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FERTILIZING ABILITY OF PINZGAU BULL SPERM *IN VITRO* AFTER CRYOSTORAGE

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ABSTRACT

The aim of the study was to evaluate fertilizing ability of Pinzgau bull sperm after different periods of cryostorage. The sperm samples from 16 bulls were arranged into the three groups according to cryostorage period as follows: less than 7 years (group 1), 7 to 13 years (group 2) and 14 or more years (group 3). Straws with frozen sperm doses were thawed in a water bath and the motile fraction of sperm was selected by modified swim-up method. Motile spermatozoa (2 x 10^6.ml^{-1}) were co-incubated with matured bovine oocytes in fertilization drops (IVF-TALP medium) under mineral oil at 39 °C in presence of 25 µg/ml heparin for 20 hours. Totally, 739 oocytes were used in the *in vitro* fertilization test. Following fertilization the presumptive zygotes were stained with DAPI nuclear stain and the status of chromatin was examined under fluorescence microscope. Penetration of the sperm head into the ooplasm of oocyte and fertilization (formation of pronuclei) were evaluated. No significant difference in fertilizing ability among the experimental groups was found. The rates of penetrated plus fertilized eggs for group 1, 2 and 3 were 79.31, 76.40 and 76.14 %, respectively. Significantly higher pronuclear formation rate was observed in the group 1, where 72.9 % of eggs had two visible pronuclei at 20 hours following incubation with sperm compared to 65.72 and 47.21 % in groups 2 and 3, respectively. Also, 4 % of fertilized eggs from the group 1 reached syngamy of both pronuclei, probably due to a faster formation of pronuclei, whilst no syngamy was observed in groups 2 and 3. In conclusion, period of cryostorage had no direct influence on penetrating and fertilizing ability of Pinzgau bull sperm doses. However, it is assumed that cryostorage length can affect the speed of pronucleus formation.

Key words: Pinzgau; bull; sperm; fertilizing ability; cryopreservation

INTRODUCTION

Cryostorage of bull semen is widely used as a useful tool to improve reproduction. In dairy cattle, the majority of inseminations are done with frozen–thawed semen. Cryopreserved semen plays a major role in genetic improvement, economics of breeding programs in the livestock industry, and preservation of endangered species and breeds. Sperm cryopreservation methods are the most efficient way for storing animal genetic resources. It is well known, that cryopreservation is stressful for spermatozoa. Although the purpose of sperm cryopreservation is to preserve sperm function and fertility, the freezing-thawing process inevitably causes their damage and reducing fertility. During freezing and thawing spermatozoa are subjected to various stresses such as cold shock, osmotic and oxidative stress and intracellular ice crystal formation. These cause disruption of sperm integrity. Particularly, membrane structure alterations (Krogenaes *et al.*, 1994; Januskauskas *et al.*, 2003), and sperm function alterations after thawing (Garner *et al*., 1997; Thomas *et al*., 1998; Pons-Rejraji *et al.*, 2009) were described. In addition, excessive mitochondrial activity induces the generation of reactive oxygen species (Chatterjee and Gagnon, 2001) that affects functions of cellular compounds and organelle (Bilodeau *et al.*, 2000).

Some evidences also exist about cryodamages

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to sperm DNA integrity after cryopreservation (Gandini *et al*., 2006; Waterhouse *et al.*, 2010). These damages ultimately result in reduced sperm motility, viability and fertilizing ability (Watson, 2000). It is assumed, that once frozen semen in the liquid nitrogen can be potentially stored unlimited time. But only scarce information was published about the influence of long-term cryostorage on quality of frozen-thawed bull semen. In humans, it was proved that sperm can be useful after even more than 28 years of cryostorage (Clarke *et al.*, 2006). But some alterations may be related to long period of storage. For example the percentage of motile sperm was lower after long-term (9–13 years) compared to short-term (1–5 years) storage (Edelstein *et al.*, 2008).

The aim of the study was to evaluate fertilizing ability of Pinzgau bull sperm after different periods of cryostorage in liquid nitrogen.

MATERIAL AND METHODS

Procedure of *in vitro* fertilization (IVF)

Bovine ovaries were collected at a local abattoir and transported to the laboratory within 3 hours at 25–30 °C. Ovaries were rinsed with 70 % ethanol and a sterile saline solution. Cumulus-oocyte complexes (COCs) were acquired by follicular fluid aspiration from visible large follicles (2–10 mm in diameter) on the surface of ovaries. Collected COCs with pale homogeny cytoplasm and complete cumulus were selected and washed twice in maturation medium E199 with HEPES (Biowest). For maturation, E199 medium with glutaMAX (Gibco), sodium pyruvate (0.25 mmol.l^{-1}), gentamycin (0.05 mg.ml^{-1}), fetal bovine serum 10 % (FBS, BioWhitaker, Verviers,Belgium) and FSH/LH (1/1 I.U., Pluset, Lab. Calier, Barcelona, Spain) was used. Oocytes were matured at 39 °C in a humidified atmosphere with 5 % CO_2 in air for 24 h. Matured oocytes were partially cleaned from cumulus cells by vortexing during 3 min. Frozen-thawed insemination doses from Pinzgau bulls were used for *in vitro* fertilization of oocytes. Insemination doses from 16 bulls of a proven fertility were used in experiments. Straws were thawed in a water bath at 36 °C and the motile fraction of sperm was separated in Sperm-TALP medium using modified swim-up procedure. The sperm fraction was then resuspended in IVF-TALP medium and chosen sperm concentration was adjusted. Following maturation, the presumptive matured oocytes were placed into 100 µl drops of fertilization medium IVF-TALP under sterile mineral oil and incubated with 2×10^6.ml^{-1} spermatozoa in the presence of 25 µg/ml heparin (Sigma-Aldrich, Germany) at 39 °C in a humidified atmosphere with 5 % CO_2 in air for 20 hours.

Following this time, the presumptive zygotes were cleaned off from the excessive sperm and remaining cumulus cells by vortexing, washed twice in Sperm-TALP medium and in the PBS with 0.6 % polyvinylpyrrolidone. Zygotes were immediately fixed in 4 % formalin during 10 min and then covered with a drop of Vectashield anti-fade medium containing DAPI stain (chromatin staining; Vector Laboratories, Burlingame, CA, USA) and mounted between coverslip and microslide and 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 x20 magnification objective. Penetration of sperm head into the ooplasm of oocyte and formation of pronuclei (fertilizing ability) were evaluated.

Statistical analysis

Differences in distribution of oocytes into either the penetrated, fertilized (presence of pronuclei) or non-penetrated among the groups of different cryostorage period were analyzed by Pearson´s Chi-square test of independence. All calculations were performed using the SAS software package (SAS Institute, 2001).

RESULTS AND DISCUSSION

Fertilizing ability estimated by *in vitro* fertilization test may be a good tool to predict potential fertility of a bull (Larsson and Rodrígues-Martínez, 2000). *In vitro* fertilization test was previously used by several authors to test fertilizing ability of sperm as a homologous system with cow oocytes and bull sperm (Fazeli *et al*., 1997; Zhang *et al.*, 1998; Marquant-Le Guienne *et al*., 1990) or as heterologous system with sperm of different species using cow oocytes. Such heterologous system was, for example, used to test ram (Garcia- Alvarez *et al.* 2009; Makarevich *et al*., 2011), antelope (Roth *et al.*, 1998), stallion (Bromfield *et al.*, 2013) and dolphin (Sánchez-Calabuig *et al*., 2015) sperm fertilizing ability. Major advantage of these *in vitro* tests is that the testing is not so expensive and labor intensive procedure as testing by artificial insemination. The *in vitro* fertilization technique can be very accurate to assess the sperm fertility, because the procedure evaluates the spermatozoa-oocyte interactions occurring during fertilization process.

In our previous study (Makarevich *et al*., 2011) we used co-incubation of ram spermatozoa with bovine oocytes with intact *zona pellucida* in the *in vitro* fertilization test, where about 53–55 % of the oocytes were penetrated by ram semen and about 30–33 % of them were fertilized. Similarly García-Alvarez *et al*. (2009) documented that such heterologous *in vitro* fertilization test can be useful to predict the *in vivo* fertility of rams.

Fertilizing ability is very individual property of bull (Ax and Lent, 1987; Merckies *et al.*, 2000).

To minimize individual effect of bull and underline the effect of time we created experimental groups from minimum three bulls, according to the length of cryostorage period.

No significant difference in fertilizing ability (rate of penetrated and fertilized eggs) among the three experimental groups was found. The total rates of penetrated and fertilized eggs for group 1, 2 and 3 were 79.31; 76.40 and 76.14 %, respectively (Table 1). Similarly, period of bull sperm storage had no influence on the occurrence of polyspermy after *in vitro* fertilization. According to period of storage 6.3 %, 6.5 % and 5.1 % of fertilized eggs were polyspermic in groups 1, 2 and 3, respectively (Table 1). Similar rate of polyspermy was reported by Santos with co-workers (2008), where the polyspermy rates varied from 4.1 to 11.1 %. It was proved, that polyspermy after bovine *in vitro* fertilization is mainly influenced by individuality of a bull, heparin dose used (Marquant-Le Guienne *et al.*, 1990) and also related to oocyte quality (Santos *et* al., 2008).

On the other hand, significant differences were found in the rate of pronuclei formation. Significantly higher pronuclear formation was observed in the group 1 (stored for 1–6 years), where 72.9 % of eggs had two visible pronuclei at 20 hour after sperm addition compared to 65.72 and 47.21 % in groups 2 and 3, respectively (Table 1). Also, syngamy of both pronuclei was observed in 4 % of fertilized eggs in the group 1, whilst no syngamy was observed in groups 2 and 3. This difference may be probably due to faster pronuclear formation in the group 1.

Slower decondensation of sperm heads occurring in groups 2 and 3 may be related to chromatin structure alteration, described in cryostored human (Royere *et al*., 1991) and boar (Hamamah *et al*., 1990) spermatozoa. In particular, Royere with co-workers (1991) concluded that freezing-thawing procedure may alter the DNA/nuclear protein relationships and impair the fertilizing ability of human sperm. Similarly, Hamamah *et al*. (1990), basing on their results, hypothesize that sperm chromatin after freezing-thawing may be overcondensed; this overcondensation may be associated with the lower conception rates obtained using human and porcine semen after cryostorage.

Table 1: Fertilizing ability of Pinzgau bull sperm after cryostorage

Groups	Period of storage (years)	No. bulls	No. oocytes	Penetrated and fertilized (N) %	Non-penetrated (N) %	Oocytes with two pronuclei (N) %	Polyspermic oocytes from fertilized (n/N) %
1	1 to 6	3	203	(161) 79.3	(42) 20.7	(148) 72.9	(10/158) 6.3
2	7 to 13	7	339	(259) 76.4	(80) 23.6	(227) 67.0	(16/243) 6.5
3	14 to 18	6	197	(150) 76.1	(47) 23.9	(93) 47.2	(5/98) 5.1
total		16	739	(570) 77.1	(169) 22.9	(468) 63.3	(31/499) 6.2

CONCLUSION

In conclusion, length of cryostorage had no direct influence on *in vitro* penetrating and fertilizing ability of cryopreserved Pinzgau bull sperm. However, long-term cryostorage might affect speed of pronucleus formation and syngamy in *in vitro* fertilized eggs.

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CRYOPRESERVATION OF AMNIOTIC FLUID STEM CELLS DERIVED FROM ZOBOR RABBIT

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ABSTRACT

The aims of our preliminary study were to evaluate the viability and to investigate the occurrence of early and late apoptosis of fresh, frozen-thawed and thawed-cultured rabbit amniotic fluid stem cells (AFSCs). Rabbit AFSCs were isolated from young (4-6 months old) Zobor rabbit females (n = 3), cultured in endothelial basal medium (EBM-2) to passage 3 (P3) and frozen. One month after cryopreservation AFSCs were thawed and cultured for 72 hours. We identified the apoptotic (Annexin-V - AnV; Yo-Pro-1 - YoP) and dead (propidium iodide - PI) AFSCs using flow cytometry and evaluated the viability of fresh (F-P3), frozen-thawed (F-T) and thawed-cultured (72 hours of post-thaw *in vitro* culture – T-72h) AFSCs. To examine the phenotype of P3 AFSCs we performed an antibody-based staining of surface markers CD29, CD44 and CD45. We have found decreased ($P < 0.05$) viability in F-T cells (77.5 ± 2.2 % and 75.2 ± 0.7 % for AnV^-/PI^- and YoP^-/PI^-, respectively), compared to F-P3 (92.5 ± 2.2 and 89.45 ± 1.3 for AnV^-/PI^- and YoP^-/PI^-, respectively). However, viability of T-72h cells was similar to F-P3 (90.9 ± 1.5 % and 84.6 ± 1.7 % for AnV^-/PI^- and YoP^-/PI, respectively). Our AFSCs were both $CD44^+$ (95.98 ± 1.51 %) and $CD29^+$ (92.33 ± 4.46) positive but $CD45^-$ (0.73 ± 0.55 %) negative, according to defined MSCs phenotype.

Key words: rabbit; amniotic fluid; stem cells; viability; cryopreservation

INTRODUCTION

Stem cells are the focus of great interest because of their potential for therapy in a wide variety of conditions. Conditions possibly treatable by stem-cell transplants include genetic defects, tissue and organ replacement, autoimmune disease and malignancies (Dziadosz *et al*., 2016). These cells with high differentiation capacities are able to give rise to tissue derivatives of three germ layers; ecto-, meso- and endoderm lineages. They can be spontaneously differentiated through the formation of embryoid bodies (Valli *et al.*, 2010), and can also undergo targeted differentiation under appropriate culture conditions (De Coppi *et al*., 2007a).

Amniotic fluid collected routinely via amniocentesis allows us to bank stem cells. These AFSCs can be proliferated in culture as needed and offer a convenient source for autologous transplantations (Abdulrazzak *et al.*, 2013). They can be isolated from mid-term amniotic fluid (AF) of both human and rodents and cultured up to 300 passages (De Coppi *et al*., 2007b; Phermathai *et al.*, 2010). Many authors declare that human AFSC are cultured easily, remain viable over many passages and tolerate cryopreservation very well (Da Sacco *et al*., 2011; De Coppi *et al*., 2007a; Young *et al.*, 2015; Chen *et al.*, 2014). Besides human, they have been isolated from various species including dogs, pigs, horses and buffaloes (Chen *et al.*, 2011; Choi *et al*.,2013; Dev *et al.*, 2013; Filioli Uranio *et al*., 2011; Iacono *et al*., 2012; Yadav *et al.*, 2011).

Stem cells are characterized by the presence of surface markers that are associated with self-renewal without differentiation. The flow cytometry has been a primary technique for the identification of stem cells

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by detection of these markers (Dziadosz *et al.*, 2016). Previously described AFSCs also exhibit intermediate characteristics between embryonic and adult stem cells. They are devoid of ethical controversies and are safer in clinical applications (Chen *et al.*, 2011; De Coppi *et al.*, 2007b; Pozzobon *et al.*, 2010). In the past, scientists classified AFSCs as pluripotent, according to the marker expression. However, the inability to form tumors upon transplantation positioned AFSCs as broad multipotent (Canazi and De Coppi, 2012) or potentially pluripotent stem cells (Gao *et al.*, 2013). Moreover, primary cultures of amniotic fluid contain a heterogeneous population of cells due to the direct contact of the fluid and the fetus (Cremer *et al.*, 1981; Ferdaos and Nordin, 2012; Joo *et al.*, 2012). Paebst *et al.* (2014) showed method for selection of specific cells prior to the cultivation by sorting of $CD44^+$ cells from amniotic fluid.

The use of preclinical models is promising for future therapeutic possibilities (Dziadosz *et al.*, 2016). For example, Klein *et al.* (2011) transplanted AFSCs to fetal lambs and reported more rapid healing time of skin wounds. Sun *et al.* (2015) differentiated human AFSCs into keratinocytes that improved epidermal regeneration of intentionally excised wounds in mouse model. De Konick *et al.* (2015) observed improved lung density and function in rabbit model with congenital diaphragmatic hernia after intratracheal injection of human AFSCs.

The aims of our preliminary study were to evaluate the viability and to investigate the occurrence of early and late apoptosis of fresh, frozen-thawed and thawed-cultured rabbit AFSCs.

MATERIAL AND METHODS

Cells isolation and culture

Amniotic fluid was obtained from humanely sacrificed Zobor rabbits (n = 3) at 23rd day of gravidity using pipette under sterile conditions and mixed with culture medium at the 5:6 ratio. The medium was composed of EBM-2 basal medium (CC-3156, Lonza, USA) supplemented with 20 % fetal calf serum (FCS), EGM-2 SingleQuots™ Kit (CC-4176, Lonza, USA) and 1 % of penicillin and streptomycin (Life Technologies, Slovakia). Five ml of the mixture was used for seeding the cells into T25 culture flasks. Cells were cultured and medium was changed at 5th day, after the cells formed adherent colonies. Subsequently, the culture medium was changed every 2nd day. Cells were dissociated after the colonies reached 80-90 % confluency using Accutase (Invitrogen, Carlsbad, CA, USA) for 5 min at 37 °C and 5 % CO_2. AFSCs were counted and seeded into new culture flasks at density of 4-5 x 10^4 cells/cm^2. Subsequently, the cells were cultured and passaged after 3-4 days, after they reached 80-90 % confluency.

Freezing

Rabbit AFSCs were frozen in freezing solution composed of medium used for culture and 10 % DMSO (D2650, Sigma-Aldrich). 2 x 10^6 cells were mixed with 1.5 ml of freezing solution and placed into cryovials. Cryovials were frozen at slow rate by reducing the temperature by 1 °C per minute in Mr. Frosty container (Thermo Scientific Nalgene, Rochester, NY, USA) and stored for 24 hours at -80 °C. After 24 hours the cryovials were immersed into liquid nitrogen and stored for 1 month. Thawing of the AFSCs was rapid and performed by swirling the cryovials in water bath at 37 °C for 30-60 sec. The suspension was then slowly instilled into pre-warmed culture medium to prevent osmotic shock.

Fluorescent staining

The viability of the cells was evaluated prior to cryopreservation (F-P3), immediately after thawing (F-T) and after additional culture for 72 h (T-72 h). Annexin-V (Roche Slovakia, Slovak Republic) and Yo-Pro-1 (Molecular Probes, Lucerne, Switzerland) staining was used for detection of early and late apoptosis, respectively. The dead cells were identified by propidium iodide (Molecular Probes, Eugene, Oregon, USA) staining. At least 0.5-1 x 10^6 cells were washed in AnV buffer and PBS without magnesium/calcium (Life Technologies, Slovakia) for AnV and YoP staining, respectively. Washed cells were placed in 50 µl of prepared staining solution and incubated in the dark at room temperature for 20 min. At least 1 x 10^4 cells in each sample were analyzed by flow cytometer FACS Calibur (BD Biosciences, USA).

Surface marker detection

Flow cytometry immunostaining was performed to identify the expression of the cell surface markers CD44, CD29 and CD45. Primary antibodies were as follows: anti-rabbit CD29 (mouse IgG1, clone P4G11, Abcam, United Kingdom), anti-rabbit CD44 (mouse IgG1, clone W4/86, AbD Serotec, Germany), anti-rabbit CD45 (mouse IgG1, clone L12/201, AbD Serotec, Germany). Secondary antibodies were as follows: anti-IgG1-PE, mouse (rat IgG1, clone X-56, Miltenyi Biotec, Germany) for CD44 and CD29; anti-IgG1-APC, mouse (rat IgG1, clone X-56, Milteny Biotec, Germany) for CD45. Briefly, F-P3 cells were dissociated, washed with PBS and centrifuged for 5 min at 587 x g at 4 °C. The pellet was resuspended in 50 µl of rabbit serum to block the Fc receptors. Cells were incubated with isotype controls, primary and secondary antibodies for 15 min on ice. At least 0.5 x 10^5 cells were analyzed for each CD marker.

Statistical Analysis

The statistical analysis was performed with One-Way ANOVA (Tukey-Test) for comparison of mean values ± SDs using SigmaPlot software (Systat Software Inc., Germany).

RESULTS AND DISCUSSION

Our present research was focused on isolation of rabbit AFSCs that could serve as a tool for preserving the animal genetic resources in gene bank.

Immunophenotyping of rabbit AFCS in our study revealed that they express surface molecular markers CD44 and CD29 that are commonly used to profile MSCs. The expression of CD44 was 95.98 ± 1.51 %, which fulfil criteria of the International Society for Cellular Therapy for positive MSCs marker expression (Dominici *et al.*, 2006). The expression of CD29 was slightly lower (92.33 ± 4.46 %). Nevertheless, such positivity was considered as sufficient for accepting these cells as $CD29^+$ according to other studies, which declared AFSCs as CD29 positive, without quantification (You *et al*., 2009; Paebst *el al*., 2014; Filioli Uranio *et al*., 2011).

Moreover, AFCSs in our study were more positive to CD44 marker than AFSCs isolated by Slamečka and Chrenek (2013). We suggest that more than the two-fold higher proportion of cells expressing CD44 (95.98 ± 1.51 % vs 47.8 %) might be attributed to antibodies specificity. We have used anti-rabbit antibodies, whereas Slamečka and Chrenek (2013) used antibodies designed for human AFSCs. It is necessary to say, that the markers required for defining a MSC were mainly determined for human cells, and the lack of specific antibodies for different animal species led to a great variability of approaches and results for molecular characterization of animal MSCs (Iacono *et al*., 2015). Furthermore, hematopoietic lineage marker CD45 was used as a negative control. The cells fulfilled the criteria of Dominici *et al*. (2006) for negative marker expression (0.73 ± 0.55 %).

Viability of AFSCs was evaluated by flow cytometry fluorescent staining. AnV^-/PI^- and YoP^-/PI^- cells were gated to evaluate the overall viability. We have found decreased ($P < 0.05$) viability in F-T cells (77.5 ± 2.2 % and 75.2 ± 0.7 % for AnV and YoP, respectively), compared to F-P3 control (92.5 ± 2.2 and 89.45 ± 1.3 for AnV and YoP respectively). However, viability of T-72 h cells was similar to fresh passage (90.9 ± 1.5 % and 84.6 ± 1.7 % for AnV^-/PI^- and YoP^-/PI, respectively).

Our results showed that freezing of stem cells increased the apoptotic and dead cell proportion. It has been reported that hematopoietic and other stem cells, particularly human embryonic stem cells, undergo apoptotic transformation during the cryopreservation process (Sangeetha *et al*., 2010; Stroh *et al*., 2002). MSCs also lose their viability very soon post-thaw, what can most likely be attributed to the rapid development of apoptotic processes (Berz and Colvin, 2012). Our F-T AFSCs showed more than two-fold increase in apoptotic or PI^+ cells compared to F-P3 AFSCs (9.8 ± 1.8 % vs 3.7 ± 1.2 %; 10.5 ± 4.8 % vs 4.0 ± 1.8 % for AnV^-/PI^- and YoP^-/PI^-, respectively). Pal *et al.* (2008) investigated the post-thaw behavior of MSCs and reported a rapid decrease in viability from > 80 % at 2 hours to < 40 % at 8 hours, while the cells were maintained at 4 °C.

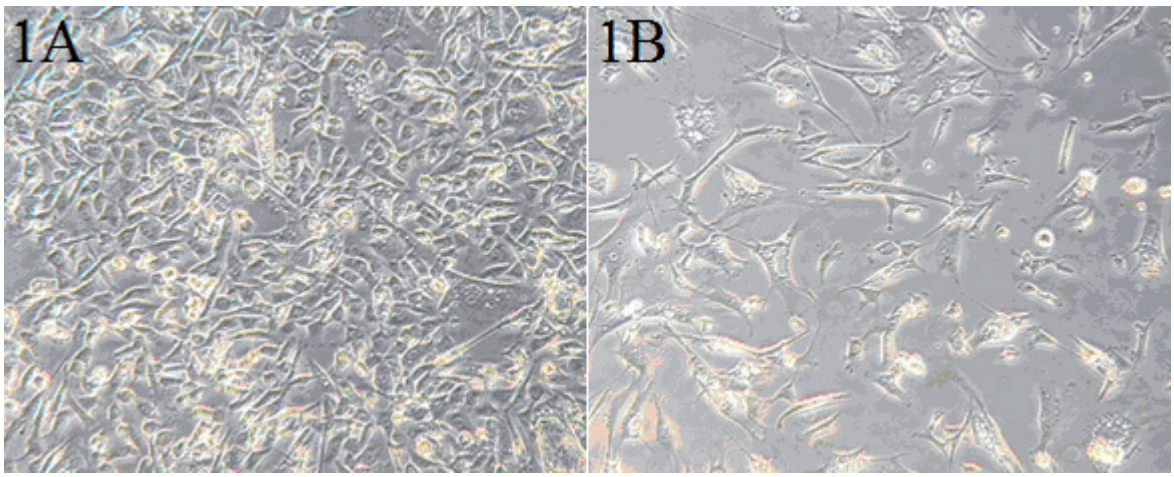

Fig. 1: Phase contrast images of the post-thaw cultured rabbit AFSCs (Zeiss Primo Vert Inverted Microscope). 1A – endothelial-like shape (10x magnification) 1B – mesenchymal-like shape (20x magnification).

Table 1: Detection of Annexin-V, Yo-Pro-1, and propidium iodide positive^{+}/negative^{-} cells expressed in %

Amniotic fluid stem cells	AnV^{-}/PI^{-}	AnV^{+}/PI^{-}	AnV^{-}/PI^{+}	AnV^{+}/PI^{+}	YoP^{-}/PI^{-}	YoP^{+}/PI^{-}	YoP^{-}/PI^{+}	YoP^{+}/PI^{+}
Fresh	92.5 ± 2.2^{a}	0.6 ± 0.3	4.1 ± 1.7^{a}	3.7 ± 1.2^{a}	89.45 ± 1.3^{a}	2.6 ± 0.9	4.6 ± 1.7	4.0 ± 1.8^{a}
Thawed	77.5 ± 2.2^{b}	2.9 ± 0.9	8.5 ± 0.9^{b}	9.8 ± 1.8^{b}	75.2 ± 0.7^{b}	3.8 ± 1.2	6.2 ± 0.8	10.5 ± 4.8^{b}
Thawed 72 h	90.9 ± 1.5^{a}	1.2 ± 1.4	4.9 ± 1.9^{ab}	4.0 ± 1.9^{a}	84.6 ± 1.7^{a}	3.6 ± 0.3	3.9 ± 1.0	5.9 ± 2.4^{ab}

Results are expressed as means ± SEM; a vs b within the same column were statistically significant at $P < 0.05$.

The decrease in viability may be associated also with DMSO addition. It is a small amphipathic molecule that penetrates via plasma membrane of cells and acts as a strong hydrogen bond disrupter and hence exerts colligative effects (Ruiz-Delgado *et al*., 2009). It affects multiple organ systems with wide spectrum of toxicities. However, a DMSO concentration of 10 % in stem cell concentrate is still considered to be standard in most centers around the world. Interestingly, the maximal possible cryopreservation time span is still unknown (Berz and Colvin, 2012).

CONCLUSION

Although the freezing of rabbit AFSCs negatively affects the viability and lead to increase in apoptotic processes, our AFSC were able of post-thaw proliferation. Moreover, after additional 72 hours of culture the proportion of dead and apoptotic cells was reduced. The AFSCs morphology did not change post-thaw and following culture. These preliminary results could potentially serve for cryopreservation of these cells with subsequent use for gene bank purposes as genetic resources or for tissue engineering.

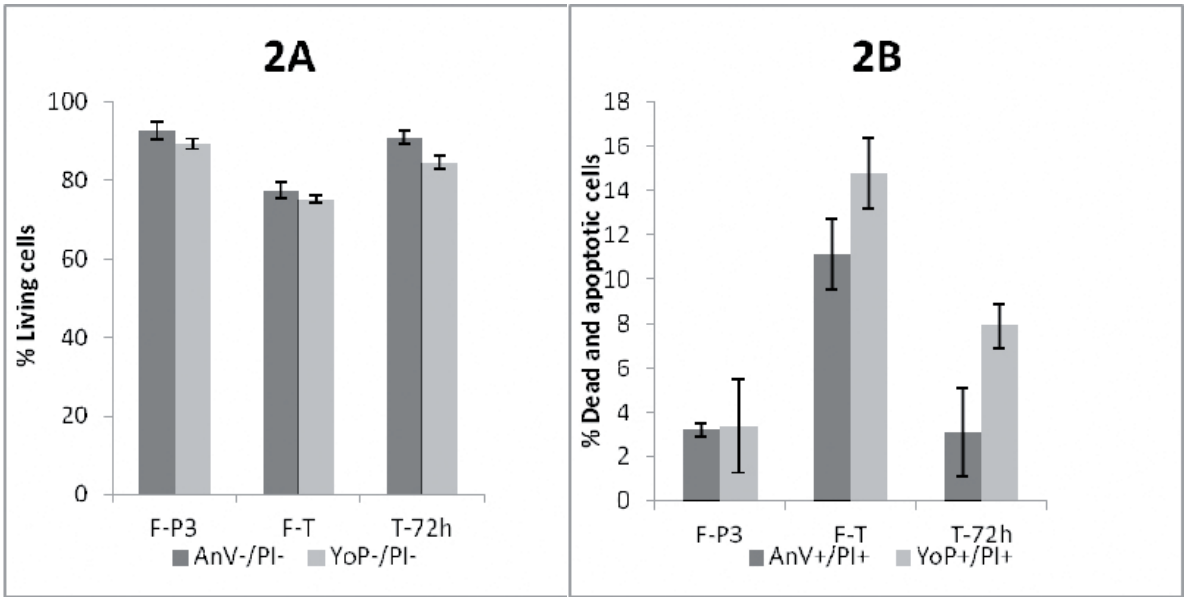

Fig. 2: Proportion of apoptotic, dead and viable AFSCs - P3 fresh (F-P3); frozen-thawed (F-T); thawed-cultured for 72 hours (T-72 h). 2A – proportion of living AnV^{-}/PI^{-} and YoP^{-}/PI^{-} AFSCs. 2B – Necrotic and apoptotic AnV^{+}/PI^{+} and YoP^{+}/PI^{+} AFSCs.

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COMPARATIVE STUDY BETWEEN ISA BROWN AND FULANI ECOTYPE CHICKENS SUPPLEMENTED WITH HUMIC ACID

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ABSTRACT

A 32 week study was carried out to determine the influence of humic acid supplementation on the growth, production and egg qualities of ISA Brown and Fulani ecotype chickens. One hundred and eighty day old chicks of 90 each of ISA Brown and Fulani ecotype were randomly distributed in a 2x3 factorial arrangement of these breeds (ISA Brown and Fulani ecotype chickens) and three treatment groups 1) control with no humic acid supplementation, 2) supplementation with 1 ml.L^{-1} humic acid and 3) 2 ml.L^{-1} humic acid in drinking water. The six groups were replicated thrice with 10 chicks in each replicate. Humic acid supplementation ($p < 0.05$) increased body weight, improved feed conversion ratio, reduced mortality, increased hen-day production and egg weight in both ISA Brown and Fulani ecotype chickens (FEC). Feed intake ($p < 0.05$) increased in ISA Brown chickens with humic acid supplementation, while it decreased in Fulani ecotype chickens at eight weeks of age. At 20 weeks of age, both breeds consumed ($p < 0.05$) less feed with humic acid supplementation. pH of the different segments of gastrointestinal tract was ($p < 0.05$) lowered by humic acid supplementation. Shell thickness was significantly ($p < 0.05$) increased at 2 ml.L^{-1} humic acid supplementation level in ISA Brown and FEC with ISA Brown groups having ($p < 0.05$) thicker shells when compared with FEC. High density lipoprotein ($p < 0.05$) increased across the treatment groups in ISA Brown, while these values ($p < 0.05$) decreased in ISA Brown groups supplemented with humic acid. Low density lipoprotein ($p < 0.05$) increased with humic acid supplementation in ISA Brown and FEC. The results of the present study indicated that the use of humic acid at 1 ml.L^{-1} improved the body weight gain, overall feed intake and hen-day production in ISA Brown and Fulani ecotype chickens. However, better nutrient utilization was observed in Fulani ecotype chickens when compared with ISA Brown chickens.

Key words: chicks; growth; laying hens; lipid profile; egg qualities; humic acid

INTRODUCTION

The poultry industry has experienced tremendous growth; this growth has been with exotic chickens. The indigenous chicken genetic resources in Nigeria have been seriously endangered owing to genetic erosion through the rapid replacement by exotic breeds. The local chicken constitutes about 80 percent of the 120 million poultry birds found in Nigeria (FMA&RD, 2006). These chickens are also known for their adaptation superiority in terms of their resistance to endemic diseases and other harsh environmental conditions (Horst, 1989). The Nigerian indigenous chickens are thought to be suitable for the development of layer strains for the tropical environment since they posses some inherent advantages which include good fertility and hatchability, flavour, colour and texture of meat and egg that is preferred by local consumers, high degree of adaptability to prevailing conditions, high genetic variance in their performance, hardiness, disease tolerance, ease of rearing and ability to breed naturally (Adebambo *et al*., 2009).

Fulani ecotype chicken (FEC) is native to the Fulani tribe in the middle belt and northern parts of Nigeria.

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They are known to be superior in live weight than any other chicken ecotype within Nigeria (Olawunmi *et al*., 2008). The indigenous chickens provide immense benefits for keepers but their productivity is significantly hindered by genetic and management problems. The Nigerian Fulani Ecotype (FEC) chicken has been reported to have great potential for genetic improvement in growth and reproductive performance (Fayeye *et al.*, 2005). The need to improve on the productivity of FEC chickens could be achieved through the use of growth promoters. Improving productivity of this chicken ecotype through growth promoter will improve the economy of the local poultry farmers.

There has been increased interest in alternative natural growth promoters due to microbial resistance and residual effect of antibiotics, one of which is organic acid (Kopecky *et al*., 2012; Sheikh *et al*., 2011).

Organic acids are non-ionised, weak acid which can penetrate the bacterial cell wall and disrupt the normal physiology of some types of bacteria (Dhawale, 2005). Humic acid is one of the main components of humic substances which include humus, humic acid, fulvic acid, ulmic acid and trace minerals and it is the most well known of the group (Yildiz *et al*., 2006). The mode of their action is related to the reduction of pH in the upper intestinal tract, interfering with the growth of undesirable bacteria and modifying the intestinal flora (Kirchgessner and Roth, 1982). Organic acid has been reported to have the beneficial effects of improving feed conversion ratio, growth performance, enhancing mineral absorption (Kral *et al*., 2011; Galik and Rolinec, 2011; Petruska *et al*., 2012). Humic substances have many beneficial effects like antibacterial, antiviral and anti-inflammatory effects. They also improve immune system, reduce odour in faeces, cause a reduction in stress and play a role in liver function (Islam *et al.*, 2005). The use of humic substances in animal brings a number of advantages for animal health and productive performance (Eren *et al*., 2000). According to FAO (2000), there is still a considerable and largely unexploited potential for increased production from local birds through improved management. The aim of this study was to determine the effects of humic acid on performance, egg traits and egg lipid profile of ISA Brown and Fulani ecotype chickens.

MATERIAL AND METHODS

Test supplement: Humic acid used is a product of Dynapharmlab Associate SDN. BHD. It contains chelated micronutrients, nitrogen 2.35 %, phosphorus 4.44 %, potassium 1.75 %, magnesium 0.36 %, iron 867 ppm, manganese 223 ppm, copper 144 ppm, zinc 153 ppm, boron 0.011 %, molybdenum 0.002 % and humic acid 0.68 %.

Experimental management and design

A total of 180 one day-old female chicks of 90 ISA Brown and 90 Fulani ecotype chicks were randomly allocated into 3 treatment groups: 1) control with no humic acid supplementation, 2) supplementation with 1 $ml.L^{-1}$ humic acid and 3) 2 $ml.L^{-1}$ humic acid supplementation in drinking water. Each treatment group was replicated thrice with 10 chicks per replicate. The experiment was conducted for 32 weeks period with all the chickens kept under uniform management conditions throughout the experimental period. Diets were formulated for the chick (0-8 weeks), grower (8-20 weeks) and layer phases of the experiment. Nutrient compositions of diets were determined according to the AOAC (1990) as shown in Table 1. Drinking water was replaced every day.

Growth performance: Birds were weighed per replicate at the beginning and on weekly basis. Feed intake was recorded weekly and calculated as g per bird; mortality was recorded as it occurred.

Gastrointestinal tract pH measurement

At the end of 8 weeks, two hens from each replicate were randomly selected and slaughtered by cervical dislocation. The gastrointestinal tract (GIT) was removed; 10 g each of contents from the crop, gizzard, duodenum, jejunum and ileum were collected in sterilized bottles (1:10 dilution) and the value of the pH for the different segments of the GIT was measured immediately using a digital pH meter.

Production performance: Between 28^{th} and 32^{nd} week of the experiment, eggs were collected daily and egg production was calculated on a hen-day basis. Eggs were weighed. Feed intake was recorded weekly and calculated as g per hen per day.

Egg quality measurement

A total of 18 eggs per treatment, 6 eggs per replicate were randomly selected on weekly basis within 24 hours of lay. The eggs were individually weighed on an electronic balance, the length and width was measured using Vernier calliper to determine the egg shape index (ESI). The eggs were broken into a flat surface where yolks were separated from the whites and then weighed. The shells were carefully washed of any adhering albumen, air dried and weighed. The thickness of each shell was determined using a micrometer screw gauge. Yolk and shell percentages were determined in relation to the egg weight. Yolk colour was determined by matching with one of the matching bands of the Roche colour fan of 12 graded colours. Albumen height

Table 1: Composition of experimental basal diet (%)

Ingredient	Starter (0 – 8 weeks)	Grower (9 – 20 weeks)	Layers
Maize	53.00	58.00	47.00
Groundnut cake	12.00	9.00	5.00
Soyabean meal	18.00	8.00	20.00
Fish meal	4.00	0.00	1.00
Wheat offal	6.90	19.00	15.80
Bone meal	3.00	3.00	2.50
Oyster shell	2.00	2.00	8.00
*Premix	0.25	0.25	0.25
Salt	0.30	0.25	0.25
Lysine	0.30	0.25	0.10
Methionine	0.25	0.25	0.10
Total	100.00	100.00	100.00
Crude protein (%)	22.80	16.93	17.06
Metabolizable energy (MJ.kg^{-1})	11.72	11.11	10.93

*Premix for chick per kg of diet: Vitamin A 10,000 iu; vitamin D3 900 iu; copper 0.1 mg; vitamin E 50.0 mg; manganese 8.5 mg; vitamin K 2.0 mg; iron 75.0 mg; vitamin B1 2.0 mg; folic acid 5.0 mg; vitamin C 26.0 mg; pantothenic acid 20.0 mg; vitamin B6 2.0 mg; choline 1200 mg; vitamin B12 0.01 mg; niacin 50 mg; zinc 70 mg; biotin 0.2 mg.

*Premix for grower per kg of diet: Vitamin A 8,000 iu; vitamin D3 1,200 iu; copper 2.0 mg; vitamin E 31.0 mg; manganese 80 mg; vitamin B2 10.0 mg; pantothenic acid 150.0 mg; iodine 1.2 mg; selenium 0.1 mg; cobalt 2 mg.

*Premix for layer per kg of diet: Vitamin A 10,000 iu; vitamin D3 200 iu; vitamin E 100 iu; vitamin K 20 mg; thiamine 15 mg; riboflavin B2 40 mg; pyridoxine B6 15 mg; niacin 150 mg; pantothenic acid 50 mg; folic acid 5 mg; biotin 0.2 mg; choline chloride 12 mg; antioxidant 1.25 g; manganese 0.8 g; zinc 0.5 g; iron 0.2 g; copper 0.5 g; iodine 0.12 g; selenium 2 mg; cobalt 2 mg.

was measured using tripod spherometer and Haugh unit was calculated according to Haugh (1937).

Yolk lipid profile: At the end of the 32nd week of the experiment, 3 eggs from each replicate were randomly chosen to determine yolk lipid profile. The eggs were hard-cooked, allowed to cool, after which the weight of the boiled eggs were noted. The yolks were carefully separated, weighed and crumbled. 1 g sample of each yolk was homogenized with 15 ml of chloroform-methanol 2:1 (v/v), thoroughly mixed and filtered. Egg homogenate filtrates were designated egg yolk samples. Total cholesterol, HDL cholesterol, total triglycerides concentrations of egg yolk were determined using RANDOX® cholesterol assay kit.

Statistical analysis

All data were subjected to analysis of variance using the General Linear Model Procedure SAS software (SAS, 2002). Treatment means were separated using the Duncan multiple range test at $p < 0.05$.

RESULTS

The effects of humic acid supplementation on the growth performance of ISA Brown and Fulani ecotype chickens (FEC) are shown in Table 2. Compared with the control group, the supplementation of humic acid resulted in significant ($p < 0.05$) increase in body weight of ISA Brown and FEC at 8 and 20 weeks of age. Irrespective of the level of humic acid supplementation, ISA Brown chicks had similar body weight gain while, the body weight gain of FEC was significantly ($p < 0.05$) higher at 2 ml.L^{-1} supplementation level.

Feed intake ($p < 0.05$) increased with humic acid supplementation in ISA Brown chicks, while it decreased in FEC at 8 weeks. However at 20 weeks of age, feed intake ($p < 0.05$) decreased in both ISA Brown and FEC with humic acid supplementation. Fulani ecotype chickens consumed ($p < 0.05$) less feed with or without humic acid supplementation when compared with the ISA Brown groups at 8 and 20 weeks.

Feed conversion ratio (FCR) was similar in all ISA Brown groups and FEC control group while the ratio decreased as the level of humic acid supplementation increased in FEC at 8 weeks. The highest FCR at 20 weeks was obtained from ISA Brown pullets without humic acid supplementation while FEC supplemented with 2 ml.L^{-1} humic acid had the least value. Similar FCR values were recorded from the other treatment groups. Significant ($p < 0.05$) percentage mortality was recorded from ISA Brown and FEC without supplementation

Table 2: Growth performance of ISA Brown and Fulani ecotype chickens supplemented with humic acid

	ISA Brown			Fulani ecotype				p-value		
	Humic acid supplementation (ml.L^{-1})									
Parameter	0	1	2	0	1	2	SEM	HA	B	HAxB
Initial weight (g/bird)	33.17	33.19	33.16	33.19	33.19	33.16	0.01	0.1470	0.4103	0.4410
Final weight at 8 wks (g/bird)	562.34^{cd}	575.76^{ab}	578.26^{ab}	553.86^{d}	569.61^{bc}	584.697^{a}	2.76	0.0001	0.4026	0.0005
Weight gain at 8 weeks (g/bird)	529.18^{cd}	542.57^{ab}	545.10^{ab}	520.67^{d}	536.42^{bc}	551.53^{a}	2.76	0.0001	0.4011	0.0005
Feed intake at 8 wks (g/bird)	1729.26^{b}	1761.52^{a}	1764.53^{a}	1685.04^{c}	1648.00^{d}	1629.80^{d}	13.01	0.7723	0.0001	0.4011
Feed conversion ratio	3.27^{a}	3.25^{a}	3.24^{a}	3.24^{a}	3.07^{b}	2.96^{c}	0.03	0.0067	0.0003	<.0001
Mortality at 8 wks (%)	6.67^{a}	0.00^{b}	0.00^{b}	3.33^{ab}	0.00^{b}	0.00^{b}	0.90	0.0252	0.4752	0.1357
Body weight at 20 wks (g/bird)	1383^{c}	1500^{a}	1500^{a}	1410^{bc}	1463^{ab}	1490^{a}	13.73	0.0018	0.7344	0.0164
Weight gain at 20 wks (g/bird)	820.99^{b}	924.24^{a}	921.74^{a}	856.14^{ab}	893.73^{ab}	905.30^{a}	12.15	0.0118	0.8429	0.0589
Feed intake 8-20 wks (g/bird)	6399.41^{a}	6270.48^{b}	6251.14^{b}	5869.96^{c}	5603.00^{d}	5558.20^{d}	81.16	<.0001	<.0001	<.0001
Feed conversion ratio	7.82^{a}	6.79^{b}	6.78^{b}	6.86^{b}	6.27^{bc}	6.16^{c}	0.15	0.0005	0.0004	0.0006

^{a,b,c} Means on the same row with different superscript are different ($p < 0.05$)
SEM = Standard error of mean
HA = Humic acid, B = Breed

alone; there was no mortality among ISA Brown and FEC supplemented with humic acid.

The pH of the gastrointestinal tract (GIT) of ISA Brown and FEC supplemented with humic acid as indicated in Table 3 showed that the GIT was significantly ($p < 0.05$) affected by humic acid supplementation and breed. The pH of the crop, gizzard, duodenum, jejunum and ileum of ISA Brown and FEC ($p < 0.05$) reduced with humic acid supplementation. The ($p < 0.05$) highest pH values in each of the GIT segment were recorded in ISA Brown chicks in the control group while, FEC supplemented with 2 ml.L^{-1} had the ($p < 0.05$) least pH values in these GIT segments.

Age at first lay, hen-day production, feed intake and egg weight were significantly ($p < 0.05$) improved with humic acid supplementation in ISA Brown and FEC as revealed in Table 4. Highest ($p < 0.05$) hen-day production was obtained with FEC supplemented with humic acid while, the least was recorded from ISA Brown chickens without supplementation. Similar hen-day production percentages were obtained from the other treatment groups. Yolk percentage was significantly ($p < 0.05$) higher and similar in all FEC groups and ISA Brown not supplemented with humic acid. This was ($p < 0.05$) lower in ISA Brown chickens supplemented with humic acid.

Shell thickness was significantly ($p < 0.05$) increased at 2 ml.L^{-1} humic acid supplementation level in ISA Brown and FEC although, shell thickness from ISA Brown chicken groups was ($p < 0.05$) higher than

Table 3: Gastrointestinal pH of ISA Brown and Fulani ecotype chickens supplemented with humic acid

	ISA Brown			Fulani ecotype				p-value		
	Humic acid supplementation (ml.L^{-1})									
Parameter	0	1	2	0	1	2	SEM	Humic	Breed	HAxB
Crop	5.17^{a}	5.01^{b}	4.87^{c}	4.41^{d}	4.23^{e}	4.15^{e}	0.10	<.0001	<.0001	<.0001
Gizzard	4.87^{a}	4.75^{b}	4.68^{c}	4.16^{d}	3.66^{e}	3.38^{f}	0.14	0.0352	<.0001	<.0001
Duodenum	5.71^{a}	5.58^{b}	5.47^{c}	5.45^{cd}	5.42^{d}	5.42^{d}	0.03	0.0020	<.0001	<.0001
Jejunum	5.84^{a}	5.72^{b}	5.63^{c}	5.59^{c}	5.46^{d}	5.41^{d}	0.04	<.0001	<.0001	<.0001
Ileum	6.55^{a}	6.32^{b}	6.22^{b}	6.03^{c}	5.92^{cd}	5.83^{d}	0.06	0.0001	<.0001	<.0001

^{a,b,c} Means on the same row with different superscript are different ($p < 0.05$)
SEM = Standard error of mean
HA = Humic acid, B = Breed

Table 4: Production performance and egg qualities of ISA Brown and Fulani ecotype chickens supplemented with humic acid

	ISA Brown			Fulani ecotype				p-value		
	Humic acid supplementation (ml.L^{-1})									
Parameter	0	1	2	0	1	2	SEM	Humic	Breed	HAxB
Age at 1st egg (days)	153^{a}	146^{b}	145^{b}	142^{c}	139^{d}	139^{d}	1.17	0.0003	<.0001	<.0001
Hen-day production (%)	87.33^{c}	92.67^{b}	92.00^{b}	92.67^{b}	95.33^{a}	95.33^{a}	0.68	<.0001	<.0001	<.0001
Feed intake (g/bird/day)	113.00^{a}	110.00^{b}	109.00^{b}	91.67^{c}	90.00^{cd}	89.67^{d}	2.48	0.0007	<.0001	<.0001
Egg weight (g/egg)	56.37^{c}	59.34^{b}	64.75^{a}	40.52^{f}	43.56^{e}	46.17^{d}	2.16	<.0001	<.0001	<.0001
Yolk (%)	29.06^{a}	27.49^{b}	27.02^{b}	28.90^{a}	29.19^{a}	29.59^{a}	0.25	0.2832	0.0025	0.0002
Shell (%)	9.49^{b}	9.41^{b}	9.30^{b}	9.86^{a}	9.57^{ab}	9.44^{b}	0.06	0.0443	0.0244	0.0570
Shell thickness (mm)	0.33^{bc}	0.34^{b}	0.35^{a}	0.32^{d}	0.32^{d}	0.33^{c}	0.03	0.0021	<.0001	0.0002
Egg shape index	0.67^{b}	0.67^{b}	0.73^{a}	0.67^{b}	0.65^{c}	0.74^{a}	0.01	<.0001	0.3262	<.0001
Haugh Unit (%)	83.06^{b}	84.12^{a}	84.30^{a}	78.75^{c}	79.26^{c}	78.92^{c}	0.61	0.0720	<.0001	<.0001
Yolk colour	8.00^{c}	9.00^{b}	10.00^{a}	6.00^{e}	7.00^{d}	7.00^{d}	0.33	<.0001	<.0001	<.0001

^{a,b,c} Means on the same row with different superscript are different ($p < 0.05$)
SEM = Standard error of mean
HA = Humic acid, B = Breed

those from the FEC groups. Haugh unit ($p < 0.05$) improved with humic acid supplementation in ISA Brown chickens while, there was no significant ($p > 0.05$) effect of humic acid on all FEC groups. Haugh unit was significantly ($p < 0.05$) higher in ISA Brown chickens when compared with FEC. Yolk colour was significantly ($p < 0.05$) higher in the groups supplemented with humic acid when compared to those in the control groups. ISA Brown chickens supplemented with 2 ml.L^{-1} produced eggs with deeper yolk colour, which was closely followed by the ISA Brown group supplemented with 1 ml.L^{-1} humic acid. The ($p < 0.05$) lowest yolk colour was obtained from FEC in the control group.

Table 5 showed the egg lipid profile of ISA Brown and FEC supplemented with humic acid. Triglycerides ($p < 0.05$) decreased in the eggs of ISA Brown and FEC supplemented with humic acid. However, egg cholesterol ($p < 0.05$) increased with humic acid supplementation. The highest cholesterol value was obtained from ISA Brown supplemented with 2 ml.L^{-1} humic acid and the least from FEC supplemented with 2 ml.L^{-1} humic acid. High density lipoprotein ($p < 0.05$) increased and decreased across the treatment groups in ISA Brown and FEC respectively. Low density lipoprotein ($p < 0.05$) increased with humic acid supplementation in ISA Brown and FEC. The highest low density lipoprotein value was recorded from ISA Brown chickens supplemented with 2 ml.L^{-1} humic acid while, the least value was obtained from FEC without humic acid supplementation.

Table 5: Lipid profile of eggs from ISA Brown and Fulani ecotype chickens supplemented with humic acid

	ISA Brown			Fulani ecotype				p-value		
	Humic acid supplementation (ml.L^{-1})									
Parameter	0	1	2	0	1	2	SEM	Humic	Breed	HAxB
Triglycerides (mg.g^{-1})	151.74^{a}	117.99^{b}	112.39^{bc}	94.46^{bc}	87.70^{c}	87.90^{c}	6.11	0.0402	0.0001	0.0008
Cholesterol (mg.g^{-1})	75.36^{d}	77.50^{c}	79.28^{a}	74.64^{d}	78.32^{b}	70.98^{e}	0.68	0.0777	0.0240	<.0001
High density lipoprotein (mg.g^{-1})	44.13^{c}	45.59^{b}	47.67^{a}	39.66^{d}	30.57^{e}	32.52^{f}	1.57	0.1018	<.0001	<.0001
Low density lipoprotein (mg.g^{-1})	24.65^{c}	25.64^{b}	26.65^{a}	21.47^{e}	23.61^{d}	24.44^{c}	0.40	<.0001	<.0001	<.0001

^{a,b,c} Means on the same row with different superscript are different ($p < 0.05$)
SEM = Standard error of mean
HA = Humic acid, B = Breed

DISCUSSION

The improved body weight gain of birds supplemented with humic acid when compared with the un-supplemented group could be due to the beneficial effect of humic acid on the gut flora. The beneficial microbiological and pH-decreasing abilities of humic acid especially at the upper part of the gastrointestinal tract might have had resulted in the inhibition of intestinal bacteria leading to the reduced metabolic needs, thereby increasing the availability of nutrients to the host. Humic acid stimulates the immune system receptors in the gut lining to protect against pathogens to promote growth (Kocabagli *et al.*, 2002; Karaoglu *et al.*, 2004). By modifying the intestinal pH, Owing *et al*. (1990) reported that organic acid improves the solubility of the feed ingredients, digestion and absorption of nutrients. Sheikh *et al.* (2010) revealed that organic acid supplementation facilitated nutrient absorption to a greater extent and thus boosted growth.

The acid anion of humic acid has been shown to complex with calcium, phosphorus, magnesium and zinc which results in an improved digestibility of these minerals and serves as substrate in the intermediary metabolism (Kishi *et al.*, 1999).

The increase in feed intake of ISA Brown chicks and decrease in FEC with humic acid supplementation may be as a result of the differences in breed. Kucukersan *et al.* (2005) showed that the average daily feed consumption of hen fed diets with humic acid was significantly decreased compared with the control group. While some researchers reported that dietary humate supplementation increased feed intake (Hayirli *et al*., 2005). The improvement in the FCR among FEC supplemented with humic acid could be possibly due to better utilization of nutrients resulting in increased body weight. Supplementation of humic acid at levels of 1 and 2 $g.kg^{-1}$ were reported to improve feed efficiency when compared with hens fed control diet (Yoruk *et al*., 2004). Organic acids improve the absorption and conversion of nutrients in the body, and improve overall gastric function (Park *et al*., 2009).

In this study, humic acid was able to improve the survivability of the birds; this could be attributed to the reduction in the intestinal pH and the ability to inhibit the growth of microbial pathogens. Humic acid was observed to show antibacterial, antiviral, antithyroidal and anti-inflammatory effects in animals, thus improves the immune system (Islam *et al*. 2005).

Humic acid has the ability to influence in particular the metabolism of protein carbohydrates of microbes by catalytic means. This leads to a direct devastation of bacterial cells or virus particles (Huck *et al*., 1991; Ricke, 2003).

The use of humic acids in animal feeds improved animal health forming a protective film on the mucous epithelium of the membrane and tract against infection and toxin (Kucukersan *et al*., 2005). The lower pH values in GIT of birds supplemented with humic acid is a confirmation of the pH decreasing ability of humic acid. The addition of acidifiers to the diet for broilers lowered the pH of the crop and gizzard content (Andrys *et al*., 2003).

Egg production and egg weight among the groups supplemented with humic acid is indicative of better utilization of nutrients. Egg production values of laying hens fed 1 and 2 $g.kg^{-1}$ humic acid were higher than those of the control group (Tancho, 1999; Yoruk *et al*., 2004). However, some studies found that humic acid had no effect on egg production of laying hens and laying quails (Kucukersan *et al.*, 2005; Yalcın *et al*., 2005). Soltan (2008) found that organic acid supplementation increased egg production by about 5.77 % compared to untreated group. Park *et al.* (2002) reported a positive effect of organic acid on laying performance. Dietary humic substances improved egg weight as reported by Wang *et al.* (2007).

The increased egg shell quality in eggs produced by birds supplemented with humic acid might be as a result of the presence of calcium in humic acid and increase in calcium absorption. Increased permeability allowed easier transfer of minerals from the blood o the bone and cells (Enviromate, 2002). Organic acid is reported to improve utilization of mineral feed additives, increasing the availability of calcium and other minerals (Omogbenigun *et al*., 2003). The pH reducing and antimicrobial effects of the organic acid may assist in gut acidifiers and appear to be a good solution for poor egg shell quality (Dibner and Butin, 2002; Dhawale, 2005). Thick egg shell is essential for protection against the penetration of pathogenic bacteria (Swiatkiewicz *et al*., 2010).

Egg lipid profile showed certain interaction between breeds and their capability to take up dietary nutrients and transfer them to their products. Sarica *et al.* (2009) reported higher cholesterol in the eggs of commercial layers and lower in local pure breeds. However, Rizzi and Chiericato, (2010) revealed that yolk cholesterol was found at higher concentration in local Mediterranean laying hen breeds compared with commercial laying hybrids.

CONCLUSION

Supplementing ISA Brown and Fulani ecotype chickens with humic acid increased body weight, egg production, egg weight, shell thickness and improved feed conversion. Humic acid at the rate 1 $ml.L^{-1}$ can be used for production efficiency in ISA Brown and Fulani ecotype chickens.

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EFFECTS OF SEASONAL CHANGES ON THE NUTRITIVE QUALITY OF *MORINGA OLEIFERA* AND *PANICUM MAXIMUM* SILAGE

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ABSTRACT

The experiment was carried out to evaluate the effects of season and plant types on the chemical composition and *in vitro* fermentation of *Moringa oleifera* and *Panicum maximum* (local) silage. Leaf samples of *M. oleifera* plants and *P. maximum* were randomly collected during the rainy and dry seasons from Federal University of Agriculture, Abeokuta, Nigeria. The design of this study was 2 × 5 factorial arrangements with 3 replicates (2 seasons and 5 mixture ratios). The forage samples were both chopped into 2-3 cm lengths and divided into different proportions of 100 % : 0 %, 0 % : 100 %, 30 % : 70 %, 50 % : 50 % and 70 % : 30 % and were carefully packed into 30 laboratory bottles (960 ml) silos after mixing thoroughly. Season x plant species interactions were observed for some chemical and *in vitro* fermentation parameters. The CP content of the samples was significantly higher ($p < 0.05$) and ranged from 69.9 to 165.5 g.kg DM^{-1} and fibre concentrations ranged from 246.3 to 710.5 g.kg DM^{-1}, 193.2 to 578.0 g.kg DM^{-1} and 63.5 to 133.3 g.kg DM^{-1} for NDF, ADF and ADL respectively. 100 % Moringa + 0 % Panicum silage recorded the highest ($p < 0.05$; 165.5 g.kg DM^{-1}) CP content in all the silages evaluated. All the silages had high calcium contents (7.0-11.7 g.kg DM^{-1}). Values obtained for the *in vitro* fermentation characteristics of the forage species indicated the presence of potentially degradable nutrients. 100 % Moringa + 0 % Panicum and all the forage mixtures silages have potentials that could be harnessed as feed supplements for ruminants both in the rainy and dry seasons.

Key words: Moringa; nutritive value; Panicum; plant types; season; silage

INTRODUCTION

Forages provide adequate amount of nutritious feed for ruminants in the form of grasses which is preferred by these types of animals. However, in the dry season, there is feed constraint due to seasonal shortages in the quantity and quality of forage from natural pastures that provide most of the feed for animals which result into problems such as sickness and weight loss due to poor dietary profile for the animals and reduction in yield and quality of forages arises. Under this condition, browse plant is of great importance in balancing protein deficiency during the long dry season. They are effective insurance against seasonal feed shortages. The main feature of browse plants is their high crude protein (CP) and mineral contents (Awad and Elhadi, 2010).

Ruminants relish guinea grass (Babayemi and Bamikole, 2006). But such forage becomes very scarce in the dry season. It is commonly used for silage but herbage has been reported to have low content of dry matter and low concentrations of water soluble carbohydrates and crude protein (Zanine *et al*., 2006). The levels of protein content and fermentable carbohydrates can be improved through various treatments including mixing legumes with cereal crops (Phiri *et al.*, 2007).

Browse plants have special characteristics of high nitrogen value, better metabolizable energy

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and organic matter digestibility when fed with grass supplement (Ajayi and Babayemi, 2008). Forage tree leaves generally have high phosphorus concentrations (McMeniman and Little, 1974).

In dealing with lack of feed during the dry season forage conservation especially as silage is considered to be the preservation technique with the greatest potential for protein rich foliage (Man and Wiktorsson, 2002). Ensiling cereal and legumes together have been reported to be responsible in providing sufficient fermentable carbohydrates for lactic acid bacteria (Koljajic *et al*., 1998) and simultaneously the protein content of silage will be increased (Anele *et al*., 2011). In addition, mixing legumes and grasses increases biomass yield, crude protein (CP) content, nutritive value of resultant silage and soil fertility (Assefa and Ledin, 2001; Nayigihugu *et al*., 2002).

This study will therefore evaluate the chemical composition and *in vitro* fermentation of *Moringa oleifera* and *Panicum maximum* silage at different proportions and seasons.

MATERIAL AND METHODS

Experimental site

The experiment was conducted at the Department of Pasture and Range Management, University of Agriculture, Abeokuta, Nigeria. The site lies within the derived savannah zone of South Western Nigeria on latitude 7°58'N, longitude 3°20'E and 75 m above sea level with average annual rainfall of 1037 mm. Abeokuta has a bimodal rainfall pattern that typically peaks in July and September with a break of two to three weeks in August. Temperatures are fairly uniform with daytime values of 28 to 30 °C during the rainy season (April – September) and 30–34 °C during the dry season (October – March) with the lowest night temperature of around 24 °C during the harmattan period between December and February. Relative humidity is high during the rainy season with values between 63 and 96 % as compared to dry season 55–84 %. The temperature of the soil ranges from 24.5 to 31.0 °C (Source: Agromet Dept., FUNAAB).

Sample collection

P. maximum samples were harvested at 15 cm above ground level at 6 weeks after cut back from the Fadama (wetland areas) located within the University and *M. oleifera* was also harvested by hand cutting the branches followed by stripping off all leaves in July, 2011 for rainy season and December, 2011 for dry season from the Teaching and Research Farm of the University. The grass samples were chopped into pieces of 2-3 cm in length, wilted for 4 hours to reduce their moisture contents at a temperature of 23.4 °C and relative humidity of 71.2 % before ensiling and divided into different proportions of 100 % : 0 %, 0 % : 100 %, 30 % : 70 %, 50 % : 50 % and 70 % : 30 %.

Experimental design

The experiment was laid out in a 2 x 5 factorial design arrangement with 3 replicates consisting of two types of season i.e. rainy and dry, and five forage proportions i.e. 100 % Moringa + 0 % Panicum, 0 % Moringa + 100 % Panicum, 50 % Moringa: 50 % Panicum, 70 % Moringa: 30 % Panicum, and 30 % Moringa:70 % Panicum.

Data collection

The chopped and wilted plant types at different forage proportions and their sole were carefully packed into laboratory (960 ml) bottle silos after mixing thoroughly following the method described by Yokota *et al.* (1995). A total of thirty (30) bottle silos were used in the ensiling process. The forage samples were ensiled for a period of 6 weeks at an ambient temperature of 26 °C. At the expiration of the ensiling, the bottle silos were opened, the pH of the silages was determined and chemical and *in vitro* analyses of the samples were thereafter carried out.

Chemical analyses

Samples of 300 g were taken from each of the bottle silos and oven-dried to a constant weight at 65 °C. The dried foliage samples were milled through a 1mm sieve and crude protein (CP), ether extract (EE), ash and the minerals such as Phosphorus (P), Potassium (K), Calcium (Ca) and Sodium (Na) were analyzed according to the standard methods of AOAC (2000). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin were determined according to Van Soest *et al*. (1991). Hemicellulose was calculated as NDF – ADF while Cellulose was calculated as ADF – ADL. Non-fibre carbohydrates (NFC) were calculated as:

NFC = 1000 – CP - ash – EE – NDF, with all variables expressed as g.kg DM^{-1}.

In vitro gas production was determined according to Menke and Steingass (1988). Rumen fluid was collected from three bulls before morning feeding according to the method described by Babayemi and Bamikole (2006). The animals have been previously fed a mixed diet of *P. maximum* grass and concentrates to fulfil maintenance requirements. The liquor was collected into a pre warmed thermos flasks and was later filtered through three layers of cheesecloth. Two hundred milligrams of ground feed samples were weighed into 100 ml calibrated syringes with pistons lubricated with grease. A buffered mineral solution

was prepared. 30 ml of buffered rumen fluid was taken into syringes containing the feeds. The syringes were positioned vertically in a water bath with shaker kept at 39 °C fitted with plungers. Gas production rates were recorded at 3, 6, 9, 12, 24, 36 and 48 hour of incubation and each syringe was gently swirled after reading. Three blank syringes containing only 30 ml of buffered inoculum were also concurrently incubated.

The data obtained were fitted to the non-linear regression equation:

$V (ml.200\ mg.DM^{-1}) = b (1- e^{-ct})$

where V = potential gas production at time *t*, *b* = volume of gas that will evolve with time and *c* = fractional rate of gas production. Initial gas production rate (Absg) was calculated as the product of *b* and *c* (Larbi *et al*., 1996).

Organic matter digestibility (OMD) were estimated as:

OMD= 14.88 + 0.889GV+ 0.45 CP + 0.651 ash (Menke and Steingass, 1988).

Short-chain fatty acids (SCFA) were estimated as:

SCFA = 0.0239GV−0.0601 (Getachew *et al.*, 2000).

Metabolizable energy (ME) were calculated as:

$ME= 2.20 + 0.1357GV+ 0.0057\ CP + 0.0002859\ EE^2$ (Menke and Steingass, 1988).

Total gas volume (GV) were expressed as ml/200 mg DM, CP and ash as g.kg DM^{-1}, ME as MJ.kg DM^{-1} and SCFA as µmol.g DM^{-1}.

***In vitro* dry matter digestibility (IVDMD)**

After 48 hours digestion, the bottle contents were centrifuged at 15,000 rpm for 10 minutes and residues were decanted into pre weighed crucibles were oven-dried at 105 °C for 24 hours. The dry residues were weighed and digestibility calculated using the equation as follows:

$$IVDMD\ (\%) = \frac{\text{Initial DM input} - \text{DM residue-Blank x 100}}{\text{Initial DM input}}$$

Data were subjected to two-way analysis of variance using the SAS (2002) package while significant means were separated using Duncan's multiple range test ($p < 0.05$).

RESULTS

Chemical composition

The proximate composition and pH of the silages are shown in Table 1. There were significant differences ($p < 0.05$) in the DM, CP, EE, ash and NFC composition as a result of interactions between plant types and season. 100 % Moringa + 0 % Panicum silage had the lowest DM contents for both the rainy and dry seasons compared with others. Meanwhile, 100 % Moringa + 0 % Panicum silage during rainy season recorded the highest CP value (165.5 g.kg DM^{-1}) which was closely trailed behind by mixtures of 30 % Moringa : 70 % Panicum silage at rainy (129.4 g.kg DM^{-1}). 0 % Moringa + 100 % Panicum silage at dry season recorded the least value of EE (10.1 g.kg DM^{-1}). 100 % Moringa + 0 % Panicum and

Table 1: Effect of season and plant type on the composition and pH of the silage (g.kg DM^{-1})

Season	Forage mixture	DM	CP	EE	ASH	NFC	pH
				(g.kg DM^{-1})			
Rainy	100 % Moringa + 0 % Panicum	910.00^{b}	165.50^{a}	32.00^{abc}	80.4^{c}	327.20^{b}	4.48
	0 % Moringa + 100 % Panicum	945.00^{a}	74.10^{c}	25.90^{bcd}	88.9^{abc}	100.60^{e}	4.64
	Moringa 50 %: Panicum 50 %	935.00^{a}	127.90^{b}	47.10^{a}	86.6^{abc}	172.30^{cd}	4.38
	Moringa 30 %: Panicum70 %	945.00^{a}	129.40^{b}	31.20^{abc}	103.4^{ab}	144.70^{d}	4.49
	Moringa70 % : Panicum 30 %	960.00^{a}	83.90^{c}	31.00^{abc}	109.2^{a}	197.20^{cd}	4.60
Dry	100 % Moringa + 0 % Panicum	910.00^{b}	127.00^{b}	37.60^{ab}	90.1^{abc}	499.30^{a}	4.20
	0 % Moringa + 100 % Panicum	950.00^{a}	69.90^{c}	10.10^{d}	108.10^{ab}	60.90^{f}	4.50
	Moringa50 % : Panicum 50 %	940.00^{a}	82.00^{c}	20.90^{bcd}	93.10^{abc}	298.70^{c}	4.70
	Moringa 30 % : Panicum 70 %	935.00^{a}	89.90^{c}	15.40^{cd}	107.60^{ab}	241.60^{c}	4.63
	Moringa 70 % : Panicum 30 %	945.00^{a}	77.8^{c}	36.60^{ab}	84.10^{c}	410.00^{a}	4.80
	SEM	3.50	6.40	2.50	2.70	2.60	0.06

^{abcd}Means along the same column with different subscripts are significantly different ($p < 0.05$)
DM: Dry matter, CP: Crude protein, EE: Ether extract, NFC: Non Fibre Content
SEM: Standard error of mean

mixtures of 70 % Moringa: 30 % Panicum silages both during dry season recorded the highest ($p < 0.05$) overall NFC.

Table 2 shows the effects of plant types and season on the fibre composition of forages. 0 % Moringa + 100 % Panicum silage at rainy and dry seasons had the highest ($p < 0.05$) NDF while 100 % Moringa + 0 % Panicum silage at dry season had the least ($p < 0.05$) NDF, ADF and celluloses levels, respectively.

Table 3 shows the mineral composition of the silages. There were significant ($p < 0.05$) interactions between plant types, forage proportions and season for the phosphorus, potassium, calcium and sodium contents of the silages. Among the interaction of the mineral contents of the forages, P and Ca contents ranged from 2.2 to 3.6 g.kg DM^{-1} and 7.0 to 11.70 g.kg DM^{-1} respectively. Silages produced from legumes and different plant types had higher amounts of these minerals than those from grasses alone. Potassium contents of the forage mixtures during the rainy season were significantly ($p < 0.05$) higher than in the dry season.

***In vitro* digestibility**

There were significant effects of plant types and season on the volume of gas produced at different times for the silages (Table 4). 100 % Moringa + 0 % Panicum and 70 % Moringa : 30 % Panicum silages both at dry season, recorded the highest ($p < 0.05$) gas production at 3 hr of incubation while 0 % Moringa + 100% Panicum and 70 % Moringa : 30 % Panicum silages during rainy season and 0 % Moringa + 100 % Panicum silage at dry season respectively produced no gas at 3 hr of incubation. However, at 48 hr of incubation, 30% Moringa : 70 % Panicum silage at dry season had the highest gas production with 35 % while 50 % Moringa : 50 % Panicum silage at rainy season produced the least gas of 23 % at 48 hr.

Irrespective of plant types and season of ensiling, there was no significant difference ($p > 0.05$) on insoluble but degradable fraction (b) and fractional rate of gas production (c) of the ensiled forages (Table 5). However, 70 % Moringa : 30 % Panicum silage at dry season had the highest SCFA and ME values above all other plant types and season. SCFA content of the silages of different plant types was higher during dry season except for 0 % Moringa + 100 % Panicum silage. Similar trend was noticed with ME.

DISCUSSION

Chemical composition

Higher DM contents as obtained in this study have been reported to concentrate the water soluble carbohydrates and improve the effectiveness of lactate-producing bacteria, and thus improve the quality of silage (Anele *et al.*, 2011).

The CP contents of all the silages of plant types were all above the critical CP level of 8 % acceptable for ruminant performance (Norton, 1994) except the 0 % Moringa + 100 % Panicum silage which was similar to 7.8 % reported by Aganga and Tshwenyane, (2004) and lower

Table 2: Effect of season and plant type on the fibre composition of the silage (g.kg DM^{-1})

Season	Forage mixture	NDF	ADF	ADL	HEM	CELL
				(g.kg DM^{-1})		
Rainy	100 % Moringa + 0 % Panicum	394.90^{d}	347.70^{de}	91.70^{b}	47.20^{d}	256.00^{de}
	0 % Moringa + 100 % Panicum	710.50^{a}	420.20^{bcd}	133.30^{a}	290.30^{a}	286.90^{d}
	Moringa 50 %: Panicum 50 %	566.10^{bc}	467.50^{b}	63.50^{cd}	98.70^{cd}	404.00^{ab}
	Moringa 30 %: Panicum70 %	591.30^{b}	463.70^{b}	66.20^{bcd}	127.60^{bc}	397.50^{abc}
	Moringa70 % : Panicum 30 %	578.70^{b}	347.50^{de}	50.00^{d}	231.20^{a}	297.50^{cd}
Dry	100 % Moringa + 0 % Panicum	246.30^{e}	193.20^{f}	80.10^{bc}	53.00^{d}	119.90^{f}
	0 % Moringa + 100 % Panicum	751.40^{a}	578.00^{a}	130.70^{a}	173.50^{b}	447.30^{a}
	Moringa50 % : Panicum 50 %	505.30^{c}	427.30^{bc}	121.30^{a}	78.10^{cd}	332.60^{bc}
	Moringa 30 % : Panicum 70 %	545.50^{bc}	384.10^{cde}	82.00^{bc}	165.10^{b}	302.00^{bc}
	Moringa 70 % : Panicum 30 %	391.50^{d}	317.00^{e}	133.30^{a}	74.50^{cd}	183.70^{ef}
	SEM	27.3	19.2	6.0	17.0	19.6

^{abcdef}Means along the same column with different subscripts are significantly different ($p < 0.05$)
NDF: Neutral detergent fibre, ADF: Acid detergent fibre, ADL: Lignin detergent fibre, HEM: Hemicellulose
CELL: Cellulose
SEM = Standard error of mean

Table 3: Effect of season and plant type on the mineral content of the silage (g.kg DM^{-1})

Season	Forage mixture	Phosphorus (P)	Potassium (K)	Calcium (Ca) (g.kg DM^{-1})	Sodium (Na)
Rainy	100 % Moringa + 0 % Panicum	3.56^{a}	33.26^{c}	11.72^{c}	2.90^{e}
	0 % Moringa + 100 % Panicum	2.44^{g}	30.57^{e}	7.02	2.71^{g}
	Moringa 50 %: Panicum 50 %	2.51^{f}	34.38^{b}	10.94^{d}	2.08^{j}
	Moringa 30 %: Panicum70 %	2.23^{h}	31.89^{a}	8.52^{f}	2.53^{h}
	Moringa70 % : Panicum 30 %	2.82^{c}	37.06^{f}	7.69^{g}	3.28^{c}
Dry	100 % Moringa + 0 % Panicum	3.05^{b}	31.39^{d}	10.48^{a}	3.13^{d}
	0 % Moringa + 100 % Panicum	2.51^{f}	24.26^{h}	7.76^{g}	2.83^{f}
	Moringa50 % : Panicum 50 %	1.90^{i}	24.07^{j}	11.25^{b}	3.35^{b}
	Moringa 30 % : Panicum 70 %	3.05^{b}	29.71^{i}	11.70^{c}	4.52^{a}
	Moringa 70 % : Panicum 30 %	2.69^{e}	26.35^{g}	7.80^{g}	2.21^{i}
	SEM	0.08	1.39	0.71	0.12
Normal range* (%)		0.33-0.47	0.50-3.10	0.45-1.20	0.20-0.30

^{abcdefghij}Means along the same column with different subscripts are significantly different ($p < 0.05$)
SEM= Standard error of mean
* Normal range. Source: Rogers and Murphy (2000)

Table 4: Effect of season and plant type on the *in vitro* gas production of the silage (hr)

Season	Forage mixture	3	6	9	12	18	24	36	48
					(hr)				
Rainy	100 % Moringa	2.00^{c}	3.00^{dc}	8.00^{c}	10.00^{d}	12.00^{d}	17.00^{e}	23.00^{f}	25.00^{f}
	0 % Moringa + 100 % Panicum	0.00^{e}	2.00^{de}	4.00^{de}	7.00^{e}	10.00^{e}	19.00^{d}	28.00^{cd}	30.00^{d}
	Moringa 50 %: Panicum 50 %	1.00^{d}	4.00^{bc}	5.00^{d}	9.00^{d}	11.00^{de}	15.00^{f}	20.00^{g}	23.00^{g}
	Moringa 30 %: Panicum70 %	1.00^{d}	5.00^{b}	10.00^{b}	15.00^{b}	19.00^{b}	24.00^{b}	29.00^{cb}	33.00^{cb}
	Moringa70 % : Panicum 30 %	0.00^{e}	3.00^{dc}	5.00^{d}	10.00^{d}	15.00^{c}	18.00^{de}	28.00^{cd}	32.00^{c}
Dry	100 % Moringa + 0 % Panicum	4.00^{a}	7.00^{a}	10.00^{b}	15.00^{b}	19.00^{b}	21.00^{c}	26.00^{e}	28.00^{e}
	0 % Moringa + 100 % Panicum	0.00^{e}	1.00^{e}	3.00^{e}	7.00^{e}	12.00^{d}	18.00^{de}	27.00^{ed}	34.00^{ab}
	Moringa 50 % : Panicum 50 %	3.00^{b}	5.00^{b}	10.00^{b}	15.00^{b}	19.00^{b}	24.00^{b}	30.00^{ab}	34.00^{ab}
	Moringa 30 % : Panicum 70 %	2.00^{c}	5.00^{b}	10.00^{b}	13.00^{c}	19.00^{b}	25.00^{b}	30.00^{ab}	35.00^{a}
	Moringa 70 % : Panicum 30 %	4.00^{a}	8.00^{a}	15.00^{a}	19.00^{a}	21.00^{a}	27.00^{a}	31.00^{a}	34.00^{ab}
	SEM	0.28	0.41	0.67	0.72	0.74	0.72	0.62	0.75

^{abcdefg} Means along the same column with different subscripts are significantly different ($P < 0.05$)

than 9.17 % reported by Fadiyimu *et al.* (2000). Meanwhile, 100 % Moringa + 0 % Panicum silage CP contents for both seasons in this study were lower than 29.68 % reported by Fadiyimu *et al.* (2000). The differences between this study and the other authors could be because they used fresh forage sample in contrast to silage used in this study. Other factors could also be responsible for CP variation in samples. Wattiaux (1999) reported that proteolytic activities that takes place during the ensilage could bring about reductions in CP content, while other variability could be attributed to plant parts, climatic condition, harvesting regime, location, soil type and age (Norton, 1994) and leaf to petiole ratio (Bamikole *et al.*, 2004). The lower CP content of 0 % Moringa + 100% Panicum silage compared to 100 % Moringa + 0 % Panicum silage in this study support the report of Mhere *et al*. (1999) that the major shortcoming of grasses is it low CP content. Hence the need for

Table 5: Effect of season and plant type on the fermentation kinetics of the silage

Season	Forage mixture	b (ml.200 mg DM^{-1})	c (ml.hr^{-1})	Lag time (hr)	SCFA (µmol.g^{-1})	ME (MJ.kg^{-1})	OMD (%)	DMD (%)
Rainy	100 % Moringa + 0 % Panicum	33.14	0.03	1.60^{dc}	0.35^{e}	4.59^{d}	42.67^{ed}	70.00^{ab}
	0 % Moringa + 100 % Panicum	53.18	0.02	4.39^{a}	0.39^{d}	4.81^{d}	40.89^{ef}	50.00^{e}
	Moringa 50 %: Panicum 50 %	29.79	0.03	2.10d^{cb}	0.30^{f}	4.30^{e}	39.61^{f}	60.00^{cd}
	Moringa 30 %: Panicum70 %	36.86	0.05	2.53^{cb}	0.51^{b}	5.52^{b}	48.77^{a}	65.00^{cb}
	Moringa70 % : Panicum 30 %	48.33	0.32	2.55^{cb}	0.37^{de}	4.68^{d}	41.76^{ef}	65.00^{cb}
Dry	100 % Moringa + 0 % Panicum	30.31	0.06	0.83^{d}	0.44^{c}	5.11^{c}	45.13^{ed}	65.00^{cb}
	0 % Moringa + 100 % Panicum	57.20	0.30	3.16^{ab}	0.37^{de}	4.67^{d}	41.06^{ef}	50.00^{e}
	Moringa 50 % : Panicum 50 %	39.20	0.04	1.65^{dc}	0.51^{b}	5.49^{b}	45.97^{cb}	55.00^{ed}
	Moringa 30 % : Panicum 70 %	41.09	0.04	2.19^{dcb}	0.54^{b}	5.63^{b}	48.15^{ab}	75.00^{a}
	Moringa 70 % : Panicum 30 %	35.22	0.06	1.12^{dc}	0.59^{a}	5.89^{a}	47.86^{ab}	60.00^{cdo}
	SEM	2.77	0.04	0.22	0.02	0.10	0.64	1.59

^{abcdef}Means along the same column with different subscripts are significantly different ($P < 0.05$)
b: Insoluble but degradable fraction, c: Fractional rate of gas production, SCFA: Short chain fatty acid
ME: Metabolizable energy, OMD: Organic matter digestibility, DMD: Dry matter digestibility

mixture of grasses with browse plant that are high in CP, essential vitamin, minerals and amino acids is supported (Makkar and Becker, 1997, Gidamis *et al*., 2003).

The range of non-fibre carbohydrates (NFC) content of the plant types silages in this study indicated that they can be easily degraded or fermented, as NFC is a crude estimate of the carbohydrate pool that differ in digestibility from NDF. The pH of silage is one of the simplest and quickest ways of evaluating its quality. The pH values of different ensiled forage mixtures at different season fell within 4.2 (Kung and Shaver, 2001) and 4.5 and 5.5 (Meneses *et al.*, 2007) that was recommended as the normal good silage for ruminants.

A higher CP content for 100 % Moringa + 0 % Panicum silage during the rainy season compared with dry season could be due to higher moisture content and nitrogen uptake being more rapid than dry matter accumulation during the rainy season. This agrees with Bamualim *et al*. (1980) and Larbi *et al*. (1997) who reported that seasonal variations occur between plant species and between seasons with higher crude protein values reported for seasons with higher moisture levels. The higher CP content of 100 % Moringa + 0 % Panicum silage in the rainy season could be due to the continuous flush (regrowth) of leaves during this period (Anele *et al.*, 2009).

0 % Moringa + 100 % Panicum silage recorded higher NDF, ADF and ADL above other plant types which was consistent with the observation of Okoli *et al.* (2003) that the fibre contents of tropical grasses is usually higher than that for browse, shrubs and trees. The range of NDF contents in this study for 100 % Moringa + 0 % Panicum silage and all the forage mixtures for both seasons were below the range of 600-650 g.kg DM^{-1} suggested as the limit above which intake of tropical feeds by ruminant animals would be limited (Van Soest *et al*., 1991).

Silages produced from the legumes had higher Ca and P contents than those from the grasses. This is in accordance to the report of Nasrullah *et al*. (2004) and Marschner (1993) that there is a marked difference between the level of Ca and P in legumes and grasses. Since grasses have a tendency to be lower in Ca and P, hence the mixture of grasses and legumes in order to improve the nutrients available to animals. Ca is above the critical level of 3 g.kg DM^{-1} as recommended for ruminants in the warm wet climates (McDowell *et al*., 1993). This result indicates that Ca content of forage satisfied the animal requirements specified by the ARC (1980).

The P level in this study was above the critical level of 2.5 g.kg DM^{-1} for ruminant animals as reported by Muhammad *et al*. (2005). The K contents in all plant type silages at different seasons of the year were higher than 8 g.kg DM^{-1} as recorded for grazing animals (Underwood, 1981).

***In vitro* digestibility**

The value of net gas produced in this study for 100 % Moringa + 0 % Panicum silage is similar to those reported for leaves from tropical trees and shrubs (*Adansonia digitata* and *Bombax glabra*) in south western Nigeria (Ogunbosoye and Babayemi, 2010). The low digestibility observed in this study for 100 %

Moringa + 0 % Panicum silage may be due to the presence of condensed tannins which is an anti-nutritional content found in most of the plant species (Barry and McNabb, 1999). Moringa (30 %) : Panicum (70 %) silage at dry season recorded the highest gas produced which suggests that it is of higher nutritional value than the other plant types and it connotes high digestibility of the forages at that mixtures.

The highest short-chain fatty acids (SCFA) and organic matter digestibility (OMD) contents in 70 % Moringa : 30 % Panicum silage in dry season and other forage mixtures, possibly because the mixtures contain more fermentable carbohydrate which is a vital substrate for growth of ruminal microorganisms (Van Soest, 1994). The estimation of the ME values is valuable for purposes of ration formulation and to set economic value of feed for other purposes (Getachew *et al*., 2002). Gas production is a reflection of the generation of SCFA and microbial mass (Getachew *et al*., 1998).

CONCLUSION

The pH values were within the range of pH for good quality silage. 100 % Moringa + 0 % Panicum silage recorded the highest CP content and lower fibre fractions than the 0 % Moringa + 100 % Panicum silage. Mixtures of the grass and browse plants produced quality silages for both the rainy and dry seasons. This will go a long way to provide quality feed for ruminants during the dry season when forages are scarce with low quality in the natural pastureland.

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Review

ETIOLOGY OF MASTITIS IN EWES AND POSSIBLE GENETIC AND EPIGENETIC FACTORS INVOLVED

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ABSTRACT

The paper reviews the knowledge related to the possible relationship of somatic cell counts to udder health and milk yield in dairy ewes. It attempts to point out the epigenetic and genetic aspects of udder health, methods for diagnostic of mastitis and pathogens involved. The possible physiological level of somatic cell counts (SCC) to establish the limits for ewe's milk are discussed. Data from the worldwide research are compared with limited results obtained in Slovakian dairy practice. Globally, applied research has focused on understanding of the relationship between SCC and mammary gland health through the presence of microorganisms. Many milk samples with high SCC are microbiologically negative, underlining the importance of research at the molecular level. Limits for SCC indicating health problems of ewe's udder are not yet established but during the last decades the proposal for limits decreased with time. Most paper considered 0.5×10^6 cells.mL^{-1} or below for healthy udders. Data obtained from bulk milk showed that only 7.3 % of the samples were in the category below 0.5×10^6 cells.mL^{-1} under Slovakian conditions. Possible genetic and epigenetic factors are discussed in this paper in relation to SCC. The identification of a genetic marker(s) that allows the inclusion of mastitis resistance in selection programmes would help to reduce the economic impact due to this disease. Subclinical mastitis is considered as a limiting factor for milk production. Several works have been published which presented a negative correlation between SCC and milk production.

Key words: mastitis; somatic cell counts; ewes; genetics; epigenetics; pathogens

INTRODUCTION

Sheep milk production is currently the main breeding aim of many agricultural farms and privately-employed farmers in many countries. Milk plays a crucial role in the economy of cooperatives and farms. The price of milk is affected, not only in the current situation, by the sheep milk market, but it is significantly influenced by the breeders. On the farmer's side, there are legislative limits for the total number of microorganisms in the milk delivered, which cannot exceed. Equally important contributions of breeders are also hygienic safety of the milk for the consumers, especially in the marketing of milk and milk products directly sold on the farm. Some microorganisms found in milk may be a source of human health hazard (zoonoses), for example, *Staphylococcal* enterotoxins (Holečková *et al.*, 2004) which are not completely degraded in milk and milk products after pasteurization (Asao *et al.*, 2003). Total microorganisms in the delivered raw milk indicate the overall milking hygiene level and pathogenic species present in the milk thus representing the udder

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health status (Philpot and Nickerson, 1991). Moreover, microorganisms and udder health status adversely affect the technological qualities of the milk in its further processing (Leitner *et al*., 2004). Recently, improvement in the overall immune responsiveness of the dairy cow including mammary gland against mastitis through the genetic and epigenetic factors has been believed to play a crucial role in providing better disease resistance, increasing animal welfare and food quality while maintain favourable production level to feed a growing population (Thompson-Crispi *et al*., 2014).

The aim of this paper was to review the knowledge related to the possible relationship of somatic cell counts (SCC) to udder health and milk yield in dairy ewes. This work also attempts to point out the epigenetic and genetic aspects of udder health, methods for diagnosis of mastitis and pathogens involved.

Udder health status

The microorganisms present in the mammary gland represent one of the most serious diseases in dairy animals, called mastitis. The reaction of the body of ewes to the presence of microorganisms in the mammary gland is the transfer of blood white blood cells (somatic cells) into the milk. Diagnosis of mastitis is carried out by various methods, which differ in reliability, cost and complexity. The most common methods for mastitis detection in ewes are assessed on the basis of setting the number of somatic cells (SCC), bacteria culturing (McDougall *et al*., 2001; Contreras *et al*., 2007; Fragkou *et al*., 2014) and palpation of the udder (Marogna *et al*., 2010). Currently, real-time PCR is going to be intensively used for the diagnosis of mastitis pathogens (Zadoks *et al*., 2014). More than 30 % of the samples from clinical and subclinical mastitis in dairy cows appear negative for the identification of pathogens during culturing (no growth of microorganisms). This situation is problematic for all stakeholders such as laboratories, farmers and veterinarians, which highlight the need for real-time PCR methods (Taponen *et al*., 2009).

In addition to total SCC, various researchers have taken into consideration the presence of various types of white blood cells in the milk that may be related to the type of microorganisms in the udder, which allows to further specify the response of the body to the presence of various bacterial species (Ariznabarreta *et al*., 2002; Bagnicka *et al*., 2011; Leitner *et al*., 2012). The knowledge of the art of mastitis in dairy ewes is in high demand at present because it is clearly confirmed that „sheep are not small cows“ (Zadoks *et al*., 2014), because many pathogens are different from those that cause udder disease in dairy cows (Cuccuru *et al*., 2011;Gilchrist *et al*., 2013).

Physiological level of somatic cells in milk

At present in our country and across the world, individual as well as bulk samples of sheep milk are normally not analyzed for the presence of somatic cells, because the payment for the milk according to SCC is not implemented. This is due to the lack of objectively clarified factors and relationships that affect milk SCC in terms of physiological and pathological aspects (Fragkou *et al*., 2014) despite the known fact that SCC is related to the presence of microorganisms in the mammary gland (McDougal *et al*., 2002; Suarez *et al.*, 2002). In addition to the above mentioned reason, the determination of SCC is expensive for breeders of ewes (Adrias *et al*., 2012).

Across the world, applied research is focused on understanding of the relationship between SCC and mammary gland health through the presence of microorganisms. Many milk samples with high SCC are microbiologically negative, underlining the importance of research at the molecular level (Zadoks *et al*., 2014). At the physiological and pathophysiological level the SCC limit ranged from 0.25 to 1.0 $\times 10^6$ cells.mL^{-1} in the 80s, and the SCC for healthy udders was proposed at 0.5 $\times$ 10^6 cells.mL^{-1} (Gonzalo and Gaudioso Lacasa, 1985). In a later work, Berthelot *et al.* (2006) reported healthy ewes with SCC below 0.5 $\times$ 10^6 cells and infected udders with SCC greater than 1 $\times$ 10^6 cells.mL^{-1}, while at the herd level, if SCC exceeded 0.65 $\times$ 10^6 cells.mL^{-1}, they indicated up to 15 % occurrence of mastitis. In determining relationship to milk production, Arias *et al.* (2012) recommended the limit value of 0.3 $\times$ 10^6 cells.mL^{-1}. In our work with 2,632 milk samples at the Experimental station, we have observed an increase of the proportion of ewes with SCC below 0.1 $\times$ 10^6 cells.mL^{-1} from 31 % in 2010 to 56 % in 2013, and a decline in proportion of ewes with SCC less than 1 $\times$ 10^6 cells.mL^{-1} from 21 % in 2010 to 12.5 % in 2013 (Idriss *et al.*, 2015). Recently in Tsigai ewes under practical conditions only 13 % of ewes had over 0.6 $\times$ 10^6 cells.mL^{-1} (Vršková *et al*., 2015). Another study from our experimental farm reported an average of 0.45 $\times$ 10^6 cells.mL^{-1} with high variation coefficient (Margetin *et al*., 2005, 2013). Earlier results published by Margetin *et al*. (1995; 1996) depending on year of study were 0.364 $\times$ 10^6 cells mL^{-1} (1993) and 1.1 $\times$ 10^6 cells.mL^{-1} (1994) SCC during the period of suckling and milking but the data were obtained only from less than 50 animals per year. Available data from Slovakia indicate relatively good udder health of ewes but represent only very limited numbers of animals and farms. Riggio *et al*. (2013) stated that in uninfected Valle del Belice ewes, 83.7 % of the samples were in the category below 0.5 $\times$ 10^6 cells. mL^{-1} and only 2.6 % above 1 $\times$ 10^6 cells.mL^{-1}. Similarly, a high percentage of samples with bacteriologically negative milk were in the following categories of SCC:

64.5 % had less than 0.05 × 10^6 cells.mL^{-1}, 81.9 % had less than 0.25 × 10^6 cells.mL^{-1}, and 92.4 % had less than 0.5 × 10^6 cells.mL^{-1} (Pengov, 2001). Therefore the author considered the threshold of 0.25 × 10^6 cells.mL^{-1} beyond the assessment of udder health.

In spite of the very good udder health in selected farms reported by Idriss *et al*. (2015) and Vršková *et al.* (2015) the health of udder in Slovakian sheep farms seems to be much more complicated and difficult. The bulk milk analysis of 1086 samples from March to August revealed that only 7.3 % of the samples were in the category below 0.5 × 10^6 cells.mL^{-1} (Tomaška *et al*., 2015). In last mentioned report 49 % of samples were over 1× 10^6 cells.mL^{-1} which deserves more detailed research in mastitis control in dairy practice in Slovakia. In general Slovakian breeders do not test for SCC in ewes' milk, and have no information about the health status of the mammary glands and thus the potential risks of the sale of raw unpasteurized sheep milk and sheep milk products to consumers' health. Farmers lack information on risks of breeding environment for udder health, including economic losses caused by mastitis. Even though SCC as an indicator of udder health and milk quality is not part of pricing milk, countries with high level of sheep breeding pay considerable attention to udder health (Leitner *et al*., 2012; Fragkou *et al*., 2014). Research in these countries is aimed to improve the health of the animal's mammary gland, resulting in improved economy of breeding ewes, the competitiveness of enterprises and production of hygienically and nutritionally better milk quality. Slovakia is also considered as the country with well-developed traditional sheep breeding, which requires more intensive research in the production of safe and hygienic raw sheep milk in primary production. Such research will also contribute to a more effective breeding of dairy ewes.

Classification of mastitis

In terms of the most common occurrence of mastitis, in practice, this disease can be divided into clinical and subclinical mastitis. Clinical mastitis is quickly detected by the breeders on the basis of clinical symptoms, such as painful udder, edema and changes in the consistency of the milk (Marogna *et al*., 2010), where the producer may use such symptoms for disposal of such milk from delivering into the dairy and in terms of the risk of microbial contamination, also from the food chain. More detrimental to the sheep breeding are subclinical mastitis, which do not show any clinical signs at the udder and milk level. Subclinical mastitis negatively influences cheese yield and its quality (Silanikove *et al.*, 2014). Moreover, such milk is not only a source of pathogens but also their byproducts - toxins (Asao *et al.*, 2003), which have been confirmed in our conditions (Holečková *et al*., 2004; Mašlanková *et al*., 2009; Zigo *et al*., 2014). The finding that the subclinical mastitis is the most widespread in the breeding system of ewes is also important (Bergonier *et al*., 2003; Leitner *et al*., 2004). In farms with a good management, the incidence of clinical mastitis is below 5 % (McDougall *et al*., 2001), but subclinical mastitis have been detected at 15-40 % of the ewes (Kiossis, *et al*., 2007, Contreras *et al.*, 2007).

Mastitis pathogens

As indicated above, subclinical mastitis is a limiting factor for milk production. Several works have been published which present a negative correlation between SCC and milk production (Fuertes *et al*., 1998, Gonzalo *et al*., 2002). Major pathogens (infectious; *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma*) cause a significant increase in SCC and decline in milk production compared to minor microorganisms (Gonzalo *et al*., 2002; Riggio *et al.*, 2013). On the other hand, minor microorganisms (environmental; the most common coagulase negative staphylococci - CNS) are the predominant organisms in the milk of ewes in the herd (Gonzalo *et al*., 2002; Kiossis *et al*., 2007; Pilipčincová *et al.*, 2010; Marogna *et al.*, 2010, Bagnicka *et al.*, 2011) and deserve deeper attention (Leitner *et al.*, 2012). In farms of eastern Slovakia, Pilipčincová *et al*. (2010) analyzed the presence of CNS species in individual ewe's milk from farms with mostly hand-milking (94.5 %), which differed significantly from the species present in cow milk (Zadoks and Watts, 2009). Specification of CNS species increases the effectiveness of different control treatments against mastitis (Ruegg, 2009). Significant differences in detection of different CNS species between regions in Slovakia (Pilipčincová *et al*., 2010) and in other countries (Mavrogianni *et al*., 2007), as well as the specific presence of microorganisms in sheep milk versus cow milk (Zadoks *et al*., 2009) suggest the need for a deeper analysis of mastitis pathogens in ewes in Slovakia and the identification of risk factors of external and internal environment. Microscopic fungi and yeasts play a certain role in the infection of the udder of dairy cow(Scaccabarozzi *et al*., 2011; Idriss *et al*., 2013), which draw little scientific attention in sheep milk.

Mastitis and milk yield

The health status of the mammary gland, manifested by increased SCC is under considerable attention of researchers also in relation to the drop in milk production. Negative phenotypic correlation between SCC and milk production in different breeds was reported by several authors in Manchega ewes (Adrias *et al*., 2012) and in Churra ewes (Gonzalo *et al.*,

2002). Špánik *et al.* (1996) calculated the negative correlation between SCC and the yield in the experimental herd. Negative significant correlation between SCC and milk production in Tsigai ewes during both suckling and milking period was also found out by Margetin *et al*. (1996). Recently we have reported non-significant reduction of milk yield with increasing SCC in Tsigai ewes (Vršková *et al*., 2015). The differences in performance between ewes with SCC above and below 0.3×10^6 cells. mL^{-1} increased during lactation (Adrias *et al*., 2012). The authors also found that ewes with twins had a higher proportion of samples with SCC above 0.3 x 10^6 cells.mL^{-1}, but also higher milk production than ewes with one lamb (Adrias *et al*., 2012). Similarly, Olives *et al.* (2013) noted a lower milk production of infected udder by 16 % during 7 weeks of lactation, and in comparing the two halves of the udder, healthy udder-half compensated for the loss of the second half by up to 6.6 %. Similar reduction of milk yield between two quarters in the same front/rear position with different SCC was also reported in cows (Tančin and Uhrinčať, 2014).

Possible genetic markers

The identification of a genetic marker(s) that allows for the inclusion of mastitis resistance in selection programmes would help reduce the economic impact due to this disease. However, the selection of a candidate gene is a difficult task because mastitis is a complex disease influenced by many genes and environmental factors. Problems associated with breeding directly for mastitis resistance also include its low heritability, ranging from 0.02-0.10 (Nash *et al*., 2000). With the recent development of extensive high-throughput genomic tools research efforts have increasingly turned to identifying single nucleotide polymorphisms (SNPs) associated with resistance as well as quantifying the genetic control of the host-pathogen interaction (Rupp and Foucras, 2010). The solutions to improve resistance to mastitis are likely to be those that focus on information from genome – wide association studies (GWAS), or selection based on breeding values of immune responses, which take into account complex genetic interactions between the innate and adaptive host defense mechanisms without the necessity of knowing all about each individual gene. These approaches may be best suited to help alleviate mastitis, at least until we gain more knowledge about genetic and epigenetic regulation of host defense mechanisms.

Major histocompatibility complex (MHC) plays an important role in immunological defense against pathogens. MHC is a group of genes on a single chromosome that codes the MHC antigens and includes two major subfamilies: class I and class II genes. MHC molecules that function in the recognition event, which is termed "antigen presentation", are polymorphic glycoproteins found on cell membranes. The MHC participates in the development of both humoral and cell mediated immune responses. The general consensus is that these genes may be useful as genetic markers of a higher or lower risk of mastitis in cows (Conington *et al*., 2008).

In sheep, MHC is located on chromosome 20 and is called Ovar (Hediger *et al*., 1991). A complete ovine MHC sequence map was assembled by successful shotgun sequencing of 26 overlapping BAC clones (Gao *et al*., 2010). The MHC of sheep and cattle share orthologous class II DR and DQ A and B loci with rodents and primates. A single dominant and highly polymorphic DRB locus encoding the beta chain of the MHC class II DR heterodimer has been described in domestic sheep Ovar-DRB1 (Scott *et al*., 1991). The detailed genomic organization and allelic diversity of Ovar-DRB1 locus has been described (Ballingall *et al*., 2008). Recently, unusual allelic diversity has also been identified at the DRA locus in domestic sheep (Ballingall *et al*., 2010). Several studies have shown the existence of ovine class II loci that are homologous to HLA-*DQB* (Dukkipati *et al.*, 2006). As in other vertebrate species, a high degree of polymorphism is found in the Ovar-*DQB* genes, with most of the polymorphic sites located in exon 2, which encodes for the antigen-binding site.

It has been reported that alleles of different MHC genes correlate with disease resistance in sheep. Currently, relevant research on Ovar genes' polymorphism and disease resistance or susceptibility mainly concentrates on Ovar-*DRB*1 and Ovar-*DQB*. Herrmann-Hoesing *et al.* (2008) detected that Ovar-*DRB*1 alleles contribute as a host genetic factor controlling ovine pneumonia provirus levels. Larruskain *et al.* (2010) found a significant association of Ovar-*DRB*1 alleles with resistance to Maedi-Visna and pulmonary adenocarcinoma viruses. Recently, Shen *et al.* (2014) have shown that polymorphism in Ovar-*DRB*1/*DQB*1 can be used as a marker of resistance to Echinococcosis in Chinese Merino sheep.

Epigenetic factors

Udder disease is affected by a number of external environmental factors (management, manner of milk removal and milking technology, season) and internal factors (physiological status of the body, like stage and order of lactation, oestrus, udder shape and response of ewes to milking, condition) (Raynal-Ljutovac *et al.*, 2007). Other important factors include the overall level of husbandry and management aimed to reduce the risk of mastitis, such as drying ewes that are treated with antibiotics (Shwimmer *et al.*, 2008; Spanu *et al.*, 2011) and in particular the routine of machine milking (Leitner *et al*., 2008). The milking routine must be based

on biological needs of ewes for machine milking and both from the morphological as well as physiological aspects (Mačuhová *et al.*, 2008, 2012; Tančin *et al*., 2011; Antonič *et al*., 2013b). Our results from several farms showed that ewes bred in Slovakia have a relatively poor response to machine milking, which is observed as retention of milk in the udder. Holding of the milk in the udder reduces milk production (Silanikove *et al*., 2010) and increases the risk of disease of the udder. It is very important to further specify the state of health of the mammary gland of ewes bred in Slovakian conditions. Furthermore, to assess the impact of manner of milk removal (hand, machine) on udder health in relation to breed, functionality and parameters of milking equipment, ewes age, stage of lactation, milking frequency, number of lambs, organization of work in the milking process and the overall level of farming must be studied. We earlier reported an increased risk for udder health-related to weaning lambs and ewes adaptation to machine milking (Antonič *et al*., 2013a), and changes in conditions of milking (Kulinová *et al*., 2012; Jackuliaková *et al*., 2014; Tančin *et al*., 2015). It seems that hand milking induces more udder infection than machine milking and the type of milking equipment (mobile and stationary) plays a role in the risk of mastitis as well (Marogna *et al.*, 2010). More frequent health problems of udder for milking with mobile devices compared with stationary ones have been noted by Marogna *et al*. (2010), which justify the worse parameters of mobile milking equipment (vacuum stability, frequency pulsations, activity of liner). The transition from extensive to intensive dairy sheep farming system also brings more infections caused by environmental pathogens (Marogna *et al*., 2010). In Slovakian conditions these were not studied though many changes have taken place in milk removal systems in dairy practice in Slovakia.

The completeness of udder emptying and speed of milking of cows are considered among the most important indicators of the impact of technology on milk removal and handling of dairy animals (Tančin and Bruckmaier, 2001), which demonstrate not only the welfare of the animals during milking, but are one of the important reactions of the organism in reducing the risk of udder disease (Tančin *et al*., 2006, 2007). Although ewes have certain differences in milk distribution in the udder as compared to dairy cows, it is likely that the speed and completeness of milking to keep good udder health is equally important. Thus, intense transition from hand to machine milking of ewes under practical conditions of Slovakia deserves serious research on the prevention of udder diseases.

CONCLUSIONS

From the above mentioned information it could be concluded that without the knowledge related to udder health and possible risk factors of breeding systems in dairy practice in Slovakia, it would not be possible to further improve the development of sheep breeding in the country, where sheep are considered to be of high importance for animal production, sustainability of countryside and production of special milk products.

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Short communication

THE POTENTIAL USE OF CHICKEN BLASTODERMAL CELLS OF ORAVKA BREED

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ABSTRACT

This study provides information about the method of isolation as well as evaluation of morfology and viability of chicken blastodermal cells (BCs) of Oravka breed. Oravka breed belongs to a critical endangered breed under the conditions of the Slovak Republic. Due to this fact, it is necessary to preserve genetic material of Oravka breed. Frozen blastodermal cells are the potential tool for long-term storage. Chicken blastodermal cells were derived from X stage of embryos (blastoderm stage) using filter paper ring method. To detect blastodermal cells viability specific nuclear fluorochrome Yo-Pro-1 in combination with propidium iodide and DAPI were used. Under the light microscope the isolated blastodermal cells were seen as a large round cells with diameter from 13 to 18 μm and acentric nucleus of 9 μm in diameter. Fluorescent analysis revealed only viable, DAPI-positive cells in the samples. Totally, 38000 blastodermal cells of Oravka breed from each blastoderm were obtained.

Key words: Oravka breed; blastodermal cells; cryopreservation; viability

INTRODUCTION

Conservation of avian genetic resources in the gene bank is necessary for the preservation of endangered species. Cryopreserved biological material can be used for recovering of the lost variation within breeds and restoring of the breeds which have become endangered as a result of destruction of their natural conditions (Sawicka *et al.*, 2011).

Domestic chicken (*Gallus gallus domesticus*) has become an important subject of biotechnological research, particularly in relation to cryopreservation. Compared to mammals, avians have advantages, such as short reproductive cycle, small gap between generations, high laying performance and easy breeding (Li and Lu, 2010). In regards of this facts, avian embryos are a powerful model to study developmental and stem cell biology.

One of the wonderful advantages of the avian embryo is its accessibility, since the avian embryo is subjected to genetic manipulation (Ishii *et al*., 2004). Free access to bird eggs (Ishii *et al*., 2004) allows subjected birds to genetic manipulation Cyriac *et al*., 2012). This feature has been utilized to produce somatic chimeras of various types through tissue grafts, chorioallantoic grafts, parabiosis, yolk sac chimeras and neural tube chimeras (Le Douarin and McLaren, 1984). However, in order to utilize any potential of avian embryonic stem line for manipulation of the avian genome, it was necessary to develop reliable means of producing germ line chimeras analogous to that produced by blastocyst injection of murine embryos.

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National Agricultural and Food Centre, Research Institute for Animal Production Nitra (NAFC, RIAP) deals with the genetic manipulations of Oravka the local poultry breed. Oravka breed was created by crossbreeding of the local hens in the Orava region with Rhode Island, Wyandotte and New Hampshire breeds (Hanusová *et al.*, 2014) and was recognized in 1990 (Chmelničná, 2004). Weis *et al.* (2010) categorized Oravka breed as a critical endangered breed under the conditions of the Slovak Republic.

The objective of this preliminary study was to optimize the method for blastodermal cells (BCs) isolation in Oravka breed and to evaluate their morphology and viability.

MATERIAL AND METHODS

In our study, chicken eggs of Oravka breed were used as a biological material. X stage blastoderms were collected from 20 freshly laid non-incubated fertilized eggs. The eggs were collected immediately after laying and transferred to the laboratory.

X stage blastoderm was prepared with filter paper ring, the perivitelline membrane was cut around the paper and the blastoderm was removed (Petitte *et al.* 1990). Blastoderm was isolated from yolk and washed several times in calcium-magnesium-free PBS (CMF-PBS) (Applichem, Darmstadt, Germany) to remove as much yolk as possible. Then, the cells were washed and centrifuged in CMF-PBS for 3 min at 300 x g, and supernatant was discarded. The cell concentration was determined using automated cell counter (Z1 Automated Cell Counter, Beckman-Coulter, Germany).

To determine the proportion of viable, apoptotic and dead cells fluorescent staining was used. The total number of cells was determined using 4‘, 6-diamidino-2-phenylindole (DAPI) fluorescent dye (blue signal; Vector Laboratories, Burlingame, CA, USA). The number of apoptotic cells was determined using Yo-Pro-1 (green signal; Molecular Probes, Lucerne, Switzerland) and the proportion of dead cells using propidium iodide (PI) staining (red signal; Molecular Probes, Eugene, Oregon, USA). Fluorescent dyes were prepared as follows: 1 µl Yo-Pro-1 (1mmol/l) was diluted with 5 µl PI and 194 µl CMF-PBS; 3 µl of cell suspension was transferred to a tube by gentle pipetting and stained with 3µl Yo-Pro-1/PI by incubation in the dark at room temperature for 20 min. After incubation, sample was washed two times in CMF-PBS. Three µl of the stained sample were placed on a microslide into 3 µl of the Vectashield anti-fade medium containing DAPI fluorescent dye and observed under a LEICA DMI 6000 B fluorescent microscope equipped with DCF 345 FX camera.

RESULTS AND DISCUSSION

Stem cell biology is a rapidly expanding field that includes also the manipulation with stem cells. Chick stem cells can be obtained from embryos and maintained in culture. They can be derived from different sources at various stages of embryonic development. It was demonstrated that in regards to their pluripotency they can form embryoid bodies, which can differentiate into all cell types including embryonic germ layers and contribute to somatic and germline lineages in chimaeras. They are, therefore, comparable to mammalian stem cells, offering a model for studying stem cell biology, as well as being a tool for many applications.

Proper techniques of cell isolation, culture and freezing will give a broad perspective for unlimited storage of genetic recourses.

In our study we isolated, cultured and evaluated under the light microscope BCs from freshly laid non-incubated fertilized eggs of Oravka breed. For isolation we used the same filter paper ring method of BCs isolation as Pettite *et. al.* (1990) and Hamidu *et al.* (2010). Our preliminary results showed that this method of chicken BCs isolation is proper and can be used in our subsequent experiments.

Morphological characterization of freshly isolated BCs revealed large round cells with diameter from 13 to 18 µm with acentric nucleus 9 µm in diameter. Furthermore, we determined total number of viable blastodermal cells. Blastoderm of Oravka breed consists of 38000 blastodermal cells. Fluorescent microscopy revealed only living, DAPI positive BCs in the samples (blue signal). Figure 1 (a, b) represents morphology and viability of freshly isolated BCs.

However, for the future most important is the preservation of isolated BCs for long-term storage in liquid nitrogen. Published reports on this topic indicate that BCs tolerate deep-freezing and have ability to produce chicken chimeras (Naito *et al*., 1992; Bednarczyk *et al*., 2002).

Chimeras can be used to restore breeds threatened with fate to extinction. Similar studies were conducted by Kino *et al.* (1997) and Naito *et al.* (1994), where injection of BCs into recipient embryos allowed obtaining sex chimeras, and through appropriate mating enabled the restoration of the genotype of the donor. According to Carcience *et al*. (1993) and Naito *et al.* (1992) injected donor blastodermal cells can incorporate into recipient embryos and differentiate into the somatic tissues. The same method was used by Bednarczyk *et al.* (2000); blastoderm was prepared with paper filter rings and thereafter BCs were injected into the sub-germinal cavity of recipient embryo to create transgenic chickens.

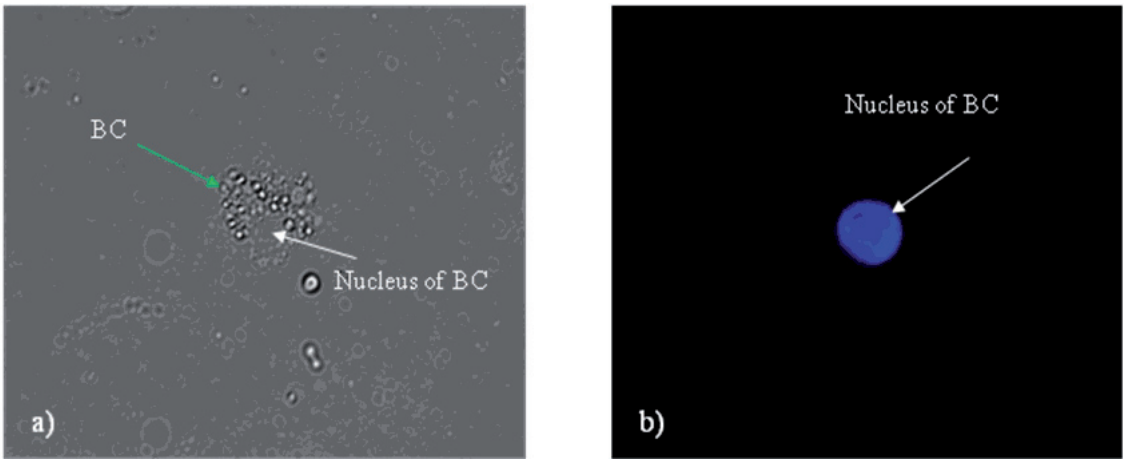

Fig. 1: Morphology and viability of freshly isolated BCs. a) light microscope; b) fluorescent microscope – DAPI staining (magnification x 20)

CONCLUSION

Our preliminary study represent only *in vitro* experiments. However, the evaluation of the BCs viability after cryoconservation should be conducted also *in vivo*. The application of isolated BCs for cryopreservation requires further development to expand its potential for avian genetic resources.

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