any significant differences. Based on our results, we can conclude that cell passaging does not affect genetic stability, since there were no changes in chromosomal counts throughout the culture. However, it was observed that there may be some instabilities during passaging that are more random. For this reason, it is recommended to monitor the stem cells' karyotype, especially if they are intended for therapeutic use.

Key words: rabbit; adipose derived stem cells; chromosomes; karyotype

INTRODUCTION

Latterly, adipose tissue has been a considerable source of mesenchymal stem cells due to its easy harvesting, accessibility and abundance of cells (Du *et al.*, 2010; Kim *et al.*, 2011). Stem cells from adipose tissue (ASCs) have many similarities, such as morphology, expression of surface markers and differentiation potential with stem cells obtained from other tissues of bone-marrow or umbilical cord origin. Mesenchymal stem cells (MSCs) from adipose tissue are nowadays considered as a widespread material for a variety of clinical applications, thus their use entails continuous safety monitoring (Kern *et al.*, 2006; Neri *et al.*, 2013).

One of the important safety points is the genetic stability that may change during culture and *in vitro* manipulation of cells. MSCs are often cultured to obtain sufficient amount for cells therapies, which increases the presumption that genetic changes may arise (Casiraghi *et al.*, 2013). There are several studies which proved that *in vitro* cell production can lower replicative potential and multipotency and that in fact leads to senescence of cells; it reduces DNA polymerase and DNA repair efficiencies, thus leading to enhanced DNA damage, such as cytogenetic alterations (deletions, duplications), mutations and epigenetic changes (Bonab *et al.*, 2006; Sperka *et al.*, 2012; Neri *et al.*, 2013). Adipose-derived stem cells in culture may be subject to chromosomal mutations and therefore changes in karyotype can be observed. It has not been specified yet in what culture conditions the mutations are the most frequent and the results

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obtained by independent research groups are often contradictory (Ferreira *et al.*, 2012).

Analyse of karyotype is very useful tool to monitor the genetic stability of ASCs during culture. Currently there is a lot of different techniques for the purpose of karyotype investigation. Each method has advantages and disadvantages in terms of sensitivity and financial point of view (Catalina *et al.*, 2007, Čurlej *et al.*, 2018). Therefore, even not very expensive method may contribute to achievement of very valuable information about genetic aspect of examined cells.

The aim of our study was to analyse the karyotype of rabbit adipose-derived stem cells in order to assess the effect of culture conditions on genetic stability of ASCs.

MATERIAL AND METHODS

Biological material

Clinically healthy rabbits (n = 3) of New Zealand White line were used in the study. Rabbits were reared in a partially air-conditioned rabbit farm hall of the Institute of Small Farm Animals at the NPPC – Research Institute for Animal Production Nitra, Slovakia. Rabbits were housed in individual cages, at a constant photoperiod of 14 hours of light a day. Temperature and humidity were recorded using a thermograph placed at the height of the cages (the average humidity and temperature during the year is maintained at 60 ± 5 % and 17 ± 3 °C). The rabbits were fed *ad libitum* a commercial feed mixture (KV, TEKRO Nitra, Slovakia) and the water was provided *ad libitum* using water feeders. Animal treatment was approved by the State Veterinary and Food Administration of the Slovak Republic no. SK CH 17016 and SK U 18016.

Harvesting and processing of the adipose tissue

Rabbits, weighting approximately 3 – 4.5 kg, were humanely sacrificed and subsequently subcutaneous fat was harvested. The collected fat samples were washed with PBS (without Ca and Mg; Biowest, USA) containing 5 % antibiotics penicillin and streptomycin (Life Technologies, Slovak Republic). Following washing, debris (blood vessels, connective tissue, muscle tissue, etc.) was removed using scissors and tweezers. Adipose tissue was cut into small pieces and was rewashed with PBS containing antibiotics. Samples were centrifuged for 5 minutes at 500 x g.

Isolation and cultivation of adipose derived stem cells

Tissue samples were incubated at 37 °C for about 2 hours with collagenase type I (Sigma Aldrich, UK) at concentration of 0.2 %. The tissue solution was neutralized with culture medium and filtered through a 100 µm filter to remove the undigested tissue. After filtration, the samples were centrifuged for 10 minutes at 1200 x g. Following centrifugation, cell pellets were resuspended in αMEM (Gibco-BRL, USA) culture medium supplemented with 20 % fetal bovine serum (Sigma Aldrich, UK) and 1 % antibiotics. Medium was changed every 3 days in order to remove non-adherent cells. Cells reached 90 % confluency in about 6-7 days after isolation. We seeded cells at a density of 12 x 10^3 / cm^2. Cells were cultured until the passage 3 (P3), as previously described in our study (Tomková *et al.*, 2018).

Karyotype analysis

Samples for chromosome analysis were processed as follows. After passaging, actively growing cells from P3 were incubated with KaryoMAX® Colcemid™ solution in HBSS (Hanks' Balanced Salt Solution, Life Technologies, Slovak Republic) for 16 h at 37 °C and 5 % CO_2. Treated cells were washed with Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, USA) and dissociated by 0.05 % trypsin (ThermoFisher, Slovak Republic). After centrifugation (200 x g for 10 min), cells were resuspended and incubated in a hypotonic solution (75 mM KCl) for 20 min at 37 °C. Following centrifugation, the harvested cells were incubated with 5 ml of chilled fixative consisting of methanol and acetic acid (3:1; v/v) for 10 minutes. Chromosome spreads were prepared by dropping the cell suspension onto pre-chilled glass slides. The air-dried cell spreads were stained by 2 % Giemsa solution (Gibco BRL, USA) and observed under a light microscope at 400 x magnification.

Statistical analysis

A normal rabbit karyotype consists of 22 pairs (2n = 44) of chromosomes. Chromosomal abnormality was defined as following: hyperploidy -> 44, hypoploidy - < 44. The final percentage of abnormal karyotype was defined as (< 2n) + (> 2n). Statistical

analysis was performed with χ^2–test for comparison of percentages using Excel software.

RESULTS AND DISCUSSION

Following 24 hours after isolation cells started to adhere to culture flasks and they showed a typical spindle-shaped morphology (Figure 1). As the cells reached the passage 3, the karyotype analysis was performed.

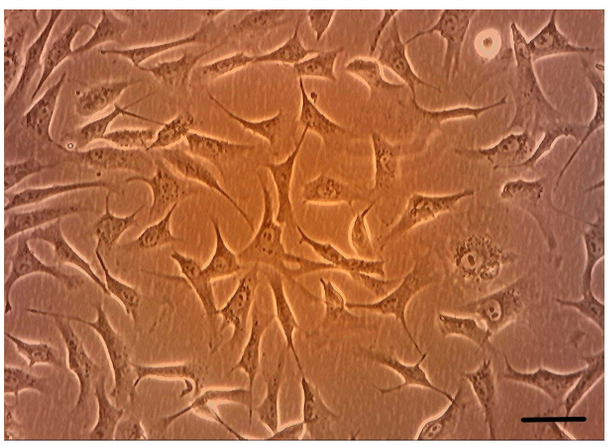

Figure 1. Spindle-shaped morphology of adipose-derived stem cells. Scale bar = 200µm

Results of karyotype analyses showed that more than 70 % of cells in all samples can maintain their chromosomal counts in each passage (Figure 2). The highest proportion of aneuploidy (30 %) was recorded in sample 2 in the third passage. In our study, we did not observe significant changes in chromosomal counts during passaging, however there were some abnormalities observed in each passage (Tables 1 – 3). The results of our study are comparable to Bellotti *et al.* (2013), who observed karyotype of adipose-derived stem cells at passages 2, 7 and 15. Abnormalities in karyotype were observed mainly at passage 15, suggesting that longer-term cultivation may result in changes in the genetic stability of cells due to cell aging.

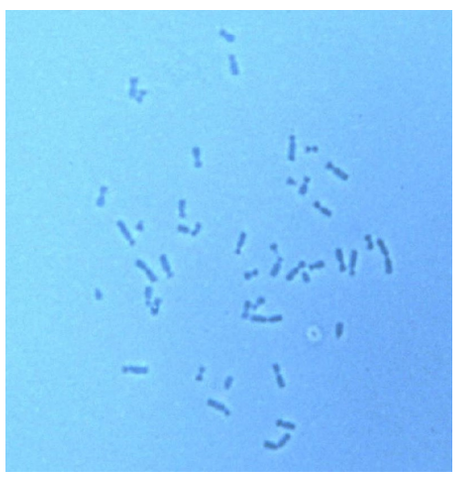

Figure 2. Normal karyotype of rabbit adipose-derived stem cells

Table 1. Chromosomal counts in passage 1

P1	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Sample 1	60	81.67 / 49	11.67 / 7	6.66 / 4	18.33
Sample 2	60	76.67 / 46	15.00 / 9	8.33 / 5	23.33
Sample 3	60	83.33 / 50	13.33 / 8	3.34 / 2	16.67

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoploidy; > 2n = aneuploidy: hyperploidy

Table 2. Chromosomal counts in passage 2

P2	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Sample 1	60	80.00 / 48	11.67 / 7	8.33 / 5	20.00
Sample 2	60	73.33 / 44	11.67 / 7	15.00 / 9	26.67
Sample 3	60	81.67 / 49	10.00 / 6	8.33 / 5	18.33

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoploidy; > 2n = aneuploidy: hyperploidy

Table 3. Chromosomal counts in passage 3

P3	Total number of cells (N)	2n % / N	< 2n % / N	> 2n % / N	Total % of abnormal karyotypes
Sample 1	60	76.67 / 46	13.33 / 8	10.00 / 6	23.33
Sample 2	60	70.00 / 42	16.67 / 10	13.33 / 8	30.00
Sample 3	60	78.33 / 47	10.00 / 6	11.67 / 7	21.67

N = number of cells; 2n = diploidy (normal); < 2n = aneuploidy: hypoploidy; > 2n = aneuploidy: hyperploidy

Based on our results we can conclude that cells passaging does not affect genetic stability, since there are no changes in chromosomal counts in culture. However, it has been already observed that during passaging there may be some instabilities that are more random. This is confirmed by study of Binato *et al.* (2012), who examined karyotype of human stem cells isolated from bone marrow. In his study, the cells maintained a stable karyotype for up to 4 passages. In the higher passages, Binato *et al.* (2012) also reported an accidental occurrence of aneuploidy.

Majority of current studies claims MSCs have stable karyotypes (Meza-Zepeda *et al.*, 2008; Ben-David *et al.*, 2011; Sensebé *et al.*, 2012; Stultz *et al.*, 2016). Similar results were also obtained in our previous studies on stem cells isolated from different tissues as bone marrow (Vašíček *et al.*, 2020), endothelial progenitor cells (Tomková *et al.*, 2017) or amniotic fluid (Kováč *et al.*, 2017; Kulíková *et al.*, 2019).

CONCLUSION

In our study we confirmed the relevance of cytogenetic studies, especially for the purpose of monitoring of numeric chromosomal aberrations. Abnormalities in chromosomal counts were detected in all samples throughout culture but no significant differences were proven so we concluded that these instabilities are more random.

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EFFECTS OF WATER SPRAY ON BODY SURFACE TEMPERATURE, MILK PRODUCTION, HAEMATOLOGICAL AND BIOCHEMICAL METABOLITES OF EGYPTIAN BUFFALOES (*BUBALUS BUBALIS*) DURING THE HOT SUMMER MONTHS IN EGYPT

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ABSTRACT

Low milk yield and impairment of reproduction and production performances of dairy buffaloes is still a major problem in tropical areas as a result of high environmental temperature. The objective of this study was to determine the effect of water spray on milk production, haematological and biochemical parameters of Egyptian buffaloes (*Bubalus bubalis*) during the summer season. Twenty Egyptian buffaloes were divided into two groups of ten animals each on the basis of body weight and lactation stage. The first group was housed in a stable without water spray (control group), while the second group was housed in a stable where a system of water tubes was provided at 1.5 meters over the animals, ended with nozzles (2 meter apart from each other) to allow spraying water over the animal body surface for 15 minutes per hour from 14:00 to 9:00 hour daily for 4 months during the summer (cooled group). Our results revealed that the rectal, udder, and milk vein temperatures were significantly ($P < 0.001$) reduced as a result of water spray treatment. Daily milk yield in buffalo cows significantly ($P < 0.001$) increased as a result of the water spray. Milk fat, lactose, and total solids were significantly increased, while milk protein was not affected. Buffaloes' blood metabolites were also affected by water spray. The results of this study suggest that providing the Egyptian buffaloes with water spray for cooling their bodies reduced the adverse effect of heat stress during the period of highest temperature humidity index, and increased the milk yield.

Key words: water spray; heat stress; milk production; haematological metabolites; Egyptian buffaloes

INTRODUCTION

In Egypt, buffaloes serve as an economically important source of meat and milk. Buffaloes produce about 66 and 43 % of the national milk and meat production, respectively (FAO, 1996). Buffaloes are well suited to hot and humid climates and muddy terrain, but exposure to direct solar radiation or high air temperature when working in the sun during hot summer months in Egypt cause buffaloes to accumulate heat due to their dark skin and sparse coat or hair in addition to poor sweating ability. Exposure of buffaloes to the hot conditions resulted in impairment of reproduction and production performances. Buffaloes were raised successfully without wallowing as long as adequate shade is available (Marai *et al*., 2009). However, dramatic climate changes during the last few years raise attention to look for ways to reduce exposure of the animal to high environmental temperature, especially in desert new reclaimed lands. During the Egyptian summer in the climate of Eastern Desert, the increase in ambient temperature resulted in an increase in rectal temperature and respiratory

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rate from 37.9 to 39.7 °C and from 23.4 to 41.0 breaths/min, in lactating buffalo cows (Kamal *et al*., 1978). To manage heat load, dairy producers provide shade, fans, and spray cooling, which improves feed intake and milk yield under hot conditions (Chen *et al*., 2013). Environmental modifications have been performed in attempts to alleviate severe heat stress in dairy cattle, for example, water spray and fans (Armstrong *et al*., 1994), and evaporative cooling (Armstrong *et al*., 1988). When given access to both feed bunks with and without water cooling, cattle preferred feed bunks with sprinklers, and the magnitude of preference increased with ambient heat load (Chen *et al*., 2013). The objective of the current study was to investigate the effect of using sprinklers mounted over the feed bunk on surface body temperatures, milk yield and composition, and blood metabolic parameters of Egyptian buffaloes. It was hypothesized that water spray application would increase the ability of heat dissipation and subsequently optimize metabolic rate and process of milk formation.

MATERIAL AND METHODS

The present study was carried out at the experimental dairy farm at the Department of Animal Production, Faculty of Agriculture, Benha University, Egypt. All experimental procedures were reviewed and approved by the Animal Ethics Committee at the Benha University. A total number of 20 buffalo cows between the first and the third parity, with age ranging from 25 to 65 months were subjected to the study. Average body weight ranged from 449 to 728 kg. Animals were kept in tie stalls in semi-open sheds with a tail-to-tail arrangement. After grazing time, animals were kept under shed offering diet up to the sundown after which all animals were moved to the stable. During summer months animals were divided into two different groups on the basis of body weight and lactation stage. In the first group, animals were allowed to graze from 9:00 to 14:00 hour then kept under shed from 14:00 to 18:00 hour the time of sundown then kept in the stable overnight (control

Figure 1. Experimental animals for stable and outdoor

group). The animals of the second group were allowed to graze during the period from 9:00 to 14:00 then kept in a stable provided with system of water tubes at 1.5 meter over the animals ended with nozzles (2 meter apart from each other) to allow spraying of water automatically over the animal body surface for 15 minutes per hour (from 14:00 until 9:00 next day) to increase dissipation of body heat by evaporation (cooled group).

Feeding and Management

The study was conducted during the summer and early fall from the middle of May to the end of December. Animals were fed a concentrate mixture, wheat, and rice straw according to their maintenance and production requirements with an allowance of 5 h grazing per day (from 09:00 to 14:00 h). All animals were offered wheat (15 kg/head), and rice hay (5 kg/head) once per day in addition to concentrated feed mixture containing protein (14 %), crude fiber (22 %), moisture (12 %), crude ash (12 %) and total digestible substances (55 %). All animals were healthy and clinically free from diseases. Buffaloes were hand--milked twice per day (at 7:00 and 14:00 h), and milk yield was recorded individually for each milking time and calculated as the total daily milk yield.

The ambient temperature and relative humidity were recorded daily every 30 min for the entire experimental period in both stables and outdoors using data loggers. The temperature humidity index (THI) was calculated according to the following equation developed by Gelade *et al*. (2007): THI = 0.72 (DBT+WBT) + 40.6, where DBT is the dry bulb temperature, and WBT is weight bulb temperature. The average monthly air temperature, relative humidity, and THI were recorded throughout the experimental period from June to September (Table 1).

Physiological Measures

1- Internal body temperature

The surface temperatures at three different body regions (rectum, udder, milk vein) were measured bi-weekly three times per day (6:00, 14:00 and 19:00 h) using an infrared thermometer (AR330, Techman®, China). The measurement range of the infrared thermometer was -50 to 330 °C with accuracy of ± 1 °C. The infrared thermometer was kept at the pen environment temperature for at least 10 min before use. The infrared thermometer was kept at 10 cm from each body region for 20 buffalo cows.

2- Milk yield and composition

Each individual buffalo was milked two times per day (at 7:00 and 14:00 h). Daily milk yield was recorded daily for each individual cow. Milk samples were collected from each milking time two times per month all over the whole experimental period and sent for laboratory analysis. Total solids, protein, fat, ash, and lactose percentages were determined for each milk sample using AOAC procedures (1984).

3- Blood sampling and analyses

Two blood samples (10 mL) were collected from each buffalo cow into tubes containing heparin as an anticoagulant (one for whole blood and another for plasma separation) through the jugular vein puncture. Blood samples were collected after

Table 1. Average monthly air temperature (T), relative humidity (RH) and temperature humidity index (THI) at the site of experimental during the experimental period from June to September

Time	0:00 – 8:00 hour			8:00 – 12:00 hour			12:00 – 20:00 hour			20:00 – 24:00 hour		
Month	T, °C	RH, %	THI, F	T, °C	RH, %	THI	T, °C	RH, %	THI, F	T, °C	RH, %	THI, F
June	30.43	48.36	78.45	34.36	31.16	74.69	26.84	59.05	80.91	24.75	81.18	73.63
July	29.44	66.07	78.80	34.33	44.55	77.68	28.03	75.28	82.96	25.75	88.97	75.53
August	30.56	60.40	79.93	34.39	46.94	77.25	27.56	77.92	83.39	25.43	87.40	74.99
September	28.78	54.23	76.86	31.04	42.81	73.35	24.93	73.65	78.37	22.61	81.05	70.76

The ambient temperature and relative humidity were recorded daily every 30 min for the entire experimental period in both stable and outdoor by using data loggers. The obtained values indicate the following: < 72 = absence of heat stress, 72 to < 74 = moderate heat stress, 74 to < 78 = severe heat stress and 78 and more = very severe heat stress.

morning milking at 7 am and monthly until the end of the experimental period. Plasma was harvested within 1 h by centrifuging the sample at 3000 g for 15 minutes. The samples were frozen at -20 °C until subsequent analysis. Total plasma protein, albumin, total lipids, total cholesterol, calcium and phosphorus, and alkaline phosphatase were analyzed using commercial kits (Biodiagnostic, Egypt). Red and white blood cells and blood hemoglobin concentration were measured within one to two hours of collection using automated analyzer.

Statistical analyses

The data were statistically analyzed by using SAS (2004) with generalized linear model including the effects of treatment. Analysis of variance and Duncan's multiple range test (Duncan, 1955). Significant results were obtained from the least squares means and F scores ($P < 0.001$).

RESULTS

Effect of water spray on body surface temperatures

Our results revealed that the rectal, udder, and milk vein temperatures significantly ($P < 0.001$, Table 2) decreased as a results of water spray treatment during the hot months in Egypt. An interaction between treatment and time were observed for udder temperature ($P = 0.002$), and for milk vein temperature ($P = 0.014$). Lowest udder and milk vein temperature was observed in treated buffalo cows at 19:00 h, but there was no interaction between treatment and time observed for rectal temperature.

Effect of water spray on milk yield and composition of buffalo cows

Daily milk yield in buffalo cows significantly ($P < 0.001$) increased due to water spray treatment (Table 2). Average daily milk yield was 5.23 kg in treated groups versus 3.41 kg in control groups. Milk ash content increased from 0.81 % in untreated buffalo cows to 1.06 % in treated ones (Table 2). However, no effect of treatment was observed for milk protein. Treating lactating buffaloes with cooling system significantly ($P < 0.001$, Table 2) increased the average of milk fat and total solids percentages.

Effect of water spray on blood metabolites of buffalo cows

Cooling of buffalo cows during hot summer months had highly significant effect ($P < 0.001$, Table 2) on haemoglobin level, red and white blood cells. The treated buffaloes had significant increase

Table 2. Effect of water spray on different measured traits (Mean ± SE)

Variables	Control group	Water spray	*P*-value
Daily milk yield, kg	3.41 ± 0.43	5.23 ± 0.14	
Milk ash, %	0.81 ± 0.05	1.06 ± 0.04	< 0.05
Milk fat, %	8.84 ± 0.26	10.37 ± 0.21	< 0.001
Milk lactose, %	5.32 ± 0.04	5.30 ± 0.03	< 0.001
Milk protein, %	3.66 ± 0.16	4.72 ± 0.12	NS
Milk total solids, %	17.66 ± 0.28	20.40 ± 0.32	< 0.001
Blood Hemoglobin, $g.dL^{-1}$	11.57 ± 1.09	16.11 ± 1.09	< 0.001
Red blood cells, x 10^6	2.4757 ± 39.30	5.3577 ± 39.30	NS
White blood cells, x 10^3	3169.44 ± 727.59	4968.52 ± 727.59	NS
Plasma total protein, $g.dL^{-1}$	7.05 ± 1.02	10.04 ± 1.02	NS
Plasma albumin, $g.dL^{-1}$	4.61 ± 0.65	7.64 ± 0.65	NS
Plasma globulin, $g.dL^{-1}$	2.44 ± 0.63	2.55 ± 0.63	NS
Plasma total lipids, $mg.dL^{-1}$	3.23 ± 0.42	4.79 ± 0.61	NS
Plasma cholesterol, $mg.dL^{-1}$	56.13 ± 13.15	113.11 ± 13.61	NS
Plasma calcium, $mg.dL^{-1}$	10.30 ± 1.23	12.15 ± 1.28	NS
Plasma phosphorus, $mg.dL^{-1}$	5.20 ± 0.47	6.11 ± 0.47	NS
Alkaline phosphates, $mg.dL^{-1}$	341.91 ± 59.81	297.96 ± 51.93	NS

in plasma total proteins, albumin, total lipids, cholesterol, calcium, and phosphorus (P < 0.05, Table 2). Although, there were no significant effects detected in the plasma level of globulin and albumin (P > 0.05, Table 2).

Effect of water spray on body surface temperature of buffalo cows

Table 3 shows the effect of water spray on body surface temperatures of Egyptian buffaloes at different times of day. The water spray group showed the body temperature was lower (33.70, 34.16 and 32.89 °C, for rectal, udder and milk vein, respectively) than the control group. The differences between means of body surface temperature due to treatment groups were significant (P < 0.05).

DISCUSSION

Results revealed that the highest THI was observed during the period from 12:00 to 20:00 h; ranging from 78.37 to 80.91. The THI has been used for several years in the USA as a guide for the use of precautionary measures, which must include considerations of handling and deterioration. Recommendations based on forecasted THI values, were categorized as follows: THI < 70 = Normal: no heat stress precautions are needed; THI 70 – 80 = Alert: to be prepared to take extra precautions and do not leave a vehicle loaded with animals standing in the sun; THI 79 – 83 = Danger: additional precautions should be taken to protect animals. Use of sprinklers and fans in loading areas is advisable. Therefore, the goal of the current study is to use sprinklers during the period of high heat stress to cool the body of buffaloes. The obtained results agree with Vaidya *et al*. (2011), who found that the average maximum temperature was observed at 14:00 h and THI of 85 during the summer season in Murrah buffaloes and Karan Fries cattle. In our study, the average daily milk was increased as a result of increasing the rate of heat dissipation by applying spray of water above the body surface of the animals. Average daily milk yield reached an average approximately equal to that obtained during winter in Egyptian buffaloes

Table 3. Effect of water spray on body surface temperatures of Egyptian buffaloes during different times of the day

Variables	Rectal temperature	Udder temperature	Milk vein temperature
Treatment			
Control	34.20 ± 0.39	34.7775 0.5086	33.4371 0.5908
Water spray	33.70 ± 0.44	34.1623 0.5837	32.8997 0.6761
P-value	0.0021	0.0009	0.0154
Day period			
Period 1 (6:00 hour)	34.53 0.4220	35.1413 0.5488	33.9641 0.6369
Period 2 (14:00 hour)	34.11 0.4230	34.7888 0.5500	33.6468 0.6383
Period 3 (19:00 hour)	33.21 0.4233	33.4796 0.5502	31.8944 0.6386
P-value	< .0001	< .0001	< .0001
Water spray × 6:00 hour		35.06 ± 0.59	33.90 ± 0.68
Water spray × 14:00 hour		34.13 ± 0.59	33.02 ± 0.69
Water spray × 19:00 hour		33.29 ± 0.59	31.77 ± 0.69
Control × 6:00 hour		35.22 ± 0.53	34.02 ± 0.62
Control × 14:00 hour		35.44 ± 0.53	34.27 ± 0.62
Control × 19:00 hour		33.66 ± 0.53	32.00 ± 0.62
Treatment × Time, P-value	0.271	0.002	0.014

Temperatures were measured using an infrared thermometer (AR330, Techman, China) at 16:00 to 17:00 h once every two days during the first 15 days after regrouping. The measurement range of the infrared thermometer was -50 to 330 °C and the accuracy was ± 1 % or ± 1 °C. The infrared thermometer was placed at least 10 min in the pen environment before use.

(4.5 kg; Farouk, 2012). Ambient temperature as well as environmental humidity and nutritional efficiency are considered the most imported non-genetic factors that affect the milk yield of dairy animals (West, 1993). Our results agree with the results obtained by Igono *et al*. (1987), who found that cows cooled with spray and fan under shade produced 2 kg/cow per day more than cows in shade alone.

Treating lactating animals with cooling system significantly increased the average of milk fat and total solids percentages but not the milk protein. It is worth mentioning that cooling buffalo cows during the hot weather of summer months increased milk fat percent to attain an average of 10.37 %, which exceeded its average value during winter, spring and autumn. This is considered a very important result from the economic point of view, since farmers raise buffaloes essentially to get fat, which is converted into butter to be sold during winter months. Buffalo milk contains less water, more total solids, more fat, slightly more lactose and more protein than cow's milk. It seems thicker than cow's because it generally contains more than 16 % total solids compared with 12-14 % for cow's milk. In addition, its fat content is usually 50-60 % (or more) higher than that of cow's milk.

Blood metabolites

Cooling animals during the hot climate of summer season increased the average of either haemoglobin level, and count of red or white blood cell to the extent of the highest values when compared to other seasons of the year. Our results agree with the results obtained by Vijayakumar *et al*. (2011), who found that Murrah buffalo heifers provided with fan and sprinkling had significantly higher haemoglobin values compared to the animals with only a fan and the control group. Many authors (Shebaita and Kamal, 1973, and Marai *et al*., 1995) found that haemoglobin concentration decreases during heat stress due to either depression or hematopoiesis.

Effect of water spray on blood constituents related to metabolic activities of buffalo cows

The highest level of total lipid and cholesterol in treated groups could be attributed to increase in feed intake as a result of cooling their bodies. These results are in agreement with Johnson (1980), who reported that water spray and wind in hot humid environments significantly increased feed intake and T3 hormone in lactating cows. Verma *et al*. (2000) showed marked decrease in blood total lipid and cholesterol concentrations of lactating Murrah buffaloes during the summer than during the winter season. Also, they mentioned that the marked decrease in cholesterol concentration during heat stress may be due to dilution as a result of the increase in total body water or to the decrease in acetate concentration, which is the primary precursor for the synthesis of cholesterol. Also, Chaudhary *et al*. (2015) observed that exposure of lactating Surti buffaloes to hot humid and hot dry weather was associated with significant decrease in glucose and cholesterol. In the present study, significant increase in levels of plasma total proteins and albumins was observed as result of water spray. However, no significant effect was detected due to the treatment applied on the plasma globulin level. In buffalo calves, the heat stress conditions induced significant decreases in total protein concentrations (Habeeb *et al*., 2007). Serum total proteins were estimated as 44 $g.L^{-1}$ in summer and 51 $g.L^{-1}$ in winter by El-Masry and Marai (1991) in Egyptian buffalo calves. In lactating Murrah buffaloes, total blood proteins were lower during the summer season than during winter (Verma *et al*., 2000). In the study of Vijayakumar *et al*. (2011), provision of Murrah buffalo with fan and sprinkling had significantly higher total protein values when compared with the control group.

CONCLUSION

Providing Egyptian buffaloes with water spray to cool their bodies reduced the adverse effect of heat stress during the period of highest THI. Milk yield and composition were affected by water spray during the hottest period of the day. Environmental modification through water spray are associated with both haematological and biochemical responses in cooled buffaloes.

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DISTRIBUTION OF LEUCOCYTES AND EPITHELIAL CELLS IN SHEEP MILK IN RELATION TO THE SOMATIC CELL COUNT AND BACTERIAL OCCURRENCE: A PRELIMINARY STUDY

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ABSTRACT

Milk somatic cell count (SCC) is a main indicator of udder health in dairy animals. Thus, increased SCC levels are usually associated with the clinical and/or subclinical intramammary infections. SCC are mainly composed of immune cells (leukocytes) and epithelial cells. Recently, several flow cytometric approaches were used to assess the distribution of these cells in the milk of ewes. Hereby, a new combined antibody panel was designed for this purpose. Briefly, milk cells were stained with specific antibodies: CD18 (leukocytes), CD21 (B cells), CD4 (Th cells), CD8 (Tc cells), CD14 (monocytes) and CD11b (polymorphonuclear cells – PMNs). CD18 negative cells were considered as epithelial cells. Moreover, a qualitative examination of bacteria species presented in the milk was carried out using MALDI-TOF MS. Analysed milk samples were divided into 5 classes according to the SCC number as follows: < 300,000 cells.ml^{-1} (SCC1), 300,000-500,000 cells.ml^{-1} (SCC2), 501,000-1,000,000 cells.ml^{-1} (SCC3), 1,001,000-2,000,000 cells.ml^{-1} (SCC4) and > 2,000,000 cells.ml^{-1} (SCC5). SCC1-2 samples were considered as normal milk samples, whereas SCC3-5 as abnormal samples. Bacteriological assessment revealed that all samples in SCC3-5 class were infected mainly by *S. epidermidis* and *S. caprae*. On the other hand, SCC2 did not exhibit a pathogen infection and in SCC1 only 22 % of samples were infected. Concerning the somatic cell composition, SCC1-2 classes comprised approximately 50:50 of leukocytes and epithelial cells. The main leukocyte subsets were PMNs. However, the number of leukocytes alongside with PMNs count significantly ($P < 0.05$) increased in SCC3, whereas the number of epithelial cells significantly ($P < 0.05$) decreased compared to SCC1-2. Similar trend, although not significant, was observed in SCC4-5 samples. The proportion of nonviable PMNs also increased ($P < 0.05$) in SCC3, however it was not markedly different in comparison to live PMNs among all SCC classes. In conclusion, described methodological approach could be effective in the more detail further research dealing with distribution of different cells of different origin (epithelial, leukocytes) in cases of subclinical mastitis caused by different mastitis pathogens.

Key words: sheep; milk; SCC; bacteria; leukocytes; flow cytometry

INTRODUCTION

Milk belongs to the basic human foodstuff. Thus, quality of milk should be thoroughly controlled before it enters the human consumption channels. The milk somatic cell count (SCC) is the basic indicator of udder health and thus milk quality, safety and technological properties of all dairy animals. Although the United States established a SCC limit for goats and sheep at 1,000,000 cells.ml^{-1}, the European Union limited the maximum SCC for cows, but not for goats and sheep (Paape *et al.*, 2007). However, Maurer and Schaeren (2007) and (Tančin *et al.*, 2017) described that SCC is highly

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associated with the infection of udder halves. Therefore, there is a general agreement that the limit of 500,000 cells.ml^{-1} could be considered as an indicator of health problems in ewe's udder (Tančin *et al.*, 2016). The SCC in small ruminants contains the different cells types presented in milk, including leukocytes and epithelial cells (Souza *et al.*, 2012). Recently, it was observed that proportion of different immune cells can be measured in milk of cows (Leitner *et al.*, 2012; Li *et al.*, 2015), goats (Bagnicka *et al.*, 2011; Boulaaba *et al.*, 2011; Leitner *et al.*, 2012) and sheep (Albenzio and Caroprese, 2011; Albenzio *et al.*, 2012; Leitner *et al.*, 2012; Świderek *et al.*, 2016) by flow cytometry using different species specific antibodies.

In this study, a novel antibody panel is used for the determination of leukocytes subsets and epithelial cells in the milk of ewes and their relationship with SCC and the presence of bacteria.

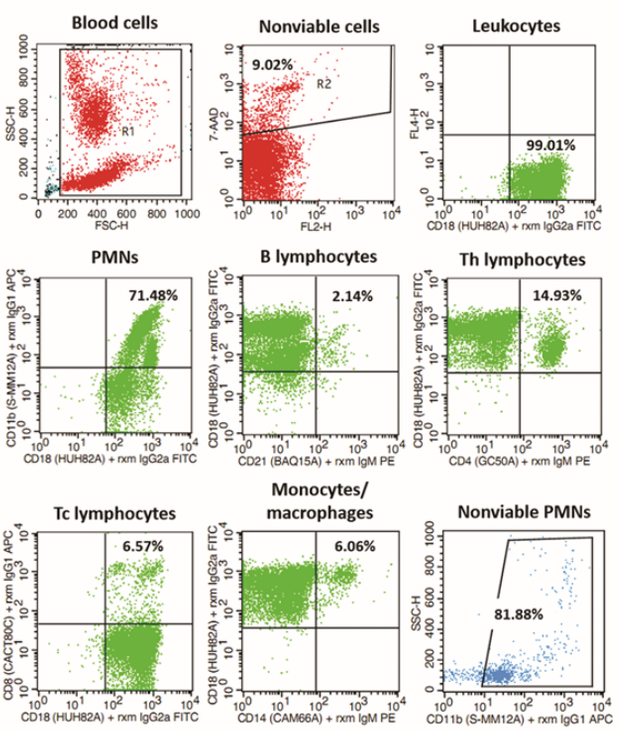
MATERIAL AND METHODS

Ten ewes of Tsigai breed from the local farm were used in the experiments. In total, 20 milk samples from each udder half were collected for bacteriological cultivation, evaluation of SCC and flow cytometry. For pathogen detection the milk samples were collected by discarding first squirts of milk and subsequently cleaning of the teat end with 70 % alcohol and milk sample from each udder halves was taken in sterile tube. The inoculum of each sample was inoculated onto blood agar (Oxoid LTD, Hamshire, UK). All plates were incubated aerobically at 37 °C and examined after 24 hours. Examination of bacteria in milk samples was performed using MALDI-TOF mass spectrometry (Bruker Daltonics, Germany). For bacterial analysis cells from a one colony of fresh culture were used for isolate to prepare samples according to the microorganism profiling ethanol-formic acid extraction procedure as recommended by the manufacturer. Samples spot was overlaid with 2 µl of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50 % acetonitrile with 2.5 % trifluoroacetic acid) and allowed to dry for 15 min (Bruker Daltonics GmbH, Germany). To identify microorganisms, the raw spectra obtained for each isolate were imported into BioTyper software version 2.0 (Bruker Daltonics). Contagious pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*) were classified as positive if one or more colony-forming unit (CFU) were found. Minor and environmental mastitis pathogens were classified as positive if at least five CFU were found. Samples were classified as contaminated if three and more pathogens were isolated from one milk samples and growth of contagious pathogens was not identified. Somatic cell count was analysed using a Somacount 150 (Bentley Instruments, Inc., Chaska, Minnesota, USA). Milk samples were divided into 5 classes according to the somatic cell count as described previously by Albenzio *et al.* (2012): < 300,000 cells/ml (SCC1), 300,000-500,000 cells.ml^{-1} (SCC2), 501,000-1,000,000 cells.ml^{-1} (SCC3), 1,001,000-2,000,000 cells.ml^{-1} (SCC4) and > 2,000,000 cells.ml^{-1} (SCC5). According to the above-mentioned studies, milk samples were considered as milk with low SCC (SCC1-2) and high SCC (SCC3-5).

Milk samples for flow cytometry were processed as described by Sarikaya *et al.* (2004). Briefly, 50 ml of each milk sample were centrifuged for 30 min. and 1500 x g at 4 °C. The fat layer on the top of the tubes was carefully removed and supernatant (skim milk) was discarded. The cell pellet was washed in 5 ml of ice-cold PBS (Biowest, USA) and centrifuged for 15 min. and 460 x g at 4 °C. Automated cell counter EVE™ (NanoEntek, USA) was used to determine the cell concentration. Cells were then divided into prepared tubes and stained with the ovine specific primary mouse monoclonal antibodies (all from WSU, USA) according to the producer's manual in order to distinguish between leukocytes subsets and epithelial cells as follows: anti-CD18 (HUH82A, Ig2a) for all leukocytes, anti-CD21 (BAQ15A, IgM) for B lymphocytes, anti-CD4 (GC50A, IgM) for T helper lymphocytes, anti-CD8 (CACT80C, IgG1) for T cytotoxic lymphocytes, anti-CD14 (CAM66A, IgM) for monocytes/macrohages and anti-CD11b (S-MM12A, Ig1) for live and nonviable polymorphonuclear cells (PMNs, granulocytes). The specificity of the used monoclonal antibodies was proved by staining of ovine blood samples (Figure 1). Proper rat anti-mouse (anti-IgG2a, anti-IgM and anti-IgG1) fluorochrome conjugated (FITC, PE and APC, respectively) antibodies were used as secondary antibodies. Heat inactivated sheep serum was used to block the unspecific antibody binding.

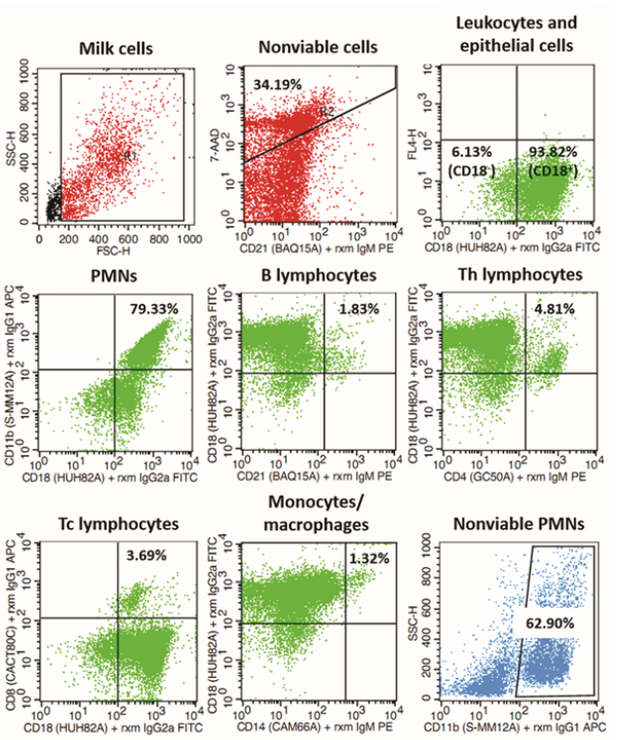
To determine the viability of analysed cells and/or to exclude the dead cells from the analysis 7-AAD (Thermo Fisher Scientific, USA) staining was used. At least 10,000 cells were analysed in each sample using flow cytometer FACSCalibur (BD Biosciences, USA). The leukocytes subsets were counted within the $CD18^+$ cells. The $CD18^-$ cells were considered as epithelial cells. The evaluation strategy is shown in Figure 2.

Obtained results were evaluated using the SigmaPlot software (Systat Software Inc., Germany) with one-way ANOVA (Holm-Sidak method) and expressed as the means ± SEM.



R1 – all leukocytes excluding cell debris and erythrocytes, R2 – all nonviable cells within R1 region, PMNs – polymorphonuclear cells, Th – T helper, Tc – T cytotoxic. Leukocytes subsets were counted within R1 region excluding the cells in R2 region. Nonviable PMNs were counted in R1 and R2 region.

Figure 1. Illustrative distribution of leukocyte subsets in ovine peripheral blood



R1 – all milk somatic cells excluding cell debris, R2 – all nonviable cells within R1 region, $CD18^+$ - leukocytes, $CD18^-$ - epithelial cells, PMNs – polymorphonuclear cells, Th – T helper, Tc – T cytotoxic. Leukocytes subsets were counted within R1 region excluding the cells in R2 region. Nonviable PMNs were counted in R1 and R2 region.

Figure 2. Illustrative distribution of leukocyte subsets and epithelial cells in milk of ewes

RESULTS AND DISCUSSION

In general, clinical mastitis resulted in low milk yield and in increased somatic cell counts (Gonzalo *et al.*, 1994; Leitner *et al.*, 2004). However, Silanikove *et al.*, (2005) also reported milk production losses caused by subclinical mastitis (SCM). The main cause of mastitis is usually an infection of different bacteria such as coagulase-negative *Staphylococcaceae* (Rupp *et al.*, 2003, Holko *et al.*, 2019). SCM and/or intramammary infection (IMI) are often indicated by the high somatic cell count. Persson *et al.* (2017) observed that a high SCC (≥ 400,000 – 500,000 cells.ml^{-1}) in the ewe's milk of mainly Swedish breeds was associated with IMI. In addition, Kern *et al.* (2013) also concluded that SCC might indicate an udder health problem. Our findings (Table 1) are in agreement with above mentioned reports, as the presence of bacteria increased

Table 1. Proportion of immune and epithelial cells in sheep milk with different somatic cell count

SCC class	SCC1 (n = 9)	SCC2 (n = 4)	SCC3 (n = 2)	SCC4 (n = 2)	SCC5 (n = 3)
SCC (x 10^3 cells.ml^{-1})	104 ± 17	347 ± 20	598 ± 81	1838 ± 33	3482 ± 583
Bacteriological--positive samples (%)	22 % (contaminated – 11 %, *S. caprae* – 11 %)	N	100 % (*S. epidermidis*)	100 % (*S. caprae*)	100 % (S. epidermidis – 33%, *S. caprae* – 67%)
Leuko – CD18$^+$ (%)	49.13 ± 9.34^a	49.01 ± 7.60^a	99.46 ± 0.25^b	86.60 ± 7.23	79.40 ± 6.35
PMNs – CD11b (%)	27.15 ± 9.84^a	18.41 ± 14.9^a	98.35 ± 0.32$^{b, A}$	60.04 ± 19.29	62.66 ± 11.59
B lym – CD21$^+$ (%)	2.47 ± 1.07	4.97 ± 3.02	0.50 ± 0.25	2.26 ± 0.43	2.27 ± 0.37
Th lym – CD4$^+$ (%)	5.19 ± 2.27	10.66 ± 8.89	0.96 ± 0.71	11.56 ± 6.75	5.62 ± 2.51
Tc lym – CD8$^+$ (%)	3.61 ± 0.95	7.20 ± 5.05	0.36 ± 0.19	8.36 ± 4.69	4.88 ± 1.43
CD4$^+$/CD8$^+$ (%)	1.40 ± 0.34	1.05 ± 0.28	2.24 ± 0.77	1.35 ± 0.05	1.08 ± 0.22
Mono/Macro – CD14$^+$ (%)	3.35 ± 1.32	4.76 ± 4.48	1.86 ± 0.47	3.02 ± 1.70	2.60 ± 0.62
Epithel – CD18$^-$ (%)	50.44 ± 9.28^a	50.19 ± 8.06^a	0.48 ± 0.22^b	13.35 ± 7.22	20.30 ± 6.15
Nonviab – 7-AAD$^+$ (%)	34.28 ± 6.05	23.02 ± 5.90	7.73 ± 1.16	42.84 ± 13.70	56.24 ± 12.88
Nonviab PMNs – CD11b$^+$ (%)	30.32 ± 9.04^{a,c}	46.03 ± 9.35^a	90.23 ± 0.84$^{b, B}$	57.77 ± 5.13	79.28 ± 3.49^d

SCC – somatic cell count, SCC1: < 300,000 cells.ml^{-1}, SCC2: 300,000-500,000 cells.ml^{-1}, SCC3: 501,000-1,000,000 cells.ml^{-1}, SCC4: 1,001,000-2,000,000 cells.ml^{-1}, SCC5: >2,000,000 cells.ml^{-1}, Leuko – leukocytes, PMNs – polymorphonuclear cells (granulocytes), B lym – B lymphocytes, Th lym – T helper lymphocytes, Tc lym – T cytotoxic lymphocytes, Mono/Macro – monocytes/macrophages, Epithel – epithelial cells, Nonviab – nonviable cells, contaminated – three and more pathogens, N – no pathogen, ^{a} vs. ^{b} and ^{c} vs. ^{d} are statistical different at $P < 0.05$ within the same row, ^{A} vs. ^{B} are statistical different at $P < 0.05$ within the same column.

in milk samples with abnormal SCC (100 % in SCC3-5) in comparison to samples with normal SCC (22 % or without pathogen in SCC1 and SCC2, respectively). The most often occurring bacteria species in the presented milk samples were coagulase negative staphylococci: *S. epidermidis* and *S. caprae*. These species are commonly reported also by others (Albenzio *et al.*, 2012; Świderek *et al.*, 2016).

Milk of dairy animals such as cows normally contain somatic cells that are usually composed of leukocytes and released glandular epithelial cells (~ 50 % of both) (Shoshani *et al.*, 2000). The ratio of polymorphonuclear cells to mononuclear (lymphocytes and monocytes/macrophages) is usually ~ 1 in the case of the health animals (Dosogne *et al.*, 2003; Mehne *et al.*, 2010). Similar observation was noticed in our study as the leukocytes and epithelial cells in the normal milk samples (SCC1-2) were presented at the ratio 1:1 (Table 1).

In this preliminary study, three different flow-cytometric approaches (Albenzio *et al.*, 2012; Leitner *et al.*, 2012; Świderek *et al.*, 2016) were combined in order to design novel and complex antibody panel for the relatively rapid evaluation of somatic cell composition of the sheep milk samples. Due to this panel, a high decrease in the number of epithelial cells was found in the all abnormal SCC classes (SCC3-5), although only value in SCC3 class was significantly different ($P < 0.05$) in comparison to normal classes (SCC1-2). In contrast, the leukocyte counts significantly increased ($P < 0.05$) in SCC3 compared to SCC1-2. This value increased also in SCC4 and 5, although not significantly. Alongside with the rise of leukocyte number, the proportion of PMNs also increased significantly ($P < 0.05$) in SCC3 and non-significantly in SCC4-5 compared to SCC1-2. On the other hand, no significant differences were observed in the proportion of B cells, T cell subsets (CD4$^+$ and CD8$^+$) or their ratio (CD4$^+$/CD8$^+$) and macrophages among the SCC classes (Table 1). Similarly, Leitner *et al.* (2012) noticed significant increase of leukocytes, PMNs and monocytes in infected samples compared to bacteria-free milk samples with no differences between CD4$^+$ and CD8$^+$ T cells. On the contrary, significant decrease in PMNs and macrophages and increase in lymphocytes in the order from SCC1 to SCC5 class was reported by Albenzio *et al.* (2012). These authors also found significant differences in proportion of T cells (both CD4$^+$ and CD8$^+$) and their ratio CD4$^+$/CD8$^+$

among the observed classes. Moreover, they suggested the nonviable PMNs for an indicator of inflammatory status of the ewe udder since their proportion significantly increased from SCC1 to SCC5, whereas number of live PMNs decreased. Our study confirmed the growing tendency of nonviable PMNs in association with increasing SCC (Table 1). However, we did not notice significant changes in live and nonviable PMNs among the studied SCC classes, except for the class SCC3 (98 % vs. 90 %, respectively). We also did not find significant differences in the number of total dead cells within the samples. Thus, the usefulness of this indicator in contrast to the live PMNs is questionable; even take into account the possible unspecific binding of the antibodies to dead cells.

In fact, IMI causes a rise in the number of milk somatic cells during lactation, due to the infiltration of leukocytes from the blood that dramatically change the proportion and distribution of leukocytes in milk. However, the immune response to IMI is not uniform, since different leukocytes are altered according to the type of infecting pathogen and the duration of infection (Leitner *et al.*, 2012). For that reason it is very important to monitor the leukocyte distribution in milk that might be affected by the number of somatic cells and the presence of pathogen.

CONCLUSION

Proposed flow-cytometric approach can easily evaluate the composition of milk somatic cells. It seems that increased leukocytes, mainly polymorphonuclear cells, in the milk of ewes might be in relationship with the rise of somatic cell count and the presence of infectious bacteria species that could finally indicate the inflammation of mammary gland. However, further experiments with a large number of samples are required in order to confirm this hypothesis.

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THE TECHNIQUE FOR CRYOPRESERVATION OF CATTLE EGGS

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ABSTRACT

The aim of this investigation was to establish a methodology of cryopreservation of cattle eggs (oocytes) under our laboratory conditions. For cryopreservation of *in vitro* matured oocytes, the freezing in minimum volume by ultra-rapid cooling technique was used. Oocytes with at least three layers of cumulus cell were placed into the equilibration solution (ES: 3 % ethylene glycol in M199-HEPES, supplemented with 10 % foetal calf serum) for 12 min. Following equilibration, the oocytes were transferred to vitrification solution (30 % EG + 1M sucrose in M199-HEPES with 10 % foetal bovine serum) at room temperature for 25 sec. Then the oocytes were placed onto nickel grid (electron microscopy grade) and plunged into liquid nitrogen. After thawing the oocytes were fertilized *in vitro*. Development of produced embryos (cleavage on day 2, and blastocyst yield on day 7) and total cell number of blastocysts after DAPI staining were determined. We obtained a relatively high cleavage rate (55.81 %) of fertilized oocytes after thawing; of them 11.24 % developed to the blastocyst stage. The quality of blastocysts obtained from vitrified oocytes was similar to the control blastocysts, as evidenced by a comparable total cell number (84.45 vs 97.29, resp.). In conclusion, the designed freezing technique proved to be suitable for cryopreservation of cattle oocytes, nevertheless further optimization is required.

Key words: bovine; oocyte; embryo; blastocyst rate; vitrification

INTRODUCTION

Vitrification has for several years been considered as a promising option in oocytes cryopreservation, because it was successfully used previously to freeze embryos. Fuku *et al.* (1992) was the first to publish successful vitrification of bovine oocytes. However, the developmental results after fertilization were not satisfactory. A significant progress in this area was achieved later when Martino *et al.* (1996) and Vajta *et al.* (1998) applied an approach of minimizing the vitrified sample to obtain a much faster cooling rate. Since then, several papers have been published worldwide describing different techniques of vitrification of bovine oocytes. Several authors deal either with an appropriate stage (GV, MII) during maturation, where it is safest to freeze oocytes (Diez *et al.*, 2005; Sprícigo *et al.*, 2014; Bulgarelli *et al.*, 2018), or with the presence/absence of cumulus cells during vitrification (Ortiz Escribano *et al.*, 2016). Moreover, various formulations of the vitrification medium were tested (Chian *et al.*, 2004; Magnusson *et al.*, 2008). Several different devices have been used for the vitrification, which specifically minimize the volume of frozen vitrification medium, such as open-pulled straw (OPS; Vajta *et al.*, 1998), micro-drop (Papis *et al.*, 2000), nylon loop (Lane a Gardner, 2001), "hemi-straw" (HS; Vanderzwalmen *et al.*, 2003), electron-microscopic grid (Martino *et al.*, 1996) or Cryotop (Kuwayama *et al.*, 2005). However, the results of all these works are considerably varied and the described protocols are often unreproducible. There is still no consistent methodology to ensure high survival of bovine oocytes after thawing and good embryo development after fertilization.

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The aim of this work was to establish a methodology of cryopreservation of female cattle gametes for the purpose of cryostorage in a bank of animal genetic resources, as well as possibilities to improve the survival of cryopreserved eggs.

MATERIAL AND METHODS

Oocyte retrieval and *in vitro* maturation (IVM)

The ovaries were isolated from undefined cows at a local abattoir and transported to the laboratory. The oocytes were recovered from antral follicles (2-8 mm) by the aspiration of follicular fluid using sterile syringe with a needle. Cumulus-oocyte complexes (COC) were collected into a Petri dish with a holding medium (M199-HEPES with 10 % fetal bovine serum-FBS) and only COC with several layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation. COCs intended for vitrification were matured for 21 hours and those selected for control group were matured for 23 hours in a maturation medium containing TCM 199 (Gibco), sodium pyruvate (0.25 mmol. L^{-1}), gentamycin (50 µg.ml^{-1}), FBS 10 % and FSH/LH (1/1 I.U., Pluset) at 38.5 °C and 5 % CO_2.

Cryopreservation of oocytes

For cryopreservation of *in vitro* matured oocytes, ultra-rapid cooling technique in minimum volume was used. Selected matured oocytes were stripped off an excessive cumulus layers by vortexing during 30 s. Oocytes with approx. three remaining cumulus cell layers were placed into equilibration solution (ES: 3 % ethylene glycol (EG) in M199-HEPES, supplemented 10 % FBS) for 12 min. Following equilibration, the oocytes were transferred to vitrification solution (30 % EG + 1M sucrose in M199-HEPES with 10 % FBS) at room temperature for 25 sec. The oocytes (10-15) in a small drop were placed with a glass micropipette onto 300 mesh nickel electron microscopy grids (EM grids; Figure 1.), an excessive medium was removed by a filtration paper and then the oocytes were immediately plunged into liquid nitrogen for storage (several weeks).

For warming, nickel grids were directly transferred into thawing solution (0.5M sucrose in M199-Hepes, at 37 ˚C) for 1 min. The warmed oocytes were transferred across the three diluent solutions (0.25M, 0.125M and 0.0625M sucrose in M199-HEPES) for 3 min in each, and then washed twice in M199-HEPES with 10 % FCS for 5 min. Oocyte survival was evaluated on the basis of the integrity of the ooplasm and the *zona pellucida* after 2 h culture post-thawing.

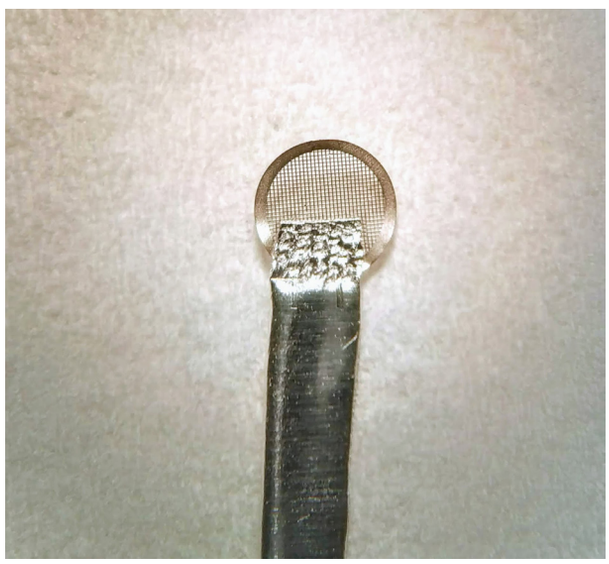

Figure 1. Electron microscopy 300 mesh grid (EM grid) fixed in a holder

***In vitro* fertilization (IVF) of vitrified-warmed oocytes and embryo culture**

Warmed morphologically good-looking oocytes were washed in IVF-TALP medium (TALP solution, 10 µg.ml^{-1} heparin, 50 µg.ml^{-1} gentamycin) and put into 100-µl droplets of IVF medium under a mineral oil, where the sperm (at 2×10^6 per ml) and PHE solution (20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine) was previously added, and incubated for 18 h at 39 °C in 5 % CO_2. Following insemination, presumptive zygotes were vortexed in centrifuge tubes containing 0.5 ml holding medium for 30 s to remove residual cumulus cells. Denuded zygotes were transferred to the dish with the Buffalo Rat Liver (BRL) – cell confluent monolayer in B2 medium with 10 % FCS.

On the Day 2 since insemination – the cleavage rate, and on Day 7, 8 and 9 – the blastocyst rate were determined.

Fluorescent staining

Randomly selected blastocysts on day 7 were fixed and stained to count the total cell number. The blastocysts were washed twice in medium and in the PBS with 0.6 % of polyvinylpirrolidone and immediately fixed in 4 % formalin for 10 min. Then the embryos were covered with a drop of Vectashield anti-fade medium containing DAPI fluorochrome (chromatin staining; Vector Laboratories, Burlingame, CA, USA), mounted between a coverslip and microslide and shortly stored at 4 °C until fluorescence analysis. Stained embryos were checked under a Leica fluorescence microscope using specific filter with wavelength for blue fluorescence and x 20 magnification objective.

RESULTS

In our study totally 378 oocytes were frozen using minimum volume vitrification technique. Fewer oocytes (25) were lost or damaged during freezing or thawing and 352 oocytes were successfully thawed. From these, 85 oocytes were excluded after 2 hours of culture, because they morphologically appeared as not surviving freezing/thawing procedures. Remaining 267 oocytes were fertilized *in vitro*. Results of cleavage and blastocyst rate of vitrified and *in vitro* fertilized oocytes are presented on Table 1.

Randomly selected blastocysts on Day 7 were fixed and stained with DAPI fluorescent nuclear dye. Total numbers of nuclei/cells were counted using a Leica fluorescence microscope. The mean total cell counts in D7 blastocysts from control and vitrified oocytes were not significantly different (Table 2).

DISCUSSION

Previously we focused our investigations on cryopreservation of eggs being in ovarian tissues from cows for the purposes of national gene bank of animal genetic resources (Makarevich *et al.*, 2017). However, using this cryopreservation strategy we did not obtain promising results, as the oocytes were damaged and did not develop further (Makarevich *et al.*, 2018). In present work we are dealing with the developing a methodology for cryopreservation of mature bovine oocyte based on published reports with the best results after fertilization. We used the technique of ultra-rapid vitrification. The critical factor for ultra-rapid freezing is the minimization of the volume of medium frozen together with the oocyte. The minimum volume of the frozen sample ensures a sufficiently rapid drop

Table 1. Development of fresh or vitrified-warmed oocytes after IVF

Groups	Oocytes totally	Oocytes vitrified	Oocytes warmed	Oocytes in IVF	Embryo cleavage n (%)	Blastocyst rate n (%)
Vitrified	378	378	352	267	149 (55.81)[a]	30 (11.24)[a]
Control	404	-	-	404	293 (72.52)[b]	93 (23.02)[b]

[a] versus [b] – difference is significant at $p < 0.05$ (Chi-square test).

Table 2. Total cell number of blastocysts *in vitro* produced from vitrified or fresh bovine oocytes

Groups	No. blastocysts	Cell number (x ± SEM)
Vitrified/warmed	11	84.45 ± 10.16
Control (fresh)	14	97.29 ± 6.43

Differences between groups is not significant (t-test).

in the temperature (cooling rate) for the creation of amorphous ice without crystal formation. While in the plastic straw, traditionally used for embryo cryopreservation, the cooling rate was set at approximately 4000 °C.min^{-1}, with the minimum volume method this cooling rate increased to 22800 °C.min^{-1} (Kuwayama *et al.*, 2005), which is sufficient for solidification without crystallization.

As a carrier enabling the volume of frozen medium to be minimized we chose an electron microscopy mesh grid (EM grid), previously reported by Martino *et al.* (1996), which we improved by adding a holder (Figure 1). The added holder allows more convenient and faster handling of the grid with oocytes. The undoubted advantage of using EM grids is the possibility of simply sucking off the excessive vitrification medium while leaving the oocytes safely retained on the grid. The whole process is done very quickly. It has been reported that the time during which the oocytes are exposed to the vitrification solution before freezing is crucial for good oocyte survival after thawing. Papis and colleagues (2000) observed that development was substantially weaker in the group of oocytes that were in contact with the vitrification medium for 45 sec before freezing versus the group that was exposed to the vitrification medium for only 30 to 32 seconds. Similarly, Vajta *et al.* (1998), although choosing another method of equilibration, obtained promising results when oocytes were exposed to the vitrification medium for only 25 seconds. Therefore, we also chose 25 seconds as the time of exposure to the vitrification medium before freezing.

A great deal of the work done later could not repeat these good results (about 25 % of blastocysts; Vajta *et al.*, 1998; Papis *et al.*, 2000). However, numerous follow-up researchers obtained only blastocyst rates below 5 % (Chian *et al.*, 2004; Zhou *et al.*, 2010; Prentice *et al.*, 2011; Sprícigo *et al.*, 2014; Wiesak *et al.*, 2017; Bulgarelli *et al.*, 2017 a.o.). Remarkably, in all mentioned reports with low blastocyst rate (up to 5 %) the oocytes were exposed to ethylene glycol at a higher concentration (mostly 7.5 %) for several (5 to 10) minutes prior to freezing.

Generally, vitrification process impairs the blastocyst formation from bovine vitrified oocytes regardless of the protocol, the cryodevices and cryoprotectants used. The decreased developmental capacity of oocytes is mainly due to the toxic action of cryoprotectants. This effect was confirmed by the decreased developmental competence of oocytes exposed to cryoprotectants without freezing (Martino *et al.*, 1996). Papis *et al.* (2000) reported a "slight" equilibration at a low ethylene glycol concentration (3 %) that is sufficient to saturate oocytes during prolonged culture/equilibration (12 min) but minimizes toxic effects and does not significantly reduce oocyte developmental competence after fertilization compared to higher concentrations. A similar strategy was successfully used by Ishii and colleagues (2018).

Another important part of our methodology is the use of three-step decreasing sucrose concentration after thawing. Sucrose solution is used to restrict water permeation into the oocytes and to prevent excessive swelling of the oocytes as the cryoprotectant leaves the cells. Nowshari *et al.* (1998) tested three dilutions of cryoprotectant in one-, two- or three-steps of descending sucrose concentrations after thawing. Their experiments demonstrate that the three-step dilution procedure resulted in the highest number of mouse oocytes cryopreserved by ultra-rapid cooling developing to blastocysts. Good results were obtained also after thawing bovine oocytes using two-step (Kuwayama *et al.*, 2005) or three-step (Ishii *et al.*, 2018) dilution procedure.

In conclusion, using previously published knowledge in the field of vitrification of bovine oocytes, we have compiled and tested ultra-rapid vitrification in very small volume of vitrification solution under conditions of our laboratory. We obtained relatively high embryo cleavage rate (55.81 %) of *in vitro* fertilized oocyte after thawing, and 11.24 % of cleaved embryos developed to the blastocyst stage. The quality of these *in vitro* produced blastocysts was similar to the control, as evidenced by a comparable total cell count. Nevertheless, further research is needed to improve efficiency of in vitro embryo production.

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PROTEIN QUALITY OF LEGUME-CEREAL MIXTURES IN RUMINANTS' NUTRITION

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ABSTRACT

Legumes are the most important crops within sustainable agriculture. In animal nutrition they represent an important source of proteins. The aim of this study was to focus legumes and legume-cereal mixtures as a suitable source of protein in ruminants' nutrition. The influence of climatic conditions on the nutritional value of legumes and legume-cereal mixtures in two years of growing were compared. Quality of feed protein was studied according to the Cornell Net Carbohydrate and Protein System (CNCPS) chemical fractionation method. The highest value of fraction A was determined in 2015 in a monoculture – as high as 39.9 % in peas with leafs, in 2017 it was highest in the mixture of peas with leafs and wheat (40.7 %). During the second harvest of 2015, the amount of soluble crude protein (A) decreased in the monoculture of leafy peas by 11 % down to 28.6 %. Important for feed quality evaluation are the values of fractions B2 and B3. These are proteins that are slowly soluble in the rumen, which increases their by-pass into the intestine as well as their digestibility. Fraction B2 content exceeded 40 % in all samples from 2015. The highest fraction B2 content 52.6 % was determined in a mixture of faba bean and oats in the first harvest. The lowest value was determined in 2017 in peas with leafs-wheat mixture in both harvests (30.6 % and 35.6 %).

Key words: legumes; legume-cereal mixtures; CNCPS

INTRODUCTION

The large diversity of species allows to grow legumes in areas with significantly different natural conditions. They have valuable agronomic characteristics. They have a positive influence on soil fertility and therefore play an important role in the crop rotation system. They are an excellent fore-crop especially for cereals; they have a phytosanitary effect. Their main physiological and biochemical advantage is their ability to synthetize large amount of protein.

Mixtures of spring cereals with legumes are considered good agricultural practice in many European countries, especially in organic and low-input farming system (Knudsen *et al*., 2004). Cultivation of mixtures contributes to the complementary use of habitat resources and compensatory growth of individual plant species, causing an increased productivity and greater stability of yield (Niggli *et al*., 2008; Doré *et al*., 2011). Mixtures have a positive effect on the soil fertility, enriching it with nitrogen through a symbiosis of legumes with nodule bacteria and in organic matter due to the huge amount of crop residue left behind (Song *et al*., 2007; Iqbal *et al*., 2018).

The cultivation of mixtures of legumes and cereals offers a number of potential agronomic benefits.

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Coming from two different plant families, legumes and cereals complement each other in the capture of resources. Cereal crops growing in the vicinity of legumes benefit from nitrogen assimilated by legume root nodule bacteria. Increasing the supply of nitrogen by applying fertilizer caused in a substantial reduction of fixation of atmospheric nitrogen by legume crops. Mixtures are particularly relevant to the exploitation of poorer soils which are unsuitable for the production of either component grown as a sole crop. Yielding of the mixtures is highly dependent on the species and proportion of component. The share of legumes in the seed mixture in terms of seed number is recommended to range from 30 to 50 %. Total seed yield of mixtures decreases with increasing share of legume seeds in sowing. Increasing the dose of nitrogen for the cultivation of mixtures usually leads to increasein the yield of cereal component, but reduces the proportion of legume seeds in the crop. Increasing the share of legume seeds at sowing increases the protein concentration, digestibility and improves the protein value of the feed made of the mixtures. Legume-cereal mixtures are a good forecrop for cereals. They reduce the negative effects associated with sowing of cereals one after another. Mixtures enrich the soil with organic matter and nutrients, but the value of their post-crop area depends on the choice of components, their share in the stand, the level of yields and soil conditions. Cultivation of cereals after legume-cereal mixtures is characterized by higher yield stability. The benefits of mixed sowings of legumes with cereals are associated with a significant reduction of weed infestation, especially in organic farming. (Staniak *et al*., 2014, Huňady and Seidenglanz, 2016).

From the complex aspect of growing, ensilaging and feeding, it is advantageous to grow legumes not only as monocultures, but in combination with cereals as cereal-legume mixtures. These mixtures play the same role in the sowing system as legumes alone (improving crop, low demand for Nitrogen fertilization). Their undeniable advantage is easier ensilaging, which is based on high content of saccharides soluble in water which are essential for lactic acid bacteria and therefore for successful fermentation process in the silage (Loučka *et al*., 2013).

Plants cultivated in mixed sowings have stronger immunity against unfavourable course of weather than in pure sowings (Ceglarek *et al*., 2002; Rudnicki, 1999).

Peas is a sustainable source of plant-based protein in livestock nutrition. It proved beneficial to sow alfalfa to the peas as a cover. This has been due to the new varieties of tendrils peas, which have leafs replaced by tendrils. Those do not cast as much shadow, they are resistant to lodging and do not repress the growth of alfalfa (Loučka *et al*., 2013). Although peas is a variable, undemanding and widely utilizable source of protein and energy, it is still underutilized.

The objective of this study was to show the possibilities for use of legume and legume-cereal mixtures' as a suitable source of protein in ruminant nutrition. Their excellent advantage is that these protein feeds can be produced by the agricultural enterprises themselves, which does not create a pressure for the primary producers to buy expensive alternative feeds.

The aim of this study was to compare the influence of climatic conditions on the nutritional value of legume-cereal mixtures in two years of growing (2015 and 2017).

MATERIAL AND METHODS

In order to compare the quality of proteins of legume-cereal mixtures, we used different types of mixtures harvested in two harvests per year and in two years (2015 and 2017) [2015 – first harvest 8 July (n = 6) and second harvest 14 July (n = 6); 2017 – first harvest 14 July (n = 6) and second 20 July (n = 6)]. Samples were grown on experimental plots in Rapotin in the district Šumperk in the Czech Republic.

- Peas with leafs 100 % monoculture (variety: PROTECTA)
- Peas with leafs 70 % + wheat 30 % (variety: PROTECTA + ZUZANA)
- Leafless peas 100 % monoculture (variety: ESO)
- Leafless peas 70 % + wheat 30 % (variety: ESO + ZUZANA)
- Faba bean 100 % monoculture (variety: MERLIN)
- Faba bean 70 % + oats 30 % (variety: MERLIN + KOROK)

Dry samples were milled on hammer mill with a 1 mm screen for chemical and solubility analysis. In the samples, dry matter content and the content of crude protein was determined in accordance with the directive of the Commission (ES) no. 152/2009 from 27th January 2009, which defines the methods of sample

collection and analysis for the purposes of official feed quality control.

N fractions were determined: N soluble in phosphate-borate buffer, non-protein N (NPN) as the ratio of total N and precipitated true protein with sodium tungstate, N bound to NDF and N bound to ADF according to Licitra *et al*. (1996). Based on the above mentioned N fractions, we calculated fractions A, B_1, B_2, B_3 and C, from which rumen degradability of crude protein is predicted.

The RDP value was calculated according to NRC (2001) as:

$$RDP = A + B_1 [kdB_1 / (kdB_1 + kp)] + B_2 [kdB_2 / (kdB_2 + kp)] + B_3 [kdB_3 / (kdB_3 + kp)]$$

Where fraction A (NPN) is the percentage of CP that is instantaneously solubilized at time zero, estimated as soluble in borate-phosphate buffer but not precipitated with the protein denaturant TCA; B_1 = Fraction B_1 is the percentage of total CP soluble in borate phosphate buffer and precipitated with TCA; B_2 = Fraction B_2 is the remaining CP and is calculated as total CP minus the sum of fractions A, B_1, B_3, and C; B_3 = Fraction B_3 is calculated as the difference between the portions of total CP recovered in NDF (i.e., NDIN) and ADF (i.e., fraction C); kdB_1, kdB_2, kdB_3 = rates of degradation of fraction B_1, B_2 and B_3 in the rumen; kp = rate of passage from the rumen. Degradation rate and passage rate of individual N fractions were available from CNCPS version 5.0 (2003).

RESULTS AND DISCUSSION

The average temperature and precipitation in the monitored years were quite different. This is illustrated by Figures 1 and 2.

Crude protein content and the nitrogen fractions in the examined samples are presented in Table 1. From the collected data, we can see the differences in crude protein content as well as in nitrogen fractions between the harvest as well as years.

The largest difference in crude protein content was between the monocultures in both years and in the first harvest it was higher in all the studied samples. We confirmed that there are large differences in the nutritive contents between types of feed but also within a given variety, which are influenced by agrotechnology as well as climatic conditions of growing (Chrenková *et al*., 2004).

Pozdíšek et al., 2018 when comparing the content of crude protein in legume-cereal (pea + barley) mixture silages, determined a decrease of crude protein concentration between the first (145.4 g.kg^{-1} DM) and second (135.64 g.kg^{-1} DM) harvest. In pea + wheat mixtures, the concentration of crude protein remained unchanged between harvests (144.6 and 144.8 g.kg^{-1} DM). In the balance experiment on heifers, coefficient of CP digestibility in pea + barley mixtures was determined to be 63 % in the first and 61.38 % in the second harvest. In wheat mixtures, higher digestibility coefficient of crude protein was determined: 66.59 % (first harvest) and 65.32 % (second harvest).

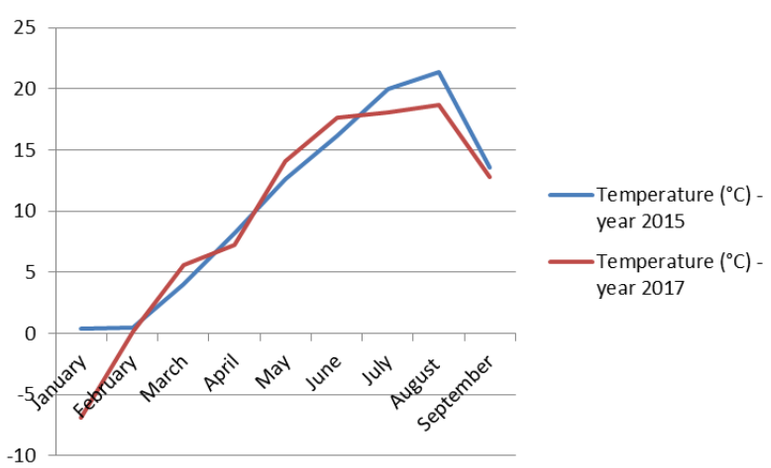

Figure 1. Average monthly temperatures in the year 2015 and 2017

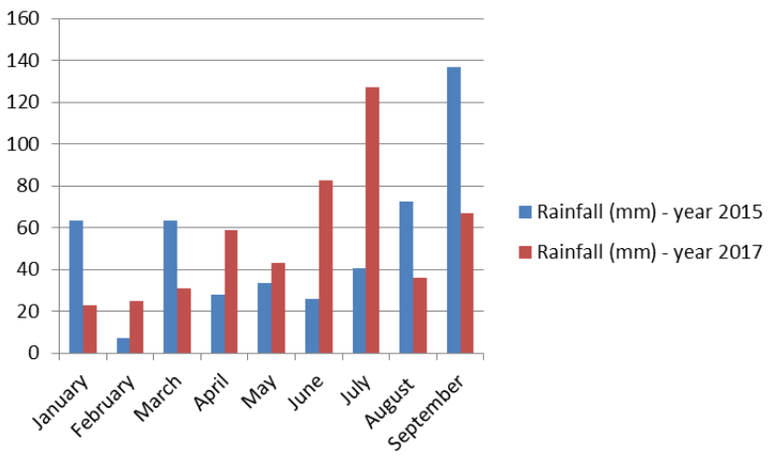

Figure 2. Average monthly rainfall in 2015 and 2017

Fraction A (crude protein very well soluble in rumen) in peas-based feeds (monoculture as well as mixtures) was higher in the first harvest in both years. It was highest in the leafy peas monoculture (39.9 %) in 2015, in 2017 it was highest in the leafy peas + wheat mixtures (40.7 %).

Fraction B_1 (rate of degradation in rumen is high) was lower in the first harvest of 2015 than in the second, while in 2017 it was reverse with the exception of peas + wheat mixtures. Total soluble crude protein content is determined by summing fractions A and B_1, which are quickly degraded in the rumen and utilized primarily by the rumen microorganisms. In the first harvest of 2017, we determined the highest content of soluble crude protein in the leafy peas monoculture (51.5 %) and the lowest in the first harvest of 2015 in the mixture of faba bean and oats (28.6 %).

Important for feed quality evaluation are the contents of B_2 and B_3 fractions. These are crude protein fractions slowly soluble in rumen, which increases their by-pass into the intestine, and also their digestibility. Fraction B_2 content was higher than 40 % in all samples in year 2015. The highest fraction B_2 content was determined in the mixture of faba bean and oats in the first harvest (52.6 %). The lowest content was determined in 2017 in leafy peas and wheat mixture in both harvests (30.6 % and 35.6 %).

Fraction C is very variable and changes based on certain technology of feed treatment (thermic treatment, incorrect ensilaging, etc.). This fraction is non-degradable in rumen and indigestible. These are crude proteins bound to acido-detergent fibre (cellulose, lignin, etc.). The fraction C content decreased with increased ripeness in both harvests in both years.

From the studied samples in 2015, the highest content of utilizable crude protein (fractions B_2 and B_3) was determined in the mixture of 70 % faba bean and 30 % oats in both harvests and in 2017 in the faba bean monoculture in the first harvest.

Our results confirmed the influence of climatic conditions on crude protein content and the values of CNCPS in the studied feed samples (Table 1).

Płaza *et al.* (2008) found in their experiments that the conditions of vegetation period significantly modified the yield of seeds and efficiency of true protein from the seeds of field pea and spring triticale mixtures. The highest yield of seeds was obtained from mixtures of field pea and spring triticale of component content 60 + 40 %, and the highest efficiency of true protein was obtained from field pea seeds cultivated in pure sowing. The mixtures of field pea with spring triticale provided more true protein from seeds than spring triticale cultivated in pure sowing.

The quantification of the main crude protein (CP) fractions during the growing period of pea and oat mixtures may be used to optimize the forage management. The determination of protein fraction could improve balancing rations for

Table 1. Fractions of crude protein according to CNCPS in years 2015 (n = 12) and 2017 (n = 12)

		% CP						
1st harvest (8.7.2015)	CP (g·kg^{-1} DM)	A	B_1	B_2	B_3	C	Insoluble CP	Soluble CP
Peas with leafs 100 %	161.7	39.9	7.1	40.9	2.1	10.1	53.1	46.9
Peas with leafs 70 % + Wheat 30 %	111.8	26.9	10.7	43.0	7.0	12.5	62.4	37.6
Leafless peas 100 %	139.9	31.4	13.4	40.2	4.6	10.5	55.3	44.7
Leafless peas 70 % + Wheat 30 %	108.1	26.5	12.1	42.7	6.4	12.4	61.5	38.5
Faba bean 100 %	206.1	33.0	9.9	44.3	5.1	7.7	57.1	42.9
Faba bean 70 % + oats 30 %	104.7	20.4	8.2	52.6	6.3	12.5	71.4	28.6
2nd harvest (14.7.2015)								
Peas with leafs 100 %	137.8	28.6	17.7	41.6	3.8	8.3	53.9	46.3
Peas with leafs 70 % + Wheat 30 %	113.5	22.6	15.1	43.1	8.6	10.7	62.3	37.7
Leafless peas 100 %	113.8	18.8	21.0	45.5	4.4	10.3	60.2	39.8
Leafless peas 70 % + Wheat 30 %	94.9	23.9	14.4	43.5	3.4	14.8	61.7	38.3
Faba bean 100 %	181.1	33.1	10.7	44.1	4.4	7.8	56.2	43.8
Faba bean 70 % + oats 30 %	106.0	23.0	10.7	45.9	10.0	10.4	66.4	33.7
		% CP						
1st harvest (14.7.2017)	CP (g·kg^{-1} DM)	A	B_1	B_2	B_3	C	Insoluble CP	Soluble CP
Peas with leafs 100 %	207.9	37.6	13.9	30.7	5.9	11.9	48.5	51.5
Peas with leafs 70 % + Wheat 30 %	197.2	40.7	10.5	30.6	5.9	12.4	48.8	51.2
Leafless peas 100 %	187.9	32.9	12.4	33.9	7.5	13.3	54.7	45.3
Leafless peas 70 % + Wheat 30 %	177.8	35.8	10.1	37.4	3.8	12.9	54.1	45.9
Faba bean 100 %	228.2	18.9	11.7	51.1	7.2	11.1	69.4	30.6
Faba bean 70 % + oats 30 %	186.4	35.8	10.9	31.7	8.7	13.0	53.3	46.7
2nd harvest (20.7.2017)								
Peas with leafs 100 %	184.3	35.7	10.5	37.4	6.8	9.6	53.8	46.2
Peas with leafs 70 % + Wheat 30 %	182.8	35.0	11.6	35.6	7.3	10.6	53.5	46.5
Leafless peas 100 %	168.0	31.8	7.2	43.1	6.9	11.0	61.0	39.0
Leafless peas 70 % + Wheat 30 %	167.9	30.7	11.1	40.3	7.1	10.8	58.2	41.8
Faba bean 100 %	217.3	34.0	9.0	42.6	4.9	9.5	57.0	43.0
Faba bean 70 % + oats 30 %	172.0	31.6	9.9	40.3	7.9	10.3	58.5	41.5

A – fraction of non-protein Nitrogen – highly soluble in rumen, B_1 – fraction of crude protein soluble in buffer and precipitated with TCA – the rate of degradation in rumen is high, B_2 – fraction of crude protein non-soluble in buffer, but soluble in neutral and acidic agents – rate of degradation in rumen is lower, B_3 – fraction of crude protein soluble in acidodetergent agent, rate of degradation is lower, C – fraction of crude protein, which are not soluble even in acidodetergent agent.

ruminants (Marković *et al*., 2017). Marković *et al*., 2017 in their work tested: The pea and oat at two different mixture rates: A_1 – 50 % pea + 50 % oat and A_2 – 75 % pea + 25 % oat and different a cutting time in three stages of growth: B_1 – a cutting of biomass at the start of flowering pea (10 % of flowering), B_2 – a cutting of biomass at forming the first pods on 2/3 plants of pea, and B_3 – cutting of biomass at forming green seeds in 2/3 pods. Stage of growth and pea-oat ratio in mixtures are significantly related to the change in the quality and chemical composition of biomass. The highest level of crude protein was obtained in pea at flowering stage (184.85 g.kg^{-1}) dry matter. The high level of easily soluble protein and non-protein nitrogen compounds (over 50 %) represent specific characteristics of the mixture. Unavailable fraction PC increased with plant maturation from 75.65 to 95.05 g.kg^{-1} of CP.

CONCLUSION

Not only the legumes represent a quality feed, but their growing and feeding is also one of the options to cover a portion of the economically demanding protein requirements in animal nutrition using domestic feed sources. An undeniable advantage of feeding green legumes is that they increase the content of digestible crude protein in the mixtures. Legumes can be utilized in a variety of ways, which is confirmed also by our results.

Legume-cereal mixtures represent an excellent fore-crop in the crop rotation system, have a positive influence on soil quality, are more resistant against diseases and pests, repress weed and enrich soil with Nitrogen. They are a suitable source of crude protein for livestock.

The quality of legumes and the studied mixtures changes not only with the vegetation period but also under the influence of climatic conditions.

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SPERM MOTILITY OF RAMS FROM TWO SLOVAK SHEEP BREEDS: SHORT COMMUNICATION

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ABSTRACT

In the present study, fresh ram sperm samples (n = 52) from the Native Wallachian (NW; n = 26) and Improved Wallachian (IW; n = 26) Slovak sheep breeds were collected from males of each breed by electro-ejaculation and analysed for motility using CASA and morphology using light microscopy assessment. Our results showed no significant differences between the breeds (P ≤ 0.05) in the sperm concentration and motility traits (Table 1). Also both these ram groups did not differ (P ≤ 0.05) in sperm morphology parameters (Table 2). Since, this is only preliminary research, additional experiments using higher number of samples (rams) as well as other evaluation approaches are required in order to compare sperm characteristics for different breeds.

Key words: Slovak rams; sperm; quality; motility; morphology

INTRODUCTION

The success of artificial insemination (AI) depends on factors related to male and female fertility, the oestrous synchronization and insemination practices (David *et al.*, 2008).

Fertility is a very complex biological function that depends on several properties of the spermatozoa, including sperm motility and morphology, which could be some of the indicators of the spermatozoa quality. CASA (computer-assisted sperm analysis) technology has been used for more objective and reproducible evaluation of sperm motility in different mammalian species (Ax *et al.*, 2000; Kubovičová *et al.*, 2011).

Native Wallachian sheep is a typical seasonally poly-oestrous breed, which were brought to the territory of Slovakia in the 13th and 14th centuries. This breed is registered as a national genetic resource since 1992. At present, 2 554 animals of this breed are kept in Slovakia.

Improved Wallachian Sheep was generated by the intentional combined crossing of NW sheep with rams of various imported semi-coarse-wool and semi-fine-wool breeds (Hamsphire, Cheviot, Texel, Lincoln and Leicester). This breed is perspective mainly for mountainous areas; in 1982 it was recognized as a new semi-coarse-wool breed. At present, 128 930 animals of this sheep breed are kept in Slovakia (Chrenek *et al.*, 2019).

Therefore, in order to gain biological material from valuable Slovak sheep breeds it is necessary to optimize appropriate methods of collection and evaluation of ram sperm for the purpose of their cryopreservation and storage in the animal gene bank. The aim of our preliminary experiments was to compare several sperm characteristics (motility, progressive motility, sperm concentration and morphology)

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of rams from two different sheep breeds – Native Wallachian (NW) and Improved Wallachian (IW), determined by CASA assay.

MATERIAL AND METHODS

Semen collection and evaluation

Clinically healthy rams of NW and IW sheep breeds aged from 1 to 7 years were used in this experiment. All rams were maintained in the flock and fed oats; water was supplied *ad libitum*. The semen samples (n = 52) were collected from four rams by electroejaculation. After collection, the semen was transported to the laboratory in a thermos flask with pre-warmed water at 37 °C.

Sperm motility and concentration

Semen was diluted in a saline (0,9 % NaCl; Braun, Germany) at a ratio of 1:40, immediately placed (2 µl) into a Leja Standard Count Analysis Chamber (depth of 20 microns; MiniTübe, Tiefenbach, Germany) and evaluated under a Zeiss AxioScope A1 microscope using the CASA software (Sperm VisionTM, MiniTübe, Tiefenbach, Germany). For each sample, six microscopic view fields were analysed for the linearity, straightness and cross wobble (LIN, STR, WOB), sperm concentration (CON; 1 x 10^9), and percentage of total motility (TM; > 5 µm.s^{-1}) and progressively moving spermatozoa (PM; > 20 µm.s^{-1}), as previously described by Kulíková *et al.* (2018).

Sperm morphology

A drop (5µl) of the ejaculate diluted with distilled water at the ratio 1:40 was placed onto the slide, covered with a coverslip and observed under a microscope with a 100x magnification objective under an immersion oil. We evaluated the morphology malformations of the sperm, such as separated tail, knob-twisted tail, torso tail, rounded tail, broken tail, retention of the cytoplasm drop, enlarged or reduced sperm head and other acrosomal sperm changes. For the determination of occurrence of morphological changes in sperm totally 400 sperm cells were examined.

Statistical analysis

The results were statistically processed by a t-test using a SigmaPlot software (Systat Software Inc., Germany). Data were expressed as the mean ± standard error of the mean (SEM). Values at $P \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

In our study 52 sperm samples from rams of NW and IW sheep breeds were analysed. There were no significant differences in the basic motility parameters such as CON, TM, PM, STR, LIN and WOB of fresh sperm samples between the tested rams (Table 1).

Common *in vitro* evaluation of sperm quality involves the subjective assessment of motility and the percentage of sperm with normal morphology (O'Hara *et al.*, 2010). The main changes that occur during semen storage include the reduction in motility, alterations in morphology and others which may result in declined fertility. However, most of these results are ambiguous.

Our results showed no difference in evaluated individual morphologic features such as separated tail, knob-twisted tail, rounded tail, etc. (Table 2).

Table 1. Concentration and motility parameters of ram sperm from two sheep breeds

RAM	CON (x10^9)	TM (%)	PM (%)	STR (%)	LIN (%)	WOB (%)
NW	1.095 ± 0.22	41.564 ± 5.4	37.642 ± 5.39	0.851 ± 0.04	0.524 ± 0.03	0.586 ± 0.03
IW	0.996 ± 0.2.	41.372 ± 5.72	36.564 ± 5.74	0.840 ± 0.04	0.503 ± 0.03	0.569 ± 0.03

The results are expressed as average ± SEM; $P \leq 0.05$.
NW – Native Wallachian sheep; IW – Improved Wallachian sheep; CON – concentration; TM – total motility; PM – progressive motility, STR – straightness; LIN – linearity; WOB – wobble.

Table 2. Occurrence of morphology malformations in ram sperm from two sheep breeds

RAM	TOTAL (%)	ST (%)	KT (%)	TT (%)	RT (%)
NW	5.288 ± 0.28	1.019 ± 0.09	0.827 ± 0.09	1.087 ± 0.12	0.702 ± 0.06
IW	5.067 ± 0.2	1.115 ± 0.08	0.760 ± 0.04	0.04 ± 0.07	0.731 ± 0.05

RAM	BT (%)	RCD (%)	SH (%)	LH (%)	ACH (%)
NW	0.983 ± 0.07	0.269 ± 0.04	0.115 ± 0.04	0.125 ± 0.04	0.154 ± 0.04
IW	0.875 ± 0.08	0.317 ± 0.06	0.067 ± 0.02	0.125 ± 0.03	0.144 ± 0.05

The results are expressed as average value ± SEM.
ST – separated tail; KT – knob-twisted tail; TT – torso tail; RT – rounded tail; BT – broken tail; RCD – retention of the cytoplasm drop; SH – small head; LH – large head; NW – Native Wallachian sheep; IW – Improved Wallachian sheep; ACH – acrosomal changes.

Kulaksiz *et al.* (2011) reported the increased percentage of abnormal spermatozoa with the increase in number of days in storage for all of the extenders tested in their study. Sperm morphology is extremely variable between even close species (Maroto-Morales *et al.*, 2010), however our results showed opposite trend, which could be due to fewer tested rams.

In conclusion, the results of this study are of preliminary character. Therefore, experiments involving higher number of samples (individuals), as well as other analytical approaches are needed in order to find the best assessment tool for evaluation of ram sperm to be stored in the national gene bank.

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