UNIVERSITATEA DE ȘTIINȚE AGRICOLE ȘI MEDICINĂ
VETERINARĂ "ION IONESCU DE LA BRAD" IAȘI

LUCRĂRI ȘTIINȚIFICE

VOL. 60
MEDICINĂ VETERINARĂ

PARTEA a 3-a

EDITURA "ION IONESCU DE LA BRAD" IAȘI 2017

on -line ISSN 2393 – 4603
ISSN–L 1454 – 7406

CONTENTS

The effect of habitat on hair copper, molybdenum, and selenium levels in cats

Gheorghe Valentin GORAN[1], Emanuela BADEA[1], Cristina ȚOCA[2], Victor CRIVINEANU[1]
[1] Faculty of Veterinary Medicine, UASVM of Bucharest, 105 Splaiul Independentei, 050097, 5th district, Bucharest, Romania, EU;
[2]IDAH of Bucharest, 63 Dr. Staicovici, 050557, 5th district, Bucharest, Romania, EU
gheorghegoran@fmvb.ro

Abstract

Cu, Mo, and Se are essential trace minerals, which maintain proper activity of some animal organisms functions. The main goal of this study was the assessment of Cu, Mo and Se levels in the hair of pet cats in an urban environment. The hair samples were collected from flank region from 20 clinically healthy pet cats. Analysis of hair Cu, Mo, and Se content of pet cats kept indoors (5 males and 5 females) and outdoors (5 males and 5 females), were performed by inductively coupled plasma mass spectrometry (ICP-MS). The mean Cu level in indoor pet cats was 19.47 mg•kg^{-1} for males and 10.58 mg•kg^{-1} for females, and in outdoor male cats was 10.33 mg•kg^{-1} and 14.32 mg•kg^{-1} for females. Generally, Mo registered lower mean levels when mean Cu levels were higher and higher levels when Cu was lower, indifferent of habitat, sex or age. The mean Se hair levels registered insignificant differences for the same habitat in pet cats below 5 years and above 5 years. In this study, the habitat statistically insignificant influenced hair Cu, Mo, and Se levels in pet cats.

Key words: *habitat, hair, cat, copper, molybdenum, selenium*

Introduction

In classification proposed by Frieden (1985), Cu, Mo, and Se are essential trace minerals, which maintain proper activity of some animal organisms functions, and deficiency in this elements leads to disorders and may prove fatal (Prashanth ET AL., 2015).

Cu is the key trace element for enzymes necessary for increasing the strength of keratin fiber, and has very important role in activity of essential enzyme systems implicated in formation, growth or repair of keratin-rich tissues. (Goluch-Koniuszy, 2016) Cu favors the intestinal absorption of iron and its incorporation into hemoglobin and is a component of many enzymes. Cu plays an important role in reducing cellular lesions caused by free radicals. Cu is also involved in the synthesis of collagen in tendons and myelin in the nervous system. Cu also participates in the synthesis of melanin, which is a hair pigment. (Collins, 2014) The Cu deficit most likely induced by zinc, through a mechanism that can be explained by the competitive absorption of zinc and Cu in enterocytes in the small intestine. Excessive zinc intake stimulates the production of metallothionein. However, Cu has a higher affinity for metallothionein, eliminating zinc, and then excreting, which leads to hypocupremia. (Green and Weaver, 2008)

Mo concentrates in the liver, kidney, bone and significant amounts are found in the dental enamel and hair, and has an essential role as cofactor of some important enzyme systems – xanthine oxidase, an enzyme involved in the formation of uric acid; aldehyde oxidase, an enzyme involved in detoxification (Mendel, 2013a), and is involved in hair health by inducing secondary Cu deficiency (Phoon et al., 2011). Mo has been shown in animals to be involved with fat, purine and sulfate metabolism, and it is also involved in detoxification and intimately involved in Cu metabolism. Symptoms associated with a Mo deficiency are represented by impaired growth, tooth decay, male impotence, xanthine stones, and also symptoms of Cu toxicity. (*Mendel, 2013b)* Mo excess could determine acute toxicity, which result in severe diarrhea, and chronic toxicity, which may cause gout. Cu deficiency symptoms may also occur, including skin problems, hair loss,

growth retardation, osteoporosis, thyroid abnormality, bone and joint abnormalities and weight loss. (*Barceloux and Barceloux, 1999*) Mo and sulfur also antagonize Cu by a ternary interaction involving the formation of Cu thiomolybdate, which results in reduced Cu absorption. Tungsten is a Mo antagonist in several oxidative enzymes that require Mo (e.g. xanthine oxidase). (Frieden, 1985)

Se is an essential component of Se proteins, which play an important role in many biological functions, such as antioxidant defense, thyroid hormone formation, DNA synthesis, fertility and reproduction (Mehdi et al., 2013; Sunde, 2014). Hair gets trace elements especially from the blood and is able to integrate Se into its matrix during keratinization. Se is part of at least 35 proteins, many of which are enzymes, and Se deficiency causes hair loss and pseudo albinism (Masumoto et al., 2007). Although much less common than Se deficiency, Se toxicity can affect individuals as a result of excessive supplementation, causing hair loss. (Fairweather-Tait et al., 2011)

Hair can be an excellent sample for assessing mineral status, especially of trace elements, in the organism, because their hair concentration is usually higher than in the blood. (Foo et al., 1993; Skibniewska et al., 2011; Skibniewska et al., 2013; Skibniewski et al., 2013)

Animal hair can be a biomarker of environmental pollution, and also for assessing animal mineral status. In recent years, as a bioindicator of metal pollution or different organ diseases, hair samples from domestic and wild species such as dog, cat, cattle, horse, goat, sheep, camel, European bison, elk, brown bear, wolf, fox, wild boar, squirrel and seal. (Combs, 1987; Medvedev, 1999; Liu, 2003; Ikemoto et al., 2004; Rashed and Soltan, 2005; Hawkins and Ragnarsdottir, 2009; Crivineanu et al., 2010; Skibniewski et al., 2010; Filistowicz et al., 2011; de Almeida Curi et al., 2012; Hernández-Moreno et al., 2013; Badea et al., 2016a; Badea et al., 2016b; Goran et al., 2017)

The main goal of this study was the assessment of Cu, Mo and Se levels in the hair of pet cats in an urban environment, using inductively coupled plasma mass spectrometry (ICP-MS).

Materials and methods

Sampling and samples preparation - The hair samples were collected from flank region from 20 clinically healthy pet cats from an urban environment. Analysis of hair Cu, Mo, and Se content of pet cats kept indoors (5 males and 5 females) and outdoors (5 males and 5 females), were performed by inductively coupled plasma mass spectrometry (ICP-MS).

Before analysis the hair samples (n=20) were weighed (approximately 0.5 g) using an analytic balance (precision 4 decimals) and placed in individual PPR vials with a capacity of 14 mL. Disintegration of the organic matter was done by cold wet mineralization, adding 5 mL of nitric acid 65% suprapur (d=1.39) and 1 mL of hydrochloric acid 30% suprapur (d=1.15) over the hair samples. The samples were disintegrated at room temperature into the fume hood for one week.

Spectrometric analysis - Digested samples were diluted to 10 mL with ultrapure water and analyzed by Perkin-Elmer Elan DRC II ICP–MS spectrometer (RF1100 W; reading time 30 s, washing time 30 s, nebulizer gas flow 0.5 $L \bullet min^{-1}$; auxiliary gas flow 0.5 $L \bullet min^{-1}$; sample injection pump flow 50 rpm). Calibration curves were developed using standard solutions of 0.001 ppm, 0.05 ppm, 0.01 ppm, 0.025 ppm, and 0.05 ppm (for low concentrations of minerals as Se), and 0.1 ppm, 1 ppm, 5 ppm, 10 ppm, and 25 ppm (for the others, as Cu and Mo) obtained by dilution from a MERCK stock standard solution for each element, containing 1000 $mg \bullet L^{-1}$ of Cu, Mo, and Se.

Statistical analysis - Statistical analysis was performed using the software of VassarStats: Website for Statistical Computation (http://vassarstats.net/). One-Way ANOVA was performed for all samples' mineral concentrations, and when ANOVA generated $p \leq 0.05$, means comparison was carried out by all-pair Tukey HSD Test.

Results and discussions

The mean Cu, Mo, and Se contents of hair samples from clinically healthy pet cats are presented in Table 1 and expressed as *mg•kg*$^{-1}$.

In this study, the effect of living conditions on Cu, Mo, and Se concentrations in the pet cat hair was observed, but habitat influenced statistically insignificant hair Cu, Mo, and Se levels in pet cats. The highest mean value of Cu was registered in the group of cats above 5 years kept indoors (26.736 mg•kg^{-1}), significantly different compared to hair Cu mean level of cats below 5 years kept in the same living conditions. Considering the animal gender, the mean content of Cu in males was higher in indoor pet cats hair (19.473 mg•kg^{-1}), and in females it was higher in outdoor pet cats hair (14.32 mg•kg^{-1}). Indifferent of gender, hair Cu mean levels were higher in pet cats above 5 years kept indoor (26.736 mg•kg^{-1}), and in outdoor pet cats below 5 years (15.064 mg•kg^{-1}). Also, it was observed that hair Cu mean levels in pet cats below 5 years registered levels significantly different reported to animals' living conditions. In this study, it was observed no significant differences between Cu mean hair levels in investigated groups of pet cats kept indoors or outdoors, independent of gender or age.

Table 1. Cu, Mo, and Se mean levels in pet cats hair samples (mg•kg^{-1})

Element			n^{o}	Cu	Mo	Se
Males		**in**	5	19.473 ^{a}	0.215 ^{a}	2.111 ^{a}
		out	5	10.330 ^{a}	0.358 ^{a}	2.279 ^{a}
Females		**in**	5	10.584 ^{a}	0.084 ^{a}	2.317 ^{a}
		out	5	14.320 ^{a}	0.160 ^{a}	2.463 ^{a}
Age	**below 5**	**in**	7	10.011*^{a}	0.087 ^{a}	2.310 ^{a}
		out	3	15.064*^{a}	0.222 ^{a}	2.257 ^{a}
	above 5	**in**	3	26.736 ^{b}	0.296 ^{a}	1.991 ^{a}
		out	7	11.288 ^{a}	0.274 ^{a}	2.420 ^{a}
Habitat		**in**	10	15.028	0.149	2.214
		out	10	12.547	0.259	2.371
All animals			20	13.853	0.204	2.293

* Significant differences at $p \leq 0.05$. The comparison can be made only between habitats for the concentration of one element and not between different elements concentrations.

^{a,b} Levels not connected by the same letter are significantly different ($p \leq 0.05$). The comparison can be made only between sex or age in the same habitat for the concentration of one element and not between different elements concentrations.

Hair Mo levels registered reversed values reported to Cu – higher levels when Cu registered lower values and lower concentrations when Cu was higher. Mo registered highest mean level in outdoors males (0.358 mg•kg^{-1}), and lowest mean level in indoors females (0.084 mg•kg^{-1}). Reported to age, Mo mean hair levels registered higher levels in above 5 years pet cats indifferent of living conditions, and higher mean levels in outdoors pet cats below 5 years and in indoors pet cats above 5 years. Insignificant differences were observed between Mo mean hair levels in pet cats kept indoors or outdoors, indifferent of gender or age.

The highest mean value of Se was registered in the group of female cats kept outdoors (2.463 mg•kg^{-1}), insignificantly different compared to hair Se mean level of indoors female cats and also males kept outdoors. The mean Se hair levels registered insignificant differences for the same habitat in pet cats below 5 years and above 5 years. Independent of habitat, hair Se mean levels were higher in females compared to males. In this study, hair Se registered higher mean levels in outdoors pet cats, independent of habitat, gender, and age, excepting pet cats below 5 years were the values registered were reversed. The lowest mean Se hair level was registered in the group of pet cats above 5 years kept indoors (1.991 mg•kg^{-1}).

In the available literature, there are no data concerning Mo content in the hair of domestic cats. The obtained results could only be compared with the data concerning Cu and Se in control groups of pet cats. While analyzing mineral and heavy metal content in the hair of cats in relationship with kidney failure, Badea et al. (2016a) obtained for clinically healthy animals (control group) the results for Cu of 0.94 mg•kg^{-1} in females and 0.92 mg•kg^{-1} in males, and also, hair Se ranged from 0.07 mg•kg^{-1} in males, and 0.15 mg•kg^{-1} in females, which are in both trace minerals, much lower compared to those registered in our study for the same groups, independent of habitat. The same pattern was observed reported to age in both trace minerals, which are also much lower compared to those registered in our study for the groups below 5 years and above 5 years, independent of habitat.

Cu and Se content in the hair of female pet cats from the group of healthy animals, which represented control group in a study on hair mineral content analysis in cats with different liver disorders amounted to 0.78 mg•kg^{-1} for Cu and 0.103 mg•kg^{-1} for Se in females above 8 years (Goran et al. 2017). These values are much lower in the case of Cu and approximately in the same range, but lower for Se than those obtained in our study.

Conclusions

Even in this study, Cu, Mo, and Se levels in pet cats hair were not significantly influenced by the living conditions, hair may be considered as an indicator for the mineral status of cats in an urban area.

Mean value of hair Cu in group of cats above 5 years kept indoors was significantly different compared to hair Cu mean level of cats below 5 years kept in the same living conditions.

Hair Cu in younger pet cats registered significantly different mean levels dependent of habitat.

Mo registered lower mean levels when mean Cu levels were higher and higher levels when Cu was lower, indifferent of habitat, sex, and age.

Hair Se registered higher mean levels in outdoors pet cats, independent of habitat, gender, and age.

References

1. Badea E., Goran G.V. Matei E., Rotaru E., Crivineanu V. (2016a). Heavy metal and mineral content in the coat of cats in relationship with kidney failure, *Lucrari Stiintifice Medicina Veterinara Timisoara* XLIX(1), 17-28.
2. Badea E., Goran G.V. Crivineanu V. (2016b). Hair mineral levels in dogs with atopic dermatitis, *Bulletin UASVM-CN* 73(2), 451-3.
3. *Barceloux D.G., Barceloux D. (1999). Molybdenum. Clinical Toxicology.* 37 *(2): 231-7.*
4. Collins J.F. (2014). Copper, *in* Modern nutrition in health and disease. 11th ed. / eds A.C. Ross, B. Caballero, R.J. Cousins, K.L. Tucker, T.R. Ziegler. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA, pp. 73-4.
5. Combs D.K. (1987). Hair analysis as an indicator of mineral status of livestock. *J Anim Sci* 65:1753-8.

6. Crivineanu V., Leonidis A., Goran G.V., Codreanu I. (2010). Blood and wool lead levels in sheep farmed near roads from province of Thessaloniki. *Lucrari Stiintifice Medicina Veterinara Timisoara* XLIII (2):140-146.
7. de Almeida Curi N.H., Brait C.H.H., Filho N.R.A., Talamoni S.A. (2012). Heavy metals in hair of wild canids from the Brazilian Cerrado. *Biol Trace Elem Res* 147:97-102.
8. Fairweather-Tait S.J., Bao Y., Broadley M.R., et al. (2011). Selenium in human health and disease. *Antioxid Redox Signal.* 14:1337–1383.
9. Filistowicz A., Dobrzanski Z., Przysiecki P., Nowicki S., Filistowicz A. (2011). Concentration of heavy metals in hair and skin of silver and red foxes (*Vulpes vulpes*). *Environmental Monitoring and Assessment* 182:477-84.
10. Foo S.C., Khoo N.Y., Heng A., Chua L.H., Chia S.E., Ong C.N., Ngim C.H., Jeyaratnam J. (1993). Metals in hair as biological indices for exposure. *Int. Arch. Occup. Environ. Health*. 65:S83-86.
11. Frieden E. (1985). New perspectives on the essential trace elements. *J Chem Educ* 62:917-23
12. Goluch-Koniuszy Z.S. (2016). Nutrition of women with hair loss problem during the period of menopause. *Menopause Review 15*(1):56-61.
13. Goran G.V. Badea E., Crivineanu V. (2017). Hair mineral content analysis in cats with different liver disorders, *Scientific Works, C Series, Veterinary Medicine* LXIII
14. Green A.L., Weaver D.F. (2008). Potential proconvulsant effects of oral zinc supplementation: a case report. *Neurotoxicology* 29:476-7.
15. Hawkins D.P, Ragnarsdottir K.V. (2009). The Cu, Mn and Zn concentration of sheep wool: Influence of washing procedures, age and colour of matrix. *Sci. Total Environ*. 407: 4140-8.
16. Hernández-Moreno D., de la Casa Resino I., Fidalgo L.E., Llaneza L., Rodríguez F.S., Pérez-López M., López-Beceiro A. (2013). Noninvasive heavy metal pollution assessment by means of Iberian wolf (*Canis lupus signatus*) hair from Galicia (NW Spain): a comparison with invasive samples. *Environ Monit Assess* 185:10421-30.
17. Ikemoto T., Kunito T., Watanabe I., Yasunaga G., Baba N., Miyazaki N., Petrov E.A., Tanabe S. (2004). Comparison of trace element accumulation in Baikal seals (*Pusa sibirica*), Caspian seals (*Pusa caspica*) and northern fur seals (*Callorhinus ursinus*). *Environ. Pollut*. 127: 83-97.
18. Liu Z.P. (2003). Lead poisoning combined with cadmium in sheep and horses in the vicinity of non-ferrous metal smelters. *Sci. Total. Environ*. 309: 117-126.
19. Masumoto K, Nagata K, Higashi M, et al. (2007). Clinical features of selenium deficiency in infants receiving long-term nutritional support. *Nutrition*. 23:782–787.
20. Medvedev N. (1999). Levels of heavy metals in Karelian wildlife 1989-91' *Environ. Monitor. Assess*., 56 (2): 177-193.
21. Mehdi Y., Hornick J.L., Istasse L., Dufrasne I. (2013). Selenium in the environment, metabolism and involvement in body functions. *Molecules*. 18:3292–311.
22. Mendel R.R. (2013a). The Molybdenum Cofactor. *J Biol Chem.* 288(19): 13165-72.
23. *Mendel R.R. (2013b). Metabolism of Molybdenum. In Metallomics and the Cell. Metal Ions in Life Sciences. Editor Banci L. 12. Springer.*
24. Phoon, X., Ander, E. L. and Plant, J. A. (2011). Essential and Beneficial Trace Elements, in Pollutants, Human Health and the Environment: A Risk Based Approach (eds J.A. Plant, N. Voulvoulis and K.V. Ragnarsdottir), John Wiley & Sons, Ltd, Chichester, UK, 73-5.
25. Prashanth L., Kattapagari K.K., Ravi Teja Chitturi R.T., Venkat Ramana Reddy Baddam V.R.R., Prasad L.K. (2015). A review on role of essential trace elements in health and disease. *Journal of Dr. NTR University of Health Sciences* 4(2): 75-85.
26. Rashed M.N., Soltan M.E. (2005). Animal hair as a biological indicator for heavy metal pollution in urban and rural areas. *Environ. Monitor. Asses*. 110: 41-53.
27. Skibniewska E., Skibniewski M., Kośla T. (2013). The effect of living conditions on vanadium bioaccumulation in cats. *Environmental Protection and Natural Resources*. 24(4): 43-5.
28. Skibniewska E.M., Skibniewski M., Kośla T., Urbańska-Słomka G. (2011). Hair zinc levels in pet and feral cats (*Felis catus*). *J. Elem.* 16(3): 481-8
29. Skibniewski M., Kośla T., Skibniewska E. (2013). Domestic cat (*Felis catus*) as a bioindicator of environmental lead contamination. *Environmental Protection and Natural Resources*. 24(4): 47-50.
30. Sunde R.A. (2014). Selenium, *in* Modern nutrition in health and disease. 11th ed. / eds A.C. Ross, B. Caballero, R.J. Cousins, K.L. Tucker, T.R. Ziegler. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA, pp. 74-5.

Copper-releasing, borate-based glasses with antibacterial properties: synthesis and *in vitro* characterization

Cristina LELESCU[1*], Aurel MUSTE[1], Marian TAULESCU[1], Gheorghe BORODI[2], Marin ȘENILĂ[3], Lucian BARBU-TUDORAN[2], Răzvan ȘTEFAN[1]

[1]Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca

[2]National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca,

[3]Reseach Institute for Analytical Instrumentation, Cluj-Napoca

*cristina.alexa.lelescu@gmail.com

Abstract

In this study, glasses within the system (60-x) B_2O_3·x ZnO·34CaO·1CuO, with x=5, 10, 15, 20, 25 mol% and with B_2O_3/ZnO ratios 11; 5; 3; 2; 1.4 have been synthesized and characterized in vitro. After being immersed in simulated body fluid (SBF) and saline solution, weight loss reduction and pH measurments, followed by inductively coupled plasma optical emission spectometry (ICP-OES), scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis were performed, in order to evaluate the changes in glass morphology. In vitro biodegradation and surface reaction were observed in all of the glasses, especially in the x=10, 15, 20 samples. SEM and XRD results revealed the presence of a hydrotalcite-like structure (double layered hydroxid) at the aqueous solution-glass surface interface, while Cu, Zn, Ca and B ions, with proangiogenic properties, were detected in the immersion fluid.

Keywords: *copper, borate glasses, in vitro, biological ions;*

Introduction

Bioactive glasses are a category of vitreous biomaterials with remarkable bioactivity and biocompatibility, which makes them highly promising in the medical applications field. In recent years, there has been a significantly growing interest in the use of borate-based bioactive glasses due to their excellent properties in bone regeneration and beyond. [4,24]. Low chemical durability, faster and more complete degradation in comparison with silicate glasses, and a controllable conversion to hydroxyapatite (HA) are among the well known characteristics of boron-containing glasses [10]. Still, an extensive and ongoing process of research is required to provide prospects for their use in soft tissue engineering [20].

A controllable degradation rate of the bioactive glass is desirable, taking into account that it should be similar to the rate of new tissue formation, and a high chemical durability results in a longer and less complete degradation rate upon immersion in aqueous solutions. [10,18,20]. This process is accompanied by dissolution of ionic compounds in the fluid in which the bioactive glass was immersed, resulting in pH and ionic concentration changes of the aqueous solution over time [6]. Also, to form a strong bond with the surrounding tissue, the glass surface must undergo a specific conversion phenomenon when immersed in simulated body fluid (SBF), leading to the fomation of a hydroxiapatite (HA)-like surface layer [8]. Therefore, *in vitro* characterization of these processes is essential to predict the *in vivo* behavior of the materials, and their applicability in tissue engineering [20].

Weight loss measurements and pH monitoring of the aqueous immersion solution are simple and relevant methods for assessing glass degradation rate, as they accompany these processes by changes in ion concentration of the *surrounding* aqueous *medium* [10]. Scanning electron microscopy (SEM) coupled with energy-dispersive X-ray analysis (EDS) is used to measure microstructural surface changes after immersion [16], while newly formed crystalline

phases can be detected by X-ray diffraction (XRD) analysis [10]. Concentration of ions released into deionized water following immersion, can be accurately measured by using inductively coupled plasma optical emission spectometry (ICP-OES), being an essential tool for predicting effects of the biomaterial on the surrounding tissue, including: cell growth stimulation, growth-factor enhancement [22], angiogenesis promotion [15] and gene expression regulation [23].

The aim of this paper was to thoroughly characterize 5 compositions of bioactive glasses derived from the B_2O_3–ZnO–CaO–CuO system, by measuring the weight loss and pH changes of the glass, after being immersed in aqueous solutions at 37°C. Additional SEM-EDS and XRD analysis were performed to characterize microstructural surface changes, while ICP-OES was completed after immersion of the glass in deionized water at 37°C. The results are intended to anticipate the biological behavior of the glasses, and their applicability in tissue engineering.

Materials and methods

Glass synthesis

Glasses within the system (60-x) B_2O_3·x ZnO·34CaO·1CuO, with x=5, 10, 15, 20, 25 ZnO mol% and with B_2O_3/ZnO ratios 11; 5; 3; 2; 1.4 were prepared with high purity raw reagent-grade chemicals. In order to obtain five different compositions of borate-based bioactive glasses, zinc oxide (ZnO), copper oxide (CuO), boric acid (H_3BO_3) and calcium carbonate ($CaCO_3$) powders were weighed, mixed and homogenized before being melted at 1230°C for 25 minutes in sintered corundum crucibles. The compositions were rapidly cooled at room temperature by quenching the molten glasses; subsequently, the samples were crashed in an agate mortar and passed through a sieve in order to finally obtain particles with a diameter (d) < 0.075 mm.

Degradation behavior and pH assessment of the borate-based glass samples in 0.9% saline solution

In order to evaluate the degradation behavior of the bioactive glasses, similarly sized samples were weighed before being soaked in 5 ml of normal saline solution (0.9%) and incubated at 37°C for 7 days. The weight reduction was assessed by measuring the relative weight loss (Δm) in all samples, using the following equation: $\Delta m = 100 \cdot (m_i - mf)/mi$, where m_i is the initial mass, found in the samples before immersion and m_f is the measured weight after 7 days of soaking in saline solution. Each sample was removed from the solution and wiped gently with filter paper to eliminate the fluid from the surface before being weighed. Consequently, the glass surface was microscopically evaluated at the end of immersion time (Olympus BX51).

Ionic dissolution assessment

100 mg of glass powder from each sample were soaked in 5 ml of deionized water, being subsequently incubated at 37°C for 5 days. The concentration of ions (copper, zinc, borate, calcium and aluminium) released during the degradation process were measured by ICP-OES analysis (FMD-07, Spectro Analytical, Germany). Multielement calibration solutions prepared from a stock solution of 1000 mg L^{-1} (Merck, Darmstadt, Germany) were used to calibrate the instrument. The estimated error in the measurement rate was +/- 5%.

Evaluation of the surface microstructural and chemical composition changes

Changes in the chemical and mirostructural properties of the samples, resulting from the interaction with aqueous solutions, were assessed after immersing the glasses in normal saline solution 0,9% for 7 days, at 37°C. The surface texture and morphological evaluation was performed by SEM, while using XRD analysis (Bruker D8 Advance), the crystalline phases were quantificated following interaction with the aqueous medium.

Results and discussions

Five glass compositions belonging to the (60-x) B_2O_3·x ZnO·34CaO·1CuO system, with x=5, 10, 15, 20, 25 ZnO mol% and with B_2O_3/ZnO ratios 11; 5; 3; 2; 1.4 were obtained by melting technique (fig. 1). Samples were rapidly cooled at room temperature, being subsequently crushed and passed through a sieve, resulting in particles with a diameter of <0.075 mm (fig. 2).

Fig. 1 Macroscopic appearance of the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=10 sample, after being quenched and crushed

Fig. 2 Macroscopic appearance of the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=5 sample, after being crushed and sieved to <0.075 mm particles

In the present study, granular forms of glass particles were obtained in order to increase the glass surface area, thereby increasing the reactivity of the sample surface in the surrounding physiological fluid.

Macroscopic aspects of the samples following immersion

After 7 days of immersion in saline solution at 37°C, a newly formed layer of approximately 0.5 mm thickness, very brittle, whitish and easily ruptured was noted at the surface of the glasses (fig. 3), especially in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x= 10; 15; 20 samples. Microscopically, the newly formed layer exhibited a crystallized appearance, while the underlying surface layer presented a smooth aspect, lacking pre-existing discontinuities (fig. 4). Clusters formed via agglomeration of particles were significantly decreased in number and size, whilst in some regions, due to the thickness of the newly formed layer and its opacity, the microscopic evaluation could not be achieved.

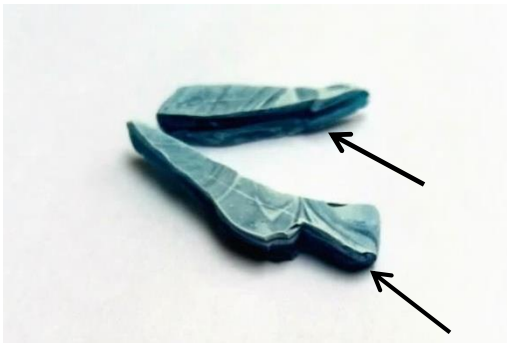


Fig. 3 Macroscopic appearance of the newly formed glass surface layer after 7 days of immersion in normal saline 0,9% in the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=10 sample

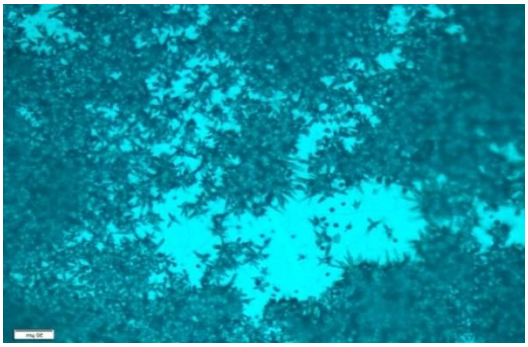


Fig. 4 Microscopic appearance of the surface layer after 7 days of immersion in normal saline 0,9% of the (60-x) B_2O_3·x ZnO·34CaO·1CuO with x=20 sample (100x)

A remarkable, dynamic process has been noted at the aqueous solution-glass surface interface on microscopic evaluation of the samples, consisting of an uninterrupted deposition of a thin layer, with a crystallized appearance (fig. 5-6).

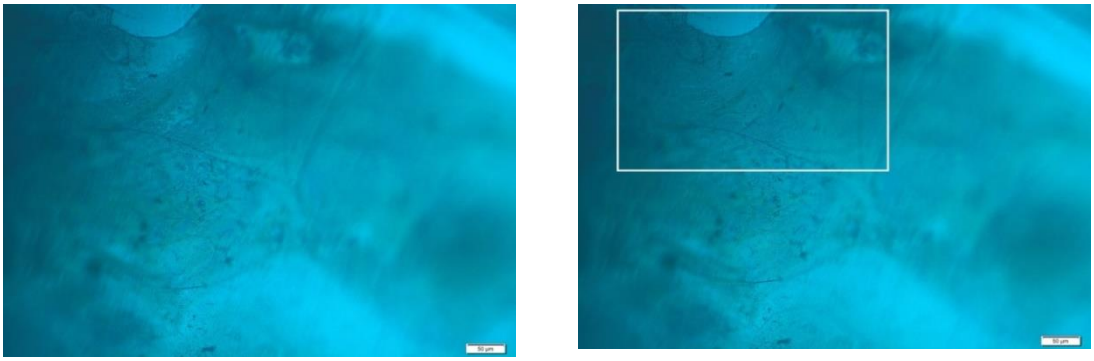


Fig. 5-6: Continuous deposition of a thin layer on the sample surfaces, after 7 days of immersion in SBF, at 37°C

Degradation behaviour and pH assessment of the bioactive glass samples

The average weight reduction (Δm) for all of the five samples after immersion in 0.9% saline solution, was found to be 0.86%. The highest weight reduction was recorded in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=10 sample (Δm= 1.76%), while the lowest weight reduction was identified in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=25 sample (Δm= 0.03%). Although weight losses are relatively low, they indicate the ocurrance of a degradation process; however, a reduced degradation and conversion rate is rather characteristic for silicate glasses, and represents a drawback by hindering the coordination between the bioactive glass degradation rate and tissue regeneration rate [7,20].

The starting pH value of the 0.9% saline solution at 37°C was 5.4. After 7 days of immersion, the most significant increase in the pH value was recorded in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=15 sample, with 3.5 units. The saline solution has undergone the smallest change in pH in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=25 sample, with 1.9 units. Changes in the pH take place due to changes in ionic concentrations, as a result of the degradation process [10,20]. A previous study has shown a correlation between increasing the B_2O_3 content in the sample and increasing pH of the immersion solution [7]. Huang [10] highlighted that, by increasing B_2O_3 content, a very rapid pH increase of the aqueous solution was caused, reaching a limit value in 50 hours; however, the final pH value was higher in the samples with a decreased B_2O_3 content, where the limit value was reached after 500 hours. In the present study, pH evaluation was performed after 168 hours of immersion and showed a lower pH increase in the sample with the highest B_2O_3 content. Increasing B_2O_3 content results in a lower chemical durability, which leads to a faster reaction with the aqueous medium, and therefore to the rapid change of the pH, but it's limiting value is detemined by the ionic concentrations released in the solution, as well as by their acidity and basicity [10].

Ionic dissolution products

In order to measure the ions concentrations released into the deionized water, the ICP-OES analysis was performed; results in all of the five samples are presented in Fig. 7.

Sample	Cu (mg/L)	Zn (mg/L)	B (mg/L)	Ca (mg/L)	Al (mg/L)
x=5	0.038	0.048	530	770	3.6
x=10	0.054	0.041	506	730	3.6
x=15	0.075	0.044	544	840	2.7
x=20	0.045	0.049	510	810	1.1
x=25	0.066	0.054	650	1270	1.2

Fig. 7: Ion concentrations of deionized water following immersion of the glass samples

The highest concentrations of B, Zn and Ca were found in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=25 sample (650 mg/L, 0.054 mg/L and 1270 mg/L respectively). Cu reached a maximum level in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=15 (0.075 mg/L) sample, while Al levels were found in all of the five samples, with the highest amounts being detected in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=5, 10 samples (3.6 mg/L). The presence of trace amounts of aluminium is explained by the high temperature and the long melting time during sample synthesis, which caused contamination from the walls of the crucible.

The biological significance of Cu is given by its anti-inflammatory, anti-infectious, antibacterial and proangiogenic properties [1]. A remarkable cellular distribution of Cu ions has been revealed in human endothelial cells, when induced to undergo angiogenesis [5]. Zn ions are required in various enzymatic activities and anti-inflammatory processes, possess a remarkable antimicrobial activity and are strongly involved in protein synthesis [2,12]. They appear to be actively involved in collagen synthesis and play a role in cell membrane stabilization, intracellular signaling and wound healing [14]. Increased levels of Zn were identified at the wound margins within the first 24 hours, while even higher levels were detected during epidermal granulation and proliferation [9,13]. Boron ions are apparently involved in the synthesis of collagen and proteoglycans, increase the turnover of extracellular matrix, and also promote protein phosphorylation [19]. Al has certain astringent, antacid and antibacterial properties, its toxic effects being dose dependent [17].

Microstructural characterization

SEM photomicrographs of the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=10, 15 samples are shown in fig. 10. Cauliflower-shaped agglomerates (fig. 8A), sphere clusters (fig. 8B), and also well defined, regular geometric shapes (fig. 8C-D) were observed at the surface of the outermost layer.

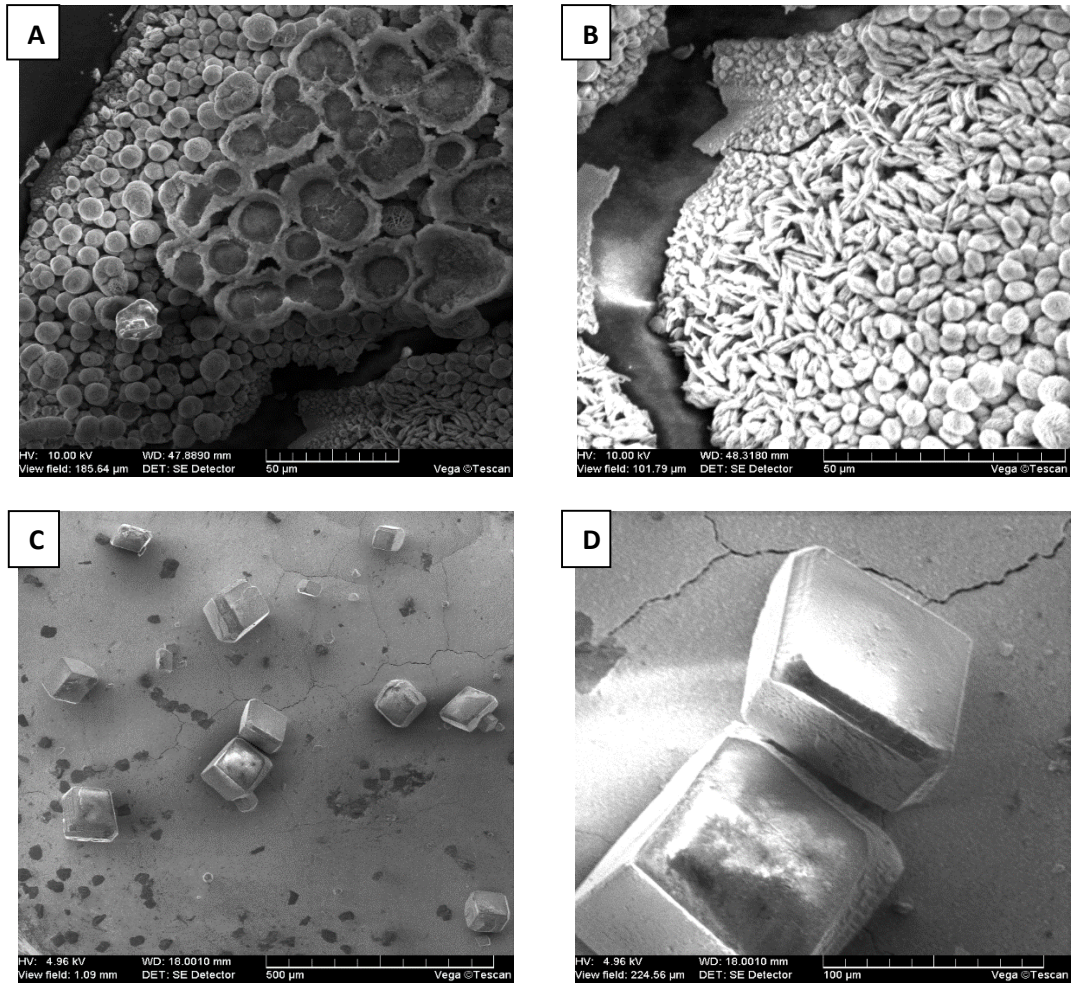


Fig. 8 SEM images of the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x=10, 15 samples.
A : x=10, scale:50 µm; **B**: x=10; scale:50 µm; **C**: x=15, scale:500 µm;
D: x= 15, scale:100 µm

Following imersion in saline solution, XRD patterns of the samples were relatively similar (fig. 12), showing peaks corresponding to zinc aluminium carbonate hydroxide hydrate [$Zn6Al_2(OH)16CO_3.4H_2O$], a hydrotalcite-like structure, also known as double layered hydroxid (strongest line at $2\theta = 11.65°$). The importance of this compound lies primarily in its catalytic behavior, particularly being a substrate for the efficient immobilization of biological materials [3,21]. The development of this interface layer is a result of the interaction between Zn and Al ions, under alkaline hydrothermal conditions [11]. In his study, Koh [11] developed this chemical compound, and successfully used it as a synthetic substrate for hexagonal-patterned ZnO nanorods.

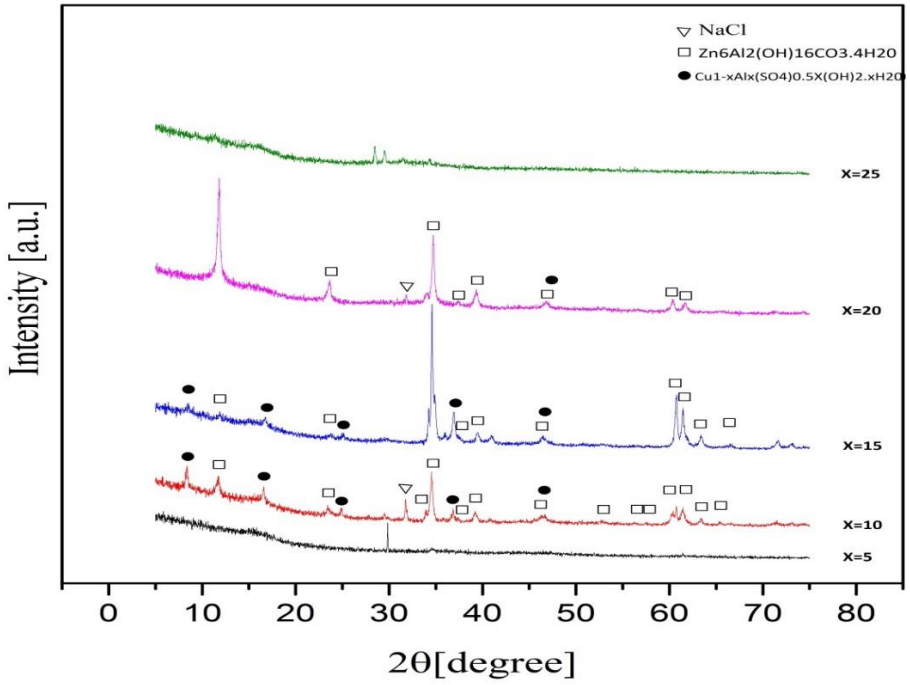


Fig. 12: X-ray diffraction patterns of the (60-x) B_2O_3·x ZnO·34CaO·1CuO system, with x=10, 15, 20 after immersion in SBF

Aluminum sulphate hydroxide hydrate is another compund detected by XRD analysis at the sample surfaces, the most intense peak being centered at 2θ = 8.31˚. The XRD scan also contained diffraction peaks corresponding to NaCl, originating form the immersion fluid.

Diffraction peaks could only be detected and assigened in the x=10, 15 and 20 samples.

Conclusions

Five different borate containg glasses with a composition in the (60-x)·B_2O_3·xZnO·34CaO·1CuO system, were synthesized and characterized by various methods, including ICP-OES, SEM-EDS, and XRD, in order to predict their applicability in tissue engineering. *In vitro* weight loss and pH measurements showed a lightly degradable behavior in all of the samples, while surface reactivity of the glasses was studied microscopically. None of these properties appeared to be linearly dependent on the boron concentration of the glass samples. SEM micrographs captured at the surface of the samples revealed the presence of various distinct, well-defined, regularly shaped structures, especially in the (60-x)·B_2O_3·xZnO·34CaO·1CuO, where x = 10; 15 samples. The XRD patterns of the samples showed the existance of a newly formed, hydrotalcite-like structure at the aqueous solution-glass surface interface, with remarkable biological properties. These results indicate a highly promising stimulatory potential of these glasses, by releasing various ions with multiple activities, related to their proangiogenic and antibacterial properties.

References

1. Barbucci R, Lamponi S, Magnani A, Piras FM, Rossi A, 2005, Role of the Hyal-Cu (II) complex on bovine aortic and lymphatic endothelial cells behavior on microstructured surfaces, *Biomacromolecules* 6: 212-219.

2. Boyd D, Carroll G, Towler MR, Freeman C, Farthing P, Brook IM, 2009, Preliminary investigation of novel bone graft substitutes based on strontium-calcium-zinc-silicate glasses, *Journal of Materials Science: Materials in Medicine* 20:413–20.
3. Cavani F, Trifiro A, Vaccari A, 1991, Hydrotalcite-type anionic clays: preparation, properties and applications 11(2):173-301.
4. Day DE, White JE, Brown RF, McMenamin KD, 2003, Transformation of borate glasses into biologically useful materials, Glass Technology44:75–81.
5. Finney L, Vogt S, Fukai T, Glesne D, 2009, Copper and angiogenesis: unravelling a relationship key to cancer progression, *Clinical and Experimental Pharmacology and Physiology* 36 (1):88e94.
6. Fu H, Fu Q, Zhou N, Huang W, Rahaman MN, Wang D, Liu X, 2009, In vitro evaluation of borate-based bioactive glass scaffolds prepared by a polymer foam replication method, *Materials Science and Engineering: C*, *29*(7), 2275-2281.
7. Fu Q, Rahaman MN, Fu H, Liu X, 2010, Silicate, borosilicate, and borate bioactive glass scaffolds with controllable degradation rate for bone tissue engineering applications. I. Preparation and in vitro degradation, *Journal of biomedical materials research.* 95(1).
8. Hench L, 1993, Bioceramics – from Concept to Clinic, *American Ceramic Society Bulletin* 72(4): 93–98.
9. Henkin RI, 1974, Zinc in wound healing, *The New England Journal of Medicine* 291:675-676.
10. Huang W, Day DE, Kittiratanapiboon K, Rahaman MN, 2006, Kinetics and mechanisms of the conversion of silicate (45S5), borate and borosilicate glasses to hydroxiapatite in dilute phosphate solution, *Journal of Materials Science: Materials in Medicine* 17: 583-96.
11. Koh YW, Loh KP, 2005, Hexagonally packed zinc oxide nanorod bundles on hydrotalcite sheets, *Journal of Materials Chemistry* 15: 2508-2514.
12. Lang C, Murgia C, Leong M, Tan L-W, Perozzi G, Knight D, 2007, Anti- inflammatory effects of zinc and alterations in zinc transporter mRNA in mouse models of allergic inflammation, *American Journal of Physiology-Lung Cellular and Molecular Physiology* 292(2):L577e84.
13. Lansdown ABG, Sampson B, Rowe A, 1999, Sequential changes in trace metal, metallothionein and calmodulin concentrations in healing skin wounds, *Journal of Anatomy* 195 (Part 3): 375–86.
14. Landsown ABG, Mirastschijski U, Stubbs N, 2007, Zinc in wound healing: Theoretical, experimental, and clinical aspects, *Wound Repair and Regeneration* 15 2-16.
15. Leach JK, Kaigler D, Wang Z, Krebsbach PH, Mooney DJ, 2006, Coating of VEGF-Releasing Scaffolds with Bioactive Glass for Angiogenesis and Bone Regeneration, *Biomaterials* 27(17): 3249–55.
16. Liu X, Xie Z, Zhang C, Pan H, Rahaman MN, Zhang X, Fu Q, Huang W, 2010, Bioactive Borate Glass Scaffolds: In Vitro and in Vivo Evaluation for Use as a Drug Delivery System in the Treatment of Bone Infection, *Journal of Materials Science: Materials in Medicine* 21(2): 575–82.
17. Lubowe II, Mecca SB, 1959, Allantoin and aluminium derivatives in dermatological applications, *Drug & cosmetic industry* 84, 36.
18. Mackovic M, Hoppe A, Detsch R, Mohn D, Stark WJ, Spiecker E, Boccaccini AR, 2012, Bioactive glass (type 45S5) nanoparticles: in vitro reactivity on nanoscale and biocompatibility, *Journal of Nanoparticle Research* 14:966.
19. Nzietchueng RM, Doussset B, Franck P, Benderdour M, Nabet P, Hess K, 2002, Mechanisms implicated in the effects of boron on wound healing, *Journal of Trace Elements in Medicine and Biology* 16, 239-244.
20. Rahaman MN, Day DE, Bal BS, Fu Q, Jung SB, Bonewald LF, Tomsia AP, 2011, Bioactive glass in tissue engineering, *Acta Biomaterialia* 7, 2355–2373.
21. Tichit D, Coq B, 2003, Catalysis by Hidrotalcites and Related Materials, *Cattech* 7(6), 206-217.
22. Xynos ID, Edgar AJ, Buttery LD, Hench LL, Polak JM, 2000, Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis, *Biochemical and Biophysical Research Communications* 276(2):461-5.
23. Xynos ID, Edgar AJ, Buttery LD, Hench LL, Polak JM, 2001, Gene-expression profiling of human osteoblasts following treatment with the ionic products of Bioglass® 45S5 dissolution, *Journal* of *Biomedical Materials Research* 55(2):151e7.
24. Ylänen, HO, 2011, Bioactive glasses. Materials, properties and applications, UK, Cambridge: Woodhead Publishing Limited.

Searches on the application of a method of induction and syncronization of estrus in cows postpartum based on two doses of GnRH and prostaglandin F2 α, with programmed insemination

Elena RUGINOSU, S.I BORȘ, Ș. CREANGĂ, D.L. DASCĂLU, Mădălina Alexandra DAVIDESCU
Stațiunea de Cercetare Dezvoltare pentru Creșterea Bovinelor Dancu
Loc. Dancu, jd. Iași
elena.ruginosu54@gmail.com

Abstract

The researches were realized in the dairy cattle farm from S.C.D.C.B. Dancu, Iasi, on an experimental lot, composed of 41 cows of Romanian Black Spotted breed (BNR), (Lot E), compared to a control group composed of 45 cows (Lot M). The purpose of this study was to determine the effectiveness of using a new protocol for the induction and synchronization of estrus at dairy cows, using the hormonal products, such as GnRH and PGF2α. The treatments applied to the cows from the experimental group were performed between 31.01.2017 and 23.06.2017, within an average value of 52 days after calving. The therapeutic protocol consisted in the following: administration of the first dose of GnRH (2.5 ml, Receptal=10 μg Buserelin, intramuscularly) on day 0, (regardless of the stage of the estrous cycle); administration of one dose PGF2α (2ml Estrumate=500 mcg Cloprostenol) on the seventh day; administration of the second dose of GnRH on the ninth day and artificial insemination at 18-24 hours after the second dose of GnRH, (without estrus detection). The application of this therapeutic protocol for estrus induction and synchronization at dairy cows with two doses of GnRH and PGF2α in the 45-80 days postpartum period has reduction effects of the intervals: calving to the first insemination and conception, with a positive impact on the reproductive management of dairy farms and the economic implications by the reduction of unproductive costs and of the workforce for the detection of cows estrus.

Key words*: dairy cows, estrus induction and synchronization, GnRH, prostaglandin F2α, fertility*

Introduction

During the last period was found a decline in reproductive efficiency, particularly in highly productive cows, due to the poor detection of the estrus, caused by the reduced intensity of heat signs, the inadequate time of observation of the animals in large farms and the deterioration of climatic conditions. Most studies indicate an association between lower fertility of cows with high milk yields and decreases concentrations in steroid hormone (estrogen-E2 and progesterone-P4), (1, 2, 9, 15, 16, 17).

Research in the field shows that treatments for cow estrus induction and synchronization may have positive effects on reproductive activity, especially in animals maintained in the free system, facilitating estrous detection and increasing the conception rate by inseminating the animals at the optimum time in relation to ovulation. Of the different protocols for estrus induction and synchronization in dairy cows and meat, the protocol named Ovsynch, which uses a combination of injections of GnRH and PGF2α, is widely used because eliminates the estrus detection and allow fixed time insemination programmed at ovulation optimum time, resulting in normal fertility after insemination at the end of treatments (1,2,3,4,5,7,9,10,11,15,16,17).

Various studies have shown that Ovsynch is an extremely economical and efficient strategy to improve reproductive performance of high performance dairy cows (3,12,13,14).

During a cow's sexual cycle, there are 2-3 waves of ovarian follicular growth, each follicular wave having an average life of 7-10 days, progressing through different stages of development, from appearance, selection, dominance, atresia or ovulation. A dominant follicle,

capable of ovulation is present only at certain times during each wave. Given the dynamic growth of ovarian follicles in a sexual cycle, the Ovsynch therapeutic protocol was developed, which aiming to achieve the following effects: reprogramming follicular waves by administering the first dose of GnRH, the luteolysis of a probable luteal body by administering of PGF2α to 7 days and determining the dominant follicle ovulation expected to be present after PGF2α by administering the second injection of GnRH at 48-56 hours later, thus facilitating fixed-time insemination, without resorting to detection of heat (1,2,6,7,9,15).

Major implications of progesterone (P4) concentration were reported at the time of the first dose of GnRH on the induced LH wave. It was established that in order to have a successful Ovsynch protocol, progesterone (P4) concentration at first GnRH1 treatment should usually be above 1 ng / ml), while in the second GnRH2 treatment, the (P4) concentration to be less than 0.5 ng / ml (5.16).

Some authors report that conception rates in lactating cows that benefited from the Ovsynch protocol were similar to those of cows that were inseminated to spontaneous postpartum estrus (Pursley et al., 1997a, b).

Other recent reports have indicated that although the use of these estrus therapeutic protocols in cows has many advantages, which would come from an effective regulation of sexual cycle and facilitating reproductive management in farms with large livestock, however, the methods of control of sexual cycle have variable results, some being contradictory and questionable (3,4,5,9,10,11,12,13,14,15,16,17).

Variability of Ovsynch protocol results consist in the proportion of non-cycling postpartum cows, the follicular dynamics of each cow in the herd or the ability of farm personnel to properly implement this protocol (7,15,16).

The purpose of present researches was to establish the efficacy of estrus induction and synchronization protocol based on the combination of injections of GnRH and PGF2α, taking into account the positive effects on reproductive management of dairy cows maintained in the free system, but also the variability of treatment outcomes, reported by various authors.

Material and methods

Research has been carried out on zootechnical base from Research and Development Station for Breeding Cattle (S.C.D.C.B.) Dancu, Iasi, on an experimental lot (Lot E), consisting of 41 cows compared to a control group of 45 cows (Lot C) in Romanian Black Spotted breed, freely maintained in a modernized shelter with a capacity of 200 heads, (Figure 1).

Figure 1. Maintenance system of the cows in the experimental group

The treatments for estrus induction and synchronization of cows from the experimental group (E lot) were performed between 31.01.2017 and 23.06.2017, the average interval from calving to treatment being 52 days (with variations between 42-70 days).

The therapeutic protocol consisted in the administration at cycling or non-cycling postpartum cows, regardless of ovarian formations (follicles or luteal body) two doses of 2.5 ml (10 μg) intramuscularly of GnRH (Receptal), separated by a dose of 2 ml prostaglandin F2α (Estrumate) intramuscularly (containing Cloprostenol 250 μg / ml, Intervet International BV, Netherlands) at 7 days after the first administration of GnRH (Receptal, 2 ml, intramuscularly). Two days after the injection of prostaglandin F2α, a second dose of GnRH (Receptal, 2 ml, intramuscularly) was given. Considering the moment of ovulation, the second injection of GnRH was given in the afternoon between 14-15 hours for the insemination to be carried out in the next morning between 8-9 hours, thus achieving the range of 18-19 hours from the second injection of GnRH, which coincides with the production of ovulation. At 18-24 hours after the second dose of GnRH, (Receptal, 2.5 ml) artificial insemination of cows was performed, without resorting to detection of heat (Figure 1).

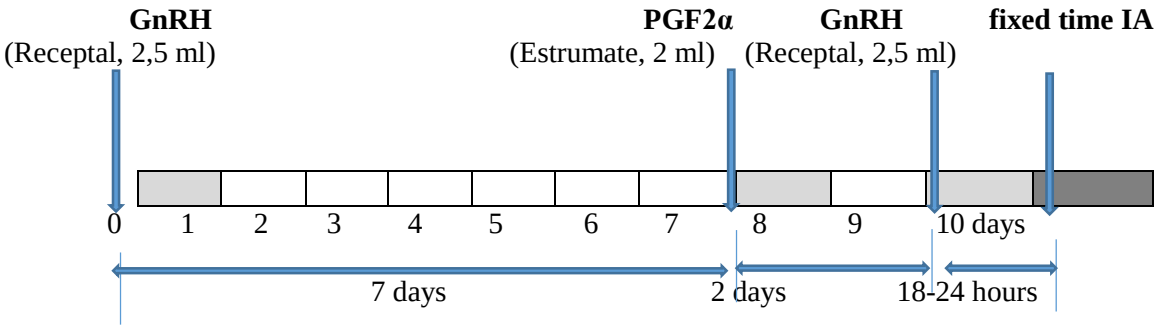


Figure 2. The protocol for induction and synchronization of estrus in cows based on 2 doses GnRH and PGF2α

The effectiveness of treatments applied to cows in the experimental group was determined by determining the intervals from treatment to conception, calving to conception, gestation index and conception rate on total insemination, as well as after the first, second, third and third insemination.

Results and discussions

The artificial insemination of cows took place after the second dose of GnRH (with or without estrus signs) in 97,56% of cases (40 of 41 cows), of which: 87,81 % (36 of 41 Cows) according to the protocol at fixed time (18-24 hours) and 9,75% (4 of 41 cows) which showed estrus were early artificially inseminated (with second administration of GnRH. One cow (2,43%) was not inseminated due to the presence of a genital inflammatory process.

The treatment-conception interval recorded an average of 32.8 ± 10.6 days (with variations between 1-110 days), (Table 1).

Table 1. The artificial insemination of cows in experimental group after the estrus induction and synchronization treatments with 2 doses GnRH and prostaglandin F2α

cows /lot E	artificially inseminated cows (IA) after treatments, of which:							
	total IA		IA at 18-24 hours after second GnRH fixed time		IA early in day of second GnRH		without IA	
n	n	%	n	%	n	%	n	%
41	40	97,56	36	87,81	4	9,75	1	2,44

The comparative analysis of cow's reproductive activity revealed that the average value for the first postpartum insemination interval was with 29 days lower in the experimental group (62 ± 1.05 days) compared to the control group (90.94 ± 8.00 days). However, there were no differences in the average conception interval , the values being close to the two batches close in the two groups (94.93 ± 10.4 days, in the experimental group and 93.72 ± 9.86 days, respectively, in control group), (Table 2).

Table 2. The reproductive activity of cows in the experimental group (n = 41 cows), after the treatments for estrus induction and synchronization treatments compared to control group without treatments (45 cows)

specification values	Intervals, (days)					
	calving-treatments	calving-the first insemination postpartum		treatments-conception	calving-conception	
	lot E	lot E	lot C	lot E	lot E	lot C
average	52±1,03	62±1,05	90,94±8,00	32,8±10,6	94,93± 10,4	93,72±9,86
minim	42	52	35	1	53	35
maxim	70	80	242	110	180	174

The analysis of the conception rate after estrus induction and synchronization treatments reveals on total insemination and after the first two inseminations with 6,39% and 8,4% higher values in the experimental group compared to control group (37, 50% - lot E vs. 31,11% - lot C and 35% - lot E, vs. 26,6% - lot C), (table 3).

Table 3. The conception rate of cows in the experimental group after treatments for estrus induction and synchronization treatments compared to control group without treatments

Cows inseminated after treatments	Pregnant cows, of which:											
	Total				at I-a IA				at I –a + a-II-a IA			
Lot E	Lot E		Lot C		Lot E		Lot C		Lot E		Lot C	
n	%	n	%	n	%	n	%	n	%	n	%	n	%
40	97,57	15	37,5	14	31,11	7	17,5	7	15,5	14	35,0	12	26,6

By applying this therapeutic protocol to cows for induction and synchronization of estrus, it was found that in 9,75% of cases showed estrus and were artificially inseminated early (in the days of the second administration of GnRH) and in 87,81% of cows did not show estrus, the artificial insemination being performed in fixed time within 18-19 hours after the second administration of GnRH.

This finding is confirmed by other authors who show that between 80 and 90% of the cows treated with this protocol are not observed in the estrus, GnRH injection causing the ovulation before the cow displays signs of estrus, approximately 26-32 hours after the second injection of GnRH (8,10).

Following the studies by Fricke et al., 1998, the ovulation of one dominant follicle, in response to the second GnRH injection occurs in about 85% of highly productive milk cows who receive this protocol.

Some authors have achieved the highest conception rate of cows that have been artificially inseminated within 8-24 hours after the second injection of GnRH, indicating a considerable flexibility in insemination time after the second injection of GnRH, the conception rates being lower only when cows were inseminated after ovulation time.

Analysis of the ovarian profile at the initiation of the estrus synchronization protocol in relation to the gestation status of the cows has shown that the conception rate after treatment depends on the trophic state of the ovaries, the phase of the estrous cycle in which it is applied and the ovarian follicles dimensions Table 4).

The influence of estrous cycle phase in cows at the time of initiation of treatments on the conception rate was also indicated by other authors, who report that the ovulation synchronization rate is higher when Ovsynch is initiated in the middle of the estrous cycle. When the Ovsynch

protocol began on day 5 through day 9 of the estrous cycle, there were several cows (> 90%) that ovulated after the first dose of GnRH. Beginning in the middle of the cycle, Ovsynch led to smaller ovulatory follicles and higher gestation rates. Synchronization rate was 91% when Ovsynch was started on day 1 to 12 of the estrous cycle and 80% when Ovsynch was started on day 1 -22 (17).

Table 4. Ovarian profile at the initiation of the estrus synchronization therapeutic protocol with 2 doses of GnRH + prostaglandin F2α in relation to gestational status after treatments

Ovarian formations at the initiation of insemination therapeutic protocol for cows estrus synchronization	Pregnant cows, at total inseminations	of which: at the first
1-2 large sized follicles (12-18 mm 1-2 medium sized follicles (8-11 mm)	1	1
1 large sized follicles (15 mm) 1 Luteal body	1	-
2 medium sized follicles (8-11 mm) 1 Luteal body	2	-
1-2 medium sized follicles (8-11 mm) 1 Ovarian hypotrophy	1	1
1-2 medium sized follicle (8-11 mm) on both ovaries	2	-
2-4 small sized follicles (4-6 mm) on both ovaries	3	1
2 small sized follicles (4-6 mm) 1 Ovarian hipotrophy	3	1
1 large sized follicles (15 mm) 1 medium sized follicles (10 mm) 2-3 small sized follicles (4-6 mm)	1	1
TOTAL pregnant cows	15	7

The research presented in this paper highlights that this therapeutic protocol based on the combination of injections of GnRH and PGF2α for the induction and synchronization of estrus in cows within the 42-70 days postpartum period has effects of decreasing the average interval between calving and the first postpartum insemination and higher conception rates, compared to the control group.

Although this protocol requires cows to be handled 3-4 times, heat detection are minimized or eliminated, making cow's artificial insemination more practical and economically feasible for dairy cows and increasing the number of cows that are inseminated in the first 60 days after calving.

Conclusions

- Cows who benefited in postpartum by estrus synchronization treatments (experimental group) were artificially inseminated after the second dose of GnRH (with or without signs of

estrus) in 97,56% of cases, of which: 87,81% in according to the protocol at fixed time (18-24 hours) and 9,75% who showed estrus and were artificially inseminated early, (in day of GnRH second administration).

-The average calving -treatment interval in the experimental group was about 52 ± 1,03 days (with variations between 42-70 days).

- The treatment-conception interval in the experimental group had an average value of 32,8 ± 10,6 days (with variations between 1-110 days).

- The conception rate in the experimental group on total inseminations had higher values with 6,39%, and after the first two inseminations with 8,4% compared to the cows in the control group (37,50% - lot E vs. 31,11% - lot C and respectively 35% - lot E vs. 26,6% - lot C).

- By the therapeutic protocol based on the combination of injections of GnRH and prostaglandin F2α for the induction and synchronization of estrus in cows the minimization or elimination of heat detection is made, making artificial insemination of cows more practical and economically feasible for dairy cattle farms and increasing the number of cows which are inseminated within the first 60 days after calving.

This work was conducted under the Sectorial Plan for Research and Development of Ministry of Agriculture and Rural Development for the years 2015-2018 "Agriculture and Rural Development - 2020 ADER", contract number 5.3.2/03.11.2015

Bibliografie

1. Ardelean V., 2008-Procedee de inducere şi sincronizare a estrului la vaci. Medicamentul veterinar / Veterinary drug nr.1
2. Cernescu H., 2004 –„Ginecologie veterinară”. Editura Cecma Partener, Timişoara.
3. Burke, J. M., R. L. de la Sota, C. A. Risco, C. R. Staples, E. J. P. Schmitt, and W. W. Thatcher. 1996- Evaluation of timed insemination using a gonadotropin-releasing hormone agonist in lactating dairy cows. J. Dairy Sci. 79:1385-1393.
4. Fricke, P. M., J. N. Guenther, and M. C. Wiltbank. 1998- Efficacy of decreasing the dose of GnRH used in a protocol for synchronization of ovulation and timed AI in lactating dairy cows. Theriogenology 50:1275-1284.
5. Giordano JO[1], Wiltbank MC, Fricke PM, Bas S, Pawlisch R, Guenther JN, Nascimento AB.- Effect of increasing GnRH and PGF2α dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows. Theriogenology. 2013 Oct 15;80(7):773-83. doi: 10.1016/j.theriogenology.2013.07.003. Epub 2013 Aug 9.
1. Gümen, A., J. N. Guenther, and M. C. Wiltbank. 2003-Follicular size and response to Ovsynch versus detection of estrus in anovular and ovular lactating dairy cows. J. Dairy Sci. 86:3184- 3194.
2. Jobst, S. M., R. L. Nebel, M. L. McGilliard, and K. D. Pelzer. 2000- Evaluation of reproductive performance in lactating dairy cows with prostaglandin F2α, gonadotropin-releasing hormone, and timed artificial insemination. J. Dairy Sci. 83:2366-2372.
3. John B. Hall, 2009- GnRH Based Estrus Synchronization Systems for Beef Cows. Extension Animal Scientist, Beef, Virginia Tech.
4. Lane E.A, Austin E.J., Crowe M.A., 2008- Oestrous synchronisation in cattle—Current options following the EU regulations restricting use of oestrogenic compounds in food-producing animals: A review Animal Reproduction Science 109 (2008) 1–16.
5. Peters, M. W. and J. R. Pursley. 2002- Fertility of lactating dairy cows treated with Ovsynch after presynchronization injections of PGF2α and GnRH. J. Dairy Sci. 85: 2403-2406.
6. Pursley JR, Mee MO, Wiltbank MC-1995- “Synchronization of ovulation in dairy cows using PGF2α and GnRH”. Theriogenology 44:915-923.
7. Pursley J.R., Wiltbank M.C.', Stevenson J.S., Ottobre J.S., Garverick H.A., Anderson L.L. –1997 a- Pregnancy Rates Per Artificial Insemination for Cows and Heifers Inseminated at a Synchronized Ovulation or Synchronized Estrus[1], Journal of Dairy Science, vol. 80.

8. Pursley J.R., Michael R. Kosorok[1], Milo C. Wiltbank, 1997 b- Reproductive Management of Lactating Dairy Cows Using Synchronization of Ovulation, Journal of dairy Science, vol 80.
9. Pursley, J. R., R. W. Silcox, and M. C. Wiltbank. 1998- Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J. Dairy Sci. 81:2139-2144.
10. Srijana Manandhar, 2016- Effect of increased doses of final gonadotropin- realeasing hormone (GnRH) of Ovsynch on luteizing hormone (LH) peak and corpus luteum (CL) function in lactating dairy cows and buffaloes. THESIS - Submitted to Michigan State University in partial fulfillment of the requirements for the degree of Animal Science- Master of Science.
11. Stevenson, J. S., Y. Kobayashi, and K. E. Thompson. 1999- Reproductive performance of dairy cows in various programmed breeding systems including OvSynch and combinations of gonadotropin-releasing hormone and prostaglandin F2α. J. Dairy Sci. 82:506-515.
12. Vasconcelos, J. L. M., R. W. Silcox, G. J. Rosa, J. R. Pursley, and M. C. Wiltbank. 1999- Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078

Vaginal smear, progesterone levels, and ultrasound examination of the ovaries as methods of determining the moment of ovulation in bitches comparative study

G. OTAVĂ, C. MIRCU, Violeta IGNA, Simona MARC ZARCULA, D. LO PRESTI
Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Banat „King Michael I of Romania" (USAMVB), Timisoara – 300645, Calea Aradului, nr. 119, gabiotava@yahoo.com

Abstract

Researches in the scientific literature reveal that the study of vaginal citology and interpretation of progesterone values do not represent certain methods to determine the ovulation time. 6 different breed have been investigated in this study (Labrador Retriever, Tibetan Mastiff, Bichon Maltese, West Highlander White Terrier and the Bucovina Shepherd). All es were monitored in terms of cyto-vaginal smear, the P4 level and ultrasound examination to determine the ovulation moment. The rapid disappearance of the follicular antrum cavity, correspondent to ovulation, was detected only with two. Although ultrasound changes during the estrous cycle were well-studied, the exact ovulation moment cannot be predicted accurately. To optimize the results of determining the ovulation moment it is recommended to collate the ultrasound examination with at least one of the other two methods of investigation.

Key words*: ovulation time, bitch , ultrasound*

Introduction

Bitches are mono-estrous animals with a single reproductive cycle. Because ovulation occurs once or twice a year and due to the very high variaty among individuals within the same breed and among breeds, it is very difficult to set a very accurate method for ovulation timing. Imagining and developing a non-invasive method of monitoring follicular development and ovulation is necessary both for research and for clinical practice. Development of some high-performance ultrasound apparata has led to substantial progress in this area lately.

Until now, the ovulation moment has been set by collating the two methods of medical investigation- determinating the progesterone level and studying the vaginal cytology . The study of literature concerning the determination of the ovulation moment by determining the progesterone level reveals vastly different data. Thus, progesterone values ranging from 4 to 10 ng/ml are set through clinical studies. In this study we wanted to obtain information about the effectiveness and coordination of the three methods of determining the ovulation moment.

Materials and methods

Six different breeds have been monitored in this study (Labrador Retriever, Tibetan Mastiff, Bichon Maltese, West Highlander White Terrier and the Bucovina Shepherd). Taking into account the very large differences among individuals of the same breed and among dog breeds,regarding the ovulation moment opposite the sexual cycle, pet owners were advised to bring the females for monitoring the estrous phase of the sexual cycle when the blood leak diminished , both chromatically and quantitatively. Thus, the owners were not called according to a fixed interval from the sexual cycle onset.

An attempt was made to investigate the vaginal cytology, the progesterone levels and ultrasound appearance of the ovaries in the females. To perform the cytovaginal smear, I needed a rod of wood to which a small amount of cotton wool was attached by rotating the rod. This improvised item was introduced into the vaginal vestibule on a nearly vertical direction from the bottom up, after a previous distance of vulvar lips performed by the same vet tehnician with the

opposite hand. After penetration into the vaginal vestibule the rod is placed in horizontal position in order to set its tip into the anterior vagina.

According to the literature there are differences concerning the interpretation based on cytovaginal smear in es between the vaginal vestibule or posterior vagina and anterior vagina. It is recommended to avoid the clitoris fossa which contains fragments of keratinizated cells which can be confused with the superficial epithelial cells obtained from the during the estrus period. Once the rod inside the vagina the vet tehnician makes movements of rotation in the opposite direction to the direction of rotating the cotton wool. One should be aware of this fact because there is a risk of cotton wool to get detached from the rod. The sample taken was displayed on a glass blade by rolling over the wool part of the rod. The smearl dyeing was made using the Diff Quick method. The examination of cytovaginal smear was done by help of the Leica microscope owned by CLC Horia Cernescu.

Preparing the animal for the blood sampling began with shaving the middle area of the forearm and antisepting it with betadine solution 4%. The blood samples for determining the progesterone level in blood was done by stinging a needle attached to a syringe into the vena cefalica antebrahiala. A quantity of 1- 2.5 ml of blood was taken.

The blood taken was transferred into a vacuum container which does not contain anticoagulant substances . The container was labelled with the owner and pet IDs. The sample thus obtained was sent, as soon as possible, at Bioclinica laboratory. The determination of the progesterone level was done by means of the chemiluminiscence method, using the Siemens Advia Centaur device. The value obtained was expressed in mmoles/l and nanograms per milliliter.

Preparing the female for the ovaries ultrasound examination consisted in trimming or shaving the flank area ,_covering the transverse processes of the lumbar vertebrae 3 and 4. The female will be positioned in lateral or dorso-lateral decubitus, either left or right depending on the ovary to be viewed. There are some situations in which, due to the presence of certain portions of the intestine in the proximity of the ovary, viewing it is more difficult. In such situations, the females can be examined in upright position. It is worth mentioning that the animal being shaved is absolutely mandatory in order to have good quality images, so it should not be left to the discretion of the owner. Many owners refuse to have the animal shaved, due to aesthetic reasons. The echography of the ovaries should be performed after a 12-hour water diet to have an empty intestin.

After applying the gel on the freshly shaved area, an attempt was made to visualize the kidney caudal pole. This represents a landmark in the case of the examination of the ovaries because they are close to the kidney. The ovary is positioned caudo-laterally towards the distal portion of the kidney. An anatomical peculiarity of female genital apparatus, namely being positioned more cranially to the kidney compared to the left kidney. Thus, the right ovary is positioned more cranially compared to the left one. The echography of the ovaries should start with the left ovary, since it is easier to locate. It should be also taken into account the fact that the ovaries are located superficially, very close under the skin. The ultrasound examination was done using a My Lab 50 Esaote, Betford Hills, NY, USA apparatus equipped with a 3-9 MHz frequency convex probe set to mode B, property of CLC Horia Cernescu..

Results and discussions

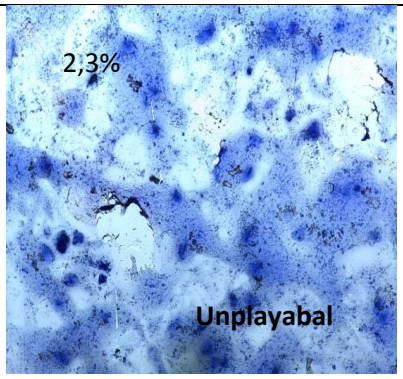
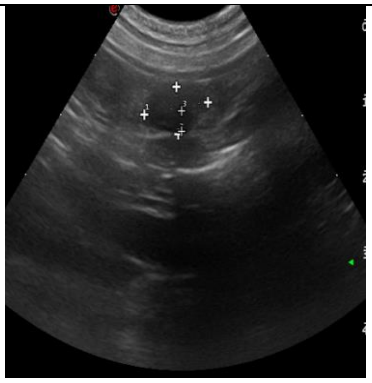
Please note that the study has been negatively impacted by a number of factors such as: the reduced availability of owners to be present at the clinic following the shedule established, the anxiety of the bitches that caused tachypnea and the tissues, and the hollow/cavitary organs containing gas.

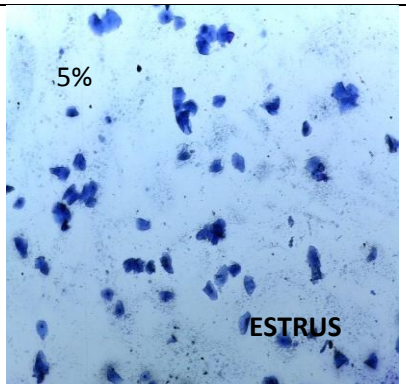
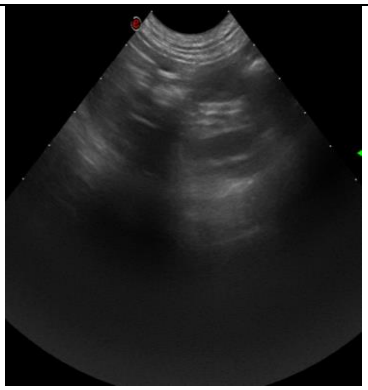
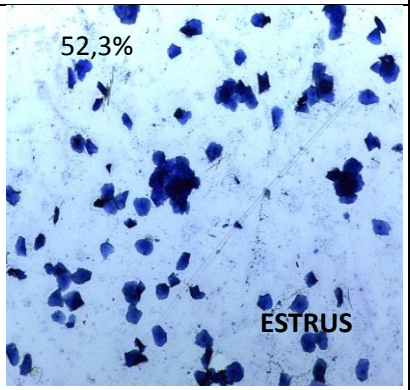
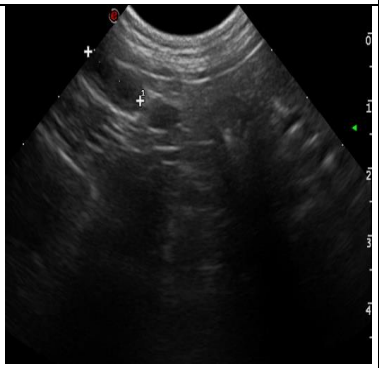
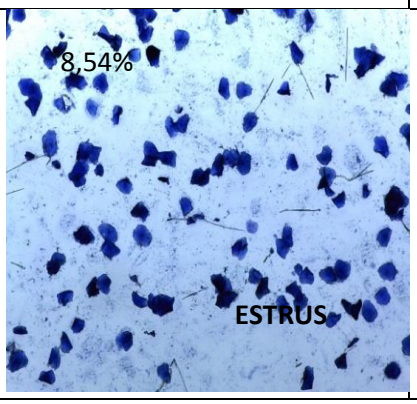
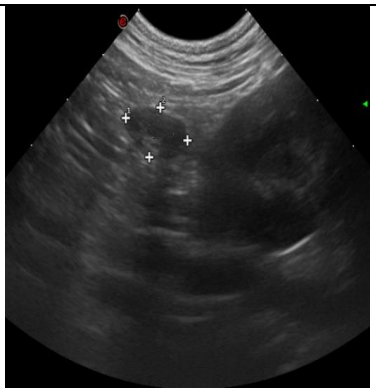
The Bucovina Shepherd beatch was investigated twice in a span of two days. At the first medical investigation the progesterone level was 1.9 ng/ml and the image of the cyto-vaginal smeard was impossible to be interpreted due to the abundant presence of the estrus mucus. The second investigation showed that the superficial cells with no nucleus of all keratenized cells cheratinizate were around 72%. According to data in the scientific literature , at the percentage of cells without nucleus of 72 per cent, the female is supposed to be on the everge of ovulation, but the progesterone level of 2.5 ng/ml does not indicate this fact. The data in the literature show a very large range (4-10ng/ml) of the progesterone levels at the time of ovulation. Related to the percentage of cells with no nucleus, ENGLAND (3) consider that it is supposed to be 75, at the ovulation moment. This demonstrates that the percentage of the keratenized cells in the cyto-vaginal smear used to determine the moment of ovulation is not a good method.

Concerning the Labrador Retriever beatch, the oestrus cycle showed a percentage of 16 cells with no nucleus. The ovary follicles had an even appearence, with thickened walls, feature specific to the follicules before ovulation. Thus, in this situation, the two medical investigation collate.

The Tibetan Mastiff female was investigated according to the cyto-vaginal smear, the progesterone level and through the ultrasound method. In this particular situation, there is no discrepancy, meaning that the ultrasound results, the percentage of the superficial cells with no nucleus and the progesterone levels showed that the famale did not ovulate. The sonograph pointed that on the ovary monitoredthere are 6 follicles.

Tabel 1. The connection among the oestrus cycle, the P4 level and the ultrasound results in a West Highlander White Terrier bitch

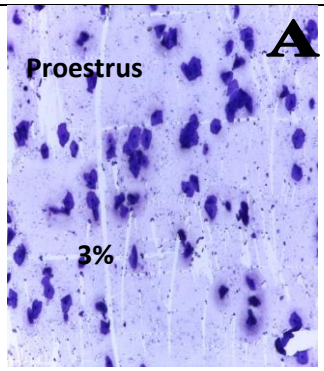
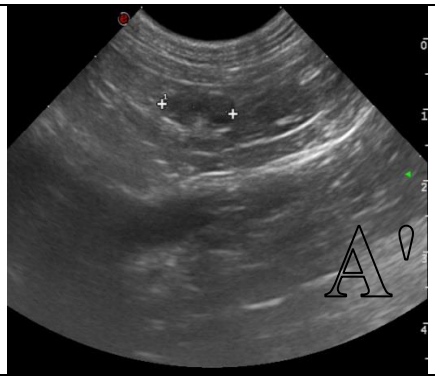
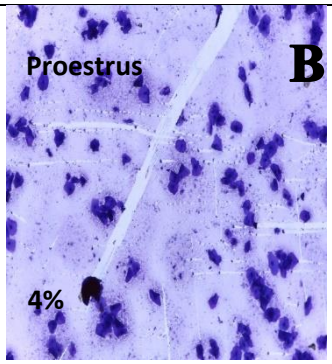
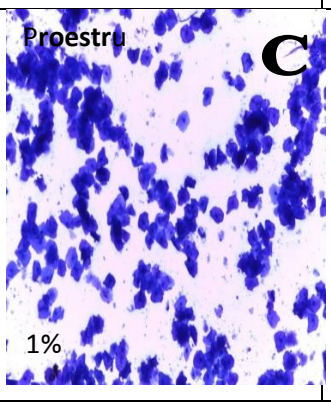
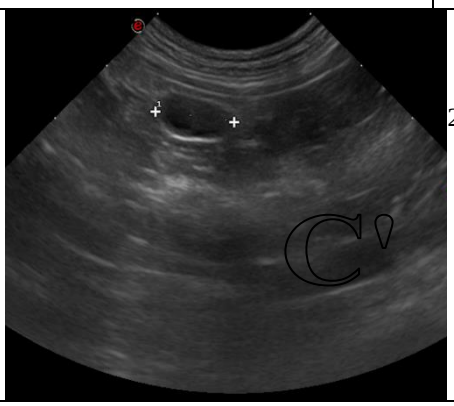
Data	The cyto-vaginal smear/The procentage of the superficial no nucleus cells	The ultrasound appearance of the ovaries	aloare nivel P_4
10.11.2015			1,1 ng/ml

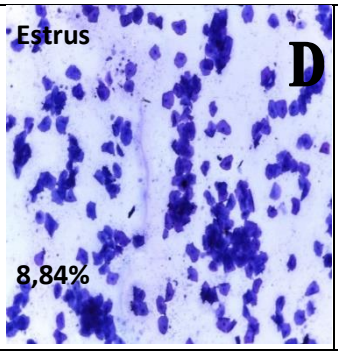
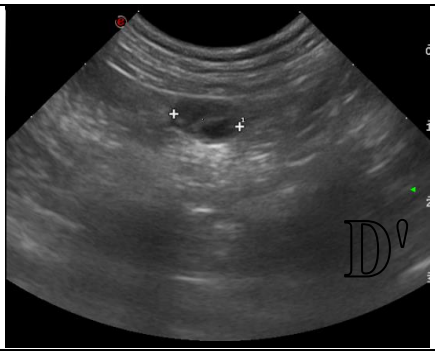
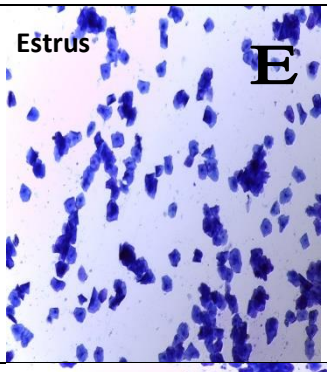
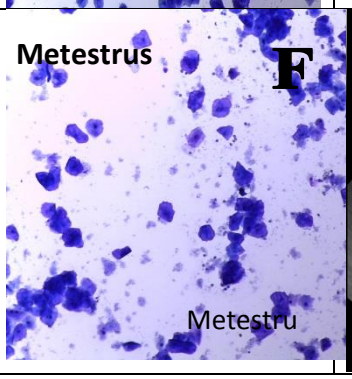
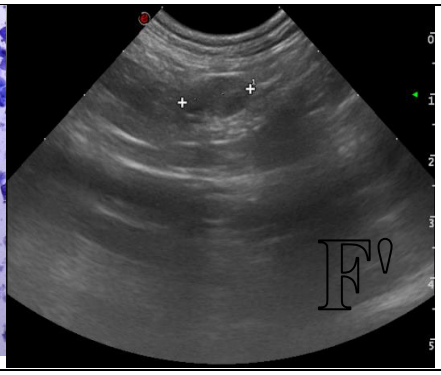
3.11.2015			0,73 ng/ml
7.11.2015			2,59 ng/ml
9.11.2015			6,4ng/ml

The West Highlander White Terrier female was investigated, using all the three methods, 4 times, every 2-4 days, according to its evolution concerning the sexual cycle. Even if the first cyto-vaginal smear was impossible to interpret (Table 1), speaking from the cytology point, the female had an ascending evolution regarding the cells without a nucleus. The same trend was found in the progesterone level. The beatch was at a basic/basal level (values between 0.73 ng/ml and 2.59 ng/ml) during the first three days of the investigation. On the last day, it was noticed that the progesterone value of 4ng/ml (value which most studies consider is an indicator for ovulation) collated neither with theultrasound image of the ovaries, nor with the vaginal cytology, . We would have expected that at a progesterone value of 6,4 ng/ml, the procentage of the cells with no nucleus to be 75%, (ENGLAND and LEVY) (2, 3) and the follicles to be well-defined.. Ultrasound follicles appearance was, however, one specific to the preovulation follicles.(Table 1). This reveals at least

a fact, namely, at a progesterone value of 6/ml, 4ng , the female did not ovulate. We consider that, out of the three methods of investigation, the ovaries ultrasound exemination brings the most precious information about the time of ovulation. The data in the scientific literature on progestrerone level at which ovulation occurs, are vastly different ranging from 4-10 ng/ml (5).

Table 2. Connection among estrous cycle, the P4 level and the ovaries ultrasound appearance in a Bichon Maltese bitch

Data	The cyto-vaginal smear/The procentage of the superficial no nucleus cells	The ultrasound appearance of the ovaries	P4 value ng/ml
3.06.2016			–
5.06.2016		—	–
6.06. 2016			2,52

8.06.2016			2,32
10.06.2016			5,39 g/ml
3.06.2016			20,24 ng/ml

The Bichon female was investigated, using all the three methods, six times, every 1-3 days, according to its evolution concerning the sexual cycle

Speaking from the cytology point, the female had an ascending evolution regarding the cells without a nucleus. From one medical exam to another, the percentage of the cells with no progressive nucleus grew. According to Table 2, when the cells with no nucleus were 23,68%, the progesterone level was 5,39 ng/ml, very close to ovulation time.. On the penultimate day of the investigation the female was exemined only hormonally and cytologically, because we had no access at the ultrasound apparatus at the weekend. On the last day of the examination, the female had a smear specific to the metestrus period beginning, and the P4 level was 20,24 ng/ml.

The data in the scientific literature show that considering the percentage of cells with no nucleus out of all keratenized cells in order to determine the ovulation time, envolves great difficulty and it is subjected to errors of interpretation. This fact is due to the cyto-vaginal smear being uneven. Thus, the percentage of keratinizated cells with no nucleus differs from one image to another.

The evolution of the progesterone levels was normal relative to the folllicular development and cytology. Thus, the female had a basal/ basic P4 level until about 3 days before ovulation. About 2 days after ovulation the P4 level was 20.24 ng/ml. The P4 value in a certain period of time after ovulation differs greatly from one bitch to another and it might be due to the number of ovarian follicles and to the number of corpus luteum. Thus, a larger number of corpus luteum will cause a higher and sudden progesterone level.

In terms of the ultrasound results, the ovarian follicles had an ecogenous appearance to the last medical examination . Since the last examination, a change in the ovaries appearance could be noticed- they turned hypo-ecogenuous. Also, the ovaries form evolved from a round shape at the beginning of the monitoring (A ', C ') to elliptical form (D ') at the end of it.

We noticed that the females belonging to large breeds and the obese ones, the exemination using the echography/ultrasound apparatus is performed with greater difficulty, situation reported also in the scientific literature (5). The easiest ultrasound examination can be done during the follicular sexual cycle. (proestrus and the estrus). During the proestrus cycle, the ultrasound appearance of the ovaries show regular and ecogenous shape, having 6-9 mm, with very thin follicula wall. The follicles grow progressively from the early follicular phase until its end. With the approaching of ovulation time, the preovulation follicle thickens its wall up to 1mm. According to the informtion in the literature a preovulating follicle can change its shape becoming flat, situation that the current study did register. At the ovulation time the follicular cavity dissapear (follicular collapse).

There is information in the literature that in half of the cases, after ovulation, soem hypo-ecogenous sstructures are still be found inside the ovary (8). These structures are different from the preovulation follicles being smaller and irregularly shaped (9).

It is rather difficult to assess the number of follicles according to the literature (5). These researchers notice that 45 % of the follicles do not ovulate. These round ecogenous structures can be seen up to three days after ovulation. This situation may mislead an inexperienced examiner. Another structure that may mislead the vet is the liquid present between the ovary and ovarian bursa, situation that can occur in 40% of cases (1, 4). A day after the ovulation, there is a blood accumulation in the follicular cavity that leads to the appearance of the hemorrhagic corpus luteum. They have a structure similar to the preovulation . It is therefore very important that the ovaries examination to take place daily in order to show the exact ovulation moment.

According to LEVY and FONTBONNE (5), detecting the ovulation moment by ultrasound investigation is just 10 percent more accurate compared to the progesterone level determination. Setting the ovulation moment is imperiously needed especially if the artificial insemination is done with chilled or frozen semen, or if the animal is susceptible of infertility (6).

The bitch ovaries are difficult, and sometimes impossible to be examined through ultrasonography, because they are small, difficult to be differentiated from the surrounding tissues and are often obscured by the intestinal gas (3). The rapid disappearance of the follicular cavity, correspondent to the ovulation , was detected only in two bitches. Although ultrasound changes during the estrous cycle were well-studied, the exact moment of ovulației cannot be predicted (6).

Conclusions

- Examining the ovaries in the follicular phase of the sexual cycle by ultrasound device is easier in the situation of a small or medium –sized female than a large one.
- The ultrasound examination is the most certain method out of the three ones described.
- Even if the sudy faced some non-ideal situations, we can conclude that the ovaries ultrasound examination is a valuable method to predict the ovulation moment in bitches.

- To optimize the results of determining the ovulation moment it is recommended to collate the ultrasound examination with at least one of the other two methods of investigation.

References

1. DOW, C. (1990) - Ovarian abnormalities in the -Journal of Comparative Pathology70,59-69.
2. ENGLAND, G.C., CONCANNON, P.W. (2003) -Determination of the optimal breeding time in the : basic considerations.In: Recent advances in small animal reproduction. Ithaca, NY: International Veterinary Information Service,2003 Disponible en www.ivis.org.
3. ENGLAND, G.C., YEAGER, A., CONCANNON, P.W. (2003) - Ultrasound imaging of the reproductive tract in the . In: recent advances in small animal reproduction. Ithaca, NY: International Veterinary Information Cervice.
4. GINTHER, O.J. (2007) - Ultrasonic imaging and Animal reproduction-color-doppler Ultrasonography
5. LEVY, X., FONTBONNE, A. (2007) - Determining the optimal time of mating in es:particularities- Rev Bras Reprod Anim, Belo Horizonte, v.31, n.1, p.128-134, jan./mar. 2007. Disponível em *www.cbra.org.br*.
6. MCENTEE, K. (1990) - Cysts in and around the ovary-In Reproductive Pathology of Domestic Mammals-San Diego,Academic Press, pp52-68
7. NYLAND, T.G., MATTOON, J.S. (2002) - Small Animal diagnostic ultrasound, second edition
8. RASKIN R.E., MEYER D.J(2001) - Atlas of canine and feline cytology. Philadelphia, WB Saunders co, 2001, pp. 277-312.
9. VERMEULEN, M.A.E. (2009) - Ovarian color-Doppler ultrasonography to ovulation in the - Research project veterinary Medicine Lousiana State University.

Effect of rozmarinic acid supplementation on *in vitro* maturation of bovine oocytes

Simona MARC, Camelia TULCAN, Oana BOLDURA, A. SOLONAR, G. OTAVĂ, G. GODJA, I. HUȚU, C. MIRCU
University of Agricultural Sciences and Veterinary Medicine of Banat "The King of Michael I" of Romania, Faculty of Veterinary Medicine, Timisoara, Romania
calinmircu@usab-tm.ro

Abstract

Antioxidants supplementation of in vitro culture media exerts the key role to reduce the effects of reactive oxidative species produced during assisted reproduction technique. The objective of the study was to determine the effect of rosmarinic acid addition to the in vitro culture media on bovine oocytes maturation rate based on morphological changes. Bovine COC's were matured according to their morphological class (class I, II and III) in two groups: control (M) and supplemented with rosmarinic acid (105 µM, AR) in TCM 199 HEPES modification media at 38.5^{0}C in 5% CO_2 humidified air atmosphere for 24h. Comparing the groups, relative to the number of COC's matured, a increase in their maturation features is observed, with 26.81% % (AR1), 21.67% (AR2) and 23.34% (AR3), respectively in groups supplemented with rosmarinic acid. The oocyte class is associated with their capacity to develop in vitro based on their morphological examination.

Key words*: antioxidants, oocyte, rosmarinic acid*

Introduction

In vitro fertilization (IVF) is an assisted reproduction technique (ART) used with good results in bovine reproduction, with 443.533 of bovine embryos obtained worldwide in the year 2012 according to statistics of the International Embrio Transfer Society (http://www.iets.org/pdf/comm_data/december2013.pdf)

Successful ART is influenced by many factors, among which reactive oxygen species (ROS) has a significant role (Agarwal et al., 2014).

Sources of ROS during ART procedures could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Agarwal et al., 2014).

Reproductive systems possess antioxidant defense mechanisms that maintain equilibrium between pro- and anti-oxidants (Roychoudhury et al., 2017; Agarwal et al., 2014); but during *in vitro* conditions, the gametes needs to be protected by supplemented antioxidants.

Studies indicates that supplementing maturation media with different antioxidants such as β-mercaptoethanol (Sadeesh et al., 2014), cysteamine (Beheshti et al., 2011); cysteine (Mircu et al., 2015), vitamin C (Sovernigo et al., 2017; Comizzoli et al. 2003; Agarwal et al., 2014), plant antioxidants – flavonoids (Kang et al., 2016, Mbemya et al., 2017) can improve oocytes maturation based on nuclear morphological changes and on gene expression.

Another natural antioxidant used in ART, especially in freezing extenders were improves sperm quality after cryopreservation, is rosmarinic acid (Malo et al., 2010; Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid derived from hydroxycinnamic acid, that belongs to polyphenols group and is found as an active compound in several medicinal plants (*Rosmarinus officinalis, Salvia officinalis, Mentha arvense, Ocimum basilicum, Thymus vulgaris* etc) (Krajcovicova et al, 2013).

Rosmarinic acid has antiinflamatory, antiviral, antibacterial, antimutagen, antidepressant, antiallergic, antioxidant effects. His antioxidant activity is supporting by enhancement of superoxide and hydroxyl scavenging (Krajcovicova et al, 2013).

Hajhosseini et al. (2013) observed that rosmarinic acid has a preventive effect on Sertoli cells apoptosis caused by electromagnetic fields.

Although in literature are data regarding beneficial effects of green tea polyphenols (Wang et al., 2007; Ly et al., 2015) and catechins (Roychoudhury et al., 2017) on reproductive health and on IVF parameters and subsequent development, there are no studies, to our knowledge, regarding the effect of rosmarinic acid supplementation on bovine oocytes *in vitro* maturation. For this reason the purpose of this present research was to evaluate the effect of rozmarinic acid added in media for in vitro bovine oocytes maturation on their maturation rate based on morphological changes.

Materials and methods

Bovine ovaries (n=18) were collected from local slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep), at 33-35^{0}C within two hours. Handling medium for COC (cumulus -oocytes- complexes) was Dulbecco-PBS (D-8662) supplemented with 100 µl Pen/Strep (17-602F, Lonza); 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021, Sigma-Aldrich). COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe.

Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^{0}C): *Ist class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, *IInd class* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III^{d} class* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm).

The maturation culture medium was prepared in our laboratory after Parrish et al. (1986) protocol with minor modifications: TCM 199 HEPES modification media, (M2520) with 10% ECS and 15 µl FSH (F8174, Sigma-Aldrich) - *group M* (control), in experimental group we added rosmarinic acid (105 µM)(536954, Sigma Aldrich) - *group AR*. Pools of 8-10 COCs were maturated in 400µl media in 4 well dishes (Nunc, Germany) covered with mineral oil at 38.5^{0}C in 5% CO_2 humidified air atmosphere for 24h. After 24h of culture, all COC were examined for maturation, signs as expansion and mucification of cumulus cells were observed. The COC's were maturated according to there their morphological class (M1, M2, M3, AR1, AR2, AR3).

Results and discussions

The results of supplementation of *in vitro* media with rosmarinic acid on bovine oocytes morphological aspects are presented in figure 1 and 2.

After *in vitro* maturation of cow's oocytes in the medium without antioxidants (M group) we noticed at the morphological assessment that 55% of class I COCs (M1), 53.33% of class COC II (M2) and 26.66% of class III COCs (M3) were matured. In the rosmarinic acid supplemented groups (AR group), 81.81% of class I COCs (AR1) were matured after 24 hours, 75% of class II (AR2) and 50% of class III (AR3).

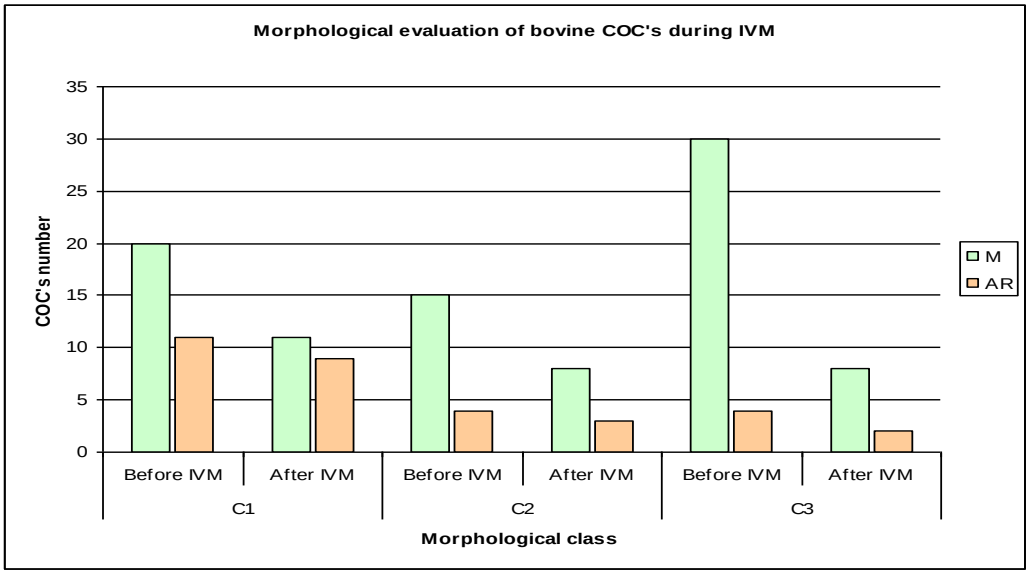


Figure 1. Morphological evaluation of bovine COC's before and after IVM

Comparing the groups, relative to the number of COC's matured, a increase in their maturation sign is observed, with 26.81% % (AR1), 21.67% (AR2) and 23.34% (AR3), respectively. Regardless of the treatment applied, the oocyte class is associated with their capacity to mature *in vitro* based on their morphological examination.

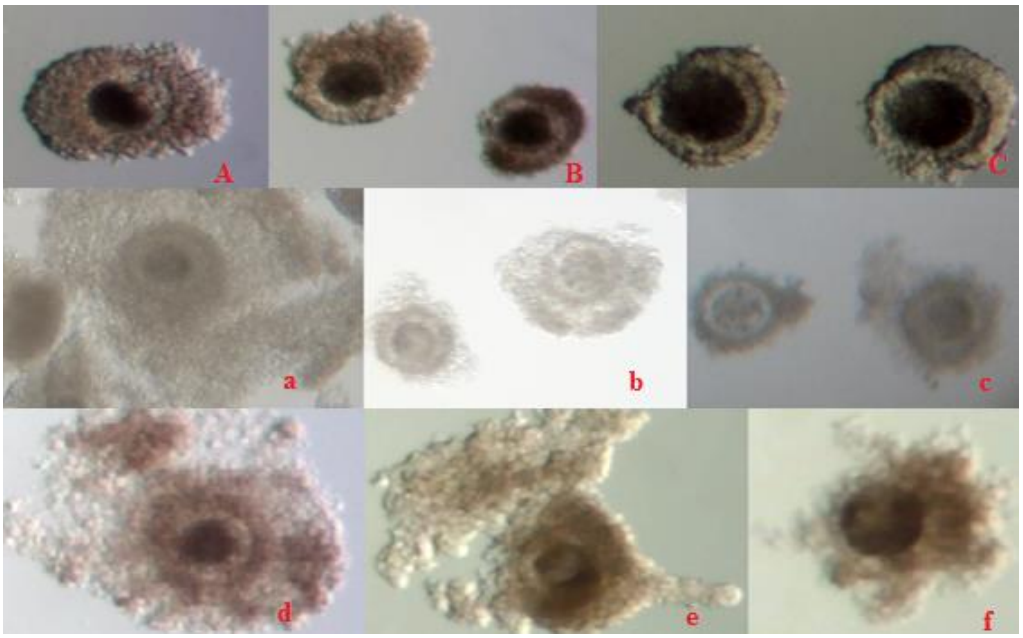


Figure 2. Aspects of COC's classification according to their morphological class and experimental group before IVM (A – Ist class, B – IInd class, C – III^{d} class), after IVM, group M (a, b, c), group AR (d, e, f)(5X)

These results are sustained also by BAX/BCL2 gene expression (unpublished data), where we observed that BCL-2 (anti-apoptotic gene) had higher levels in Ist class COC's from rosmarinic acid (AR1) groups compared with the other groups and BAX (pro-apoptotic gene) level is indirectly proportional with the quality of the oocyte, with the highest level in III^{d} class oocytes, what it means that both antioxidant supplementation and the quality of the oocyte has an important role in maintaining cellular viability.

Oxidative stress has negative effects on *in vitro* gametes and embryos (Agarwal et al., 2014; Beheshti et al, 2011) and excessive ROS production can't be controlled properly by the mammalian cells antioxidant systems (superoxide dismutase, glutathione system, thioredoxin system, catalase, thiol compounds) that scavenge ROS or prevents its formation due to the multiple potential sources of ROS, lack of physiological defense mechanisms etc (Sadeesh et al., 2014; Lu et al., 2013; Agarwal et al., 2014). That's why it's important to add antioxidants in media used in ART procedures (Sadeesh et al., 2014; Beheshti et al., 2011; Mircu et al., 2015; Sovernigo et al., 2017; Kang et al., 2016, Mbemya et al., 2017).

From literature data we know that rosmarinic acid antioxidant effects protects ovine spermatozoa during lyophilization by maintaining the sperm DNA integrity and after reconstitution of the freeze-dryed spermatozoa, they can sustain fertilization and even embryonic development (Olaciregui et al., 2017). Also in boar semen cryopreservation rosmarinic acid it is used as an antioxidant where improves the post-thaw quality of spermatozoa and the ability to fertilize (Malo et al., 2010; Luno et al., 2014; Luno et al., 2015).

Our preliminary results suggests that antioxidant properties of rosmarinic acid is effective also on bovine oocytes *in vitro* maturation. Further studies are needed to clarify the effects of rosmarinic acid used during IVM on further steps of IVF technique.

Conclusions

- Supplementation of the cow's oocyte culture media with rosmarinic acid can determine a higher quantity of bovine oocytes matured *in vitro* based on morphological evaluation.
- Quality of the COC used for *in vitro* techniques has an important role in the success of the experiment.

Aknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equipped through POSCCE 2669 program

References

1. AGARWAL, A., DURAIRAJANAYAGAM, D., PLESSIS, S. (2014) - *Utility of antioxidants during assisted reproductive techniques: an evidence based review*, Reproductive Biology and Endocrinology, 12, 112, https://doi.org/10.1186/1477-7827-12-112.
2. BEHESHTI, R., MOHAMMADI-ROSHANDEH,A., GHALEHKANDI GIASI, J., GHAEMMAGHAMI, S., HOUSHANGI, A.F. (2011) - *Effect of antioxidant supplements on in vitro maturation of bovine oocyte,* Advances in Environmental Biology, 5, 7, 473-1475.
3. COMIZZOLI,P., WILDT,D.E. PUKAZHENTHI, B.S. (2003) - *Overcoming poor in vitro nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season*, Reproduction, 126, 809-816.
4. HAJHOSSEINI, L., KHAKI, A., MERAT, E., AINEHCHI, N. (2013) – *Effect of rosmarinic acid on Sertoli cells apoptosis and serum antioxidant levels in rats after exposure to electromagnetic fields*, Afr J Tradit Complement Altern Med., 10, 6, 477-480.
5. KANG, J.T., MOON, J.H., CHOI, J.I., PARK, S.J., KIM, S.J., SAADELDIN, I.M., LEE, B.C. (2016) - *Effect of Antioxidant Flavonoids (Quercetin and Taxifolin) on In vitro Maturation of Porcine Oocytes,* Asian Australas. J.Anim.Sci., 29, 3, 352-358.

6. KRAJCOVICOVA, ZDENKA, MELUS, V. (2013) – *Bioactivity and potential health benefits of rosmarinic acid*, University Review, 7, 2, 6-14.
7. LU, J., HOLMGREN, A.(2013) – The thiroredoxin antioxidant system, Free radical biology and medicine, 66,8
8. LUÑO V, GIL L, OLACIREGUI M, GRANDIA, j., ANSO, T., DeBLAS, I (2015) - *Fertilisation rate obtained with frozen-thawed boar semen supplemented with rosmarinic acid using a single insemination timed according to vulvar skin temperature changes*, Acta Veterinaria Hungarica, 63, 1, 100-109.
9. LUÑO V, GIL L, OLACIREGUI M, GONZÁLEZ N, JEREZ RA, DE BLAS I. (2014) - *Rosmarinic acid improves function and in vitro fertilising ability of boar sperm after cryopreservation*, Cryobiology, 69, 1, 157-62.
10. LY, CHRISTINA, YOCKELL-LELIEVRE, JULIEN, FERARO, Z.M., ARNASON, J.T., FERRIER, J., GRUSLIN, A. (2015) – *The effect of dietary polyphenols on reproductive health and early development*, Human Reproduction Update, 21, 2, 228-248.
11. MALO, C., GIL, L., GONZALEZ, N., MARTINEZ, F., CANO, R., DE BLAS, I., ESPINOSA, E. (2010) – *Anti-oxidant supplementation improves boar semen characteristics and fertility after cryopreservation: comparison between cysteine and rosemary (Rosmarinus ooficinalis),* Cryobiology, 61, 1, 142-147.
12. MBEMYA, G.T., VIEIRA, L.A., CANAFISTULA, F.G., LOIOLA PESSOA, O.D., RIBEIRO RODRIGUES,A.P. (2017) – *Reports on in vivo and in vitro contribution of medical plants to improve the female reproductive function,* Reprod Clim, (http://dx.doi.org/10.1016/j.recli.2016.11.002)
13. MIRCU C., BOLDURA OANA, IGNATIADI AIKATERINI, RAȚIU ANA-MARIA, HUȚU I., POPESCU SORINA, TULCAN CAMELIA, AHMADI MIRELA, BONCA GH., MILOVANOV CORNELIA (2015) – *Effect of cysteine supplementation on sow cumulus cells and on BCL2 gene expression during in vitro maturation*, Lucr Stiin Med Vet USAMV Iasi, Ed."Ion Ionescu de la Brad", 58, 292-297.
14. OLACIREGUI, M., LUNO, VICTORIA, DOMINGO, PAULA., GONZALEZ, NOELIA, GIL, LYDIA (2017) – *In vitro developmental ability of ovine oocytes following intracytoplasmatic injection with freeze-dried spermatozoa*, Scientific reports, 7, 1096, DOI:10.1038/s41598-017-00583-0, www.nature.com/scientificreports.
15. PARRISH J.J., SUSKO-PARRISH J.L., LEIBFRIED-RUTLEDGE M.L., CRITSER E.S., EYESTONE W.H., FIRST N.L**.** (1986) -*Bovine in vitro fertilization with frozen-thawed semen*, Theriogenology, 25, 591–600.
16. ROYCHOUDHURY, S., AGARWAL, A., VIRK, G., CHO, C-L**.**, (2017) - *Potential role of green tea catechins in the management of oxidative stress-associated infertility**,*** http://dx.doi.org/10.1016/j.rbmo.
17. SAADESH, E., SHAH, F., BALHARA, A.K., THIRUMARAN, S. M. K., YADAV, S., Yadav, P. S., (2014) **-** *Effect of growth factor and antioxidant on in vitro maturation of oocytes and cleavage rates of in vitro produced Indian buffalo (Bubalus bubalis) embryos*, Veterinarski Arhiv, 84 ,5, 459-474).
18. SOVERNIGO, T.C., ADONA, P.R., MONZANI, P.S., GUEMRA, S., BARROS, F., LOPES, F.G., LEAL, C (2017) – *Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production*, Reproduction in Domestic Animals, 52, 4, 561-569.
19. WANG, Z.G., YU, S.D., XU, Z.R. (2007) – *Improvement in bovine embryo production in vitro by treatment with green tea polyphenols during in vitro maturation of oocytes*, Animal Reproduction Science, 100, 1-2, 22-31.
20. http://www.iets.org/pdf/comm_data/december2013.pdf

Evaluation of sows oocytes viability through Trypan Blue staining after vitrification

Simona MARC, C. MIRCU, Nicoleta CREȚAN, G. OTAVĂ, Camelia TULCAN, I. HUȚU
University of Agricultural Sciences and Veterinary Medicine of Banat "The King of Michael I" of Romania, Faculty of Veterinary Medicine, Timisoara, Romania
E-mail address: calinmircu@usab-tm.ro

Abstract

Along sperm and embryo cryopreservation that are used routinely also in animal assisted reproduction, studying are done also on animal oocyte cryopreservation in order to find the best conditions to preserve their viability. Vitrification is one of the methods that can be used in order to preserve oocytes. The higher reactive oxygen species that are formed during in vitro conditions can influence the success of assisted reproduction technique. The aim of this study was to evaluate the antioxidant potential of ascorbic acid (0.5mM) and rosmarinic acid (105µM) added in media for in vitro maturation on swine oocyte subjected to vitrification. COC's viability after vitrification was evaluated by 0.02% Trypan Blue staining. Comparing experimental groups C (vitamin C) and AR (rosmarinic acid) with group M (control), relative to the number of vitrified oocytes, a slight increase in their viability is observed, with 16.67% (C, class I) and 33.33% (AR, class II), respectively. Regardless of the treatment applied, the oocyte class is associated with viability (p = 0.048). Due to low number of oocytes used in each group we can concluded that supplementation of oocyte maturation media before vitrification with rosmarinic acid and ascorbic acid could produce a slight increase in viability.

Key words*: antioxidants, oocyte, vitrification*

Introduction

Vitrification of oocytes and embryos is a revolutionary cryopreservation technique used both in human (Konc et al. 2014) and in animals (Somfai et al. 2014; Spricigo et al., 2015; Yang et al. 2002; El-Sokary et al., 2013) assisted reproduction techniques (ART) performed in diferent conditions and with different results.

Vitrification is defined as the ultrarapid solidification of a solution by an extreme elevation in viscosity at low temperatures without ice crystal formation (Konc et al., 2014).

The main causes of cell death during cryopreservation is ice crystal formation and toxic concentrations of solutes. In order to reduce the negative effects of cryopreservation, cryoprotective additives (CPA) are used. They are: *intracellular/membrane-permeating* (propyleneglycol, dimethyl sulfoxide, glycerol, ethylene glycol) and *extracellular* (sucrose, trehalose, glucose, amid, ficoll, proteins and lipoproteins). The first ones displaces water via an osmotic gradient and partly occupies the place of the intracellular water, while the extracellular cryoprotective additives increases the extracellular osmolarity generating an osmotic gradient across the cell membrane supporting the dehydration of the cell before cryopreservation and also prevents the rapid entry of water into the cell after thawing. During vitrification cells are dehydrated before the ultrarapid cooling by high concentration of CPA (Konc et al., 2014; Yang et al., 2002).

Oocytes are very sensitive to cryoprotectants used during cryopreservation protocols. Although vitrification of matured porcine oocytes has high survival rates, obtaining embryos by IVF or ICSI from them is difficult (Somfai et al., 2014). Cooling/warming processes from vitrification technique of porcine oocytes at MII stage determined accumulation of reactive oxygen species (ROS), parthenogenetic activation and spindle abnormalities (Somfai et al., 2014).

Sources of ROS during ART procedures could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous

environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Mbemya et al., 2017; Agarwal et al., 2014). When is an imbalance between reactive oxygen species (ROS) and a biological system's ability to readly detoxify the reactive intermediates or repair the resulting damage, oxidative stress apears (Roychoudhury et al., 2017).

Because during *in vitro* conditions oocytes are separated from the body and do not benefit from maternal antioxidant protection, supplemented antioxidants are needed. Studies indicates that supplementing maturation media with different antioxidants such as β-mercaptoethanol (Sadeesh et al., 2014), cysteine, cysteamine (Beheshti et al., 2011), palm pollen grain extract (Salek-Abdollahi et al., 2015), quercetin and taxifolin – exogenous flavonoids (Kang et al., 2016), *Gundelia Tourneforii* leaves hydro alcoholic extract (Abedi et al., 2014) can improve oocytes maturation based on nuclear morphological changes.

Regarding vitamin C (L-ascorbic acid), a water-soluble antioxidant there are few studies to investigate it's antioxidant effects during *in vitro* maturation of oocytes (Sovernigo et al., 2017; Comizzoli et al. 2003; Tatemoto et al., 2001), most of studies were performed to emphasize it's beneficial role on freezing spermatozoa (Varo et al., 2014; Fanaei et al., 2014) or on improved motility and reduced DNA damage in post-thaw spermatozoa.

Another antioxidant used especially in freezing extenders which improved sperm quality after cryopreservation was rosmarinic acid (Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017). Rosmarinic acid is one of the first secondary metabolites produced in plant cell cultures in extremely high yields, up to 19% of the cell dry weight. Other promising biological activities of rosmarinic acid and its derivatives (rabdosiin and lithospermic acid B) are: improvement of cognitive performance, prevention of the development of Alzheimer's disease, cardioprotective effects, reduction of the severity of kidney diseases and cancer chemoprevention. (Bulgakov et al.,2012).

The purpose of this present research was to evaluate the viability of swine oocyte after *in vitro* maturated in media supplemented with ascorbic acid and rosmarinic acid and cryopreserved through vitrification.

Materials and methods

Swine ovaries (n=10) were collected from slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep), at 33-35^{0}C within two hours. Handling medium for COC (cumulus -oocytes- complexes) was Dulbecco-PBS (D-8662) supplemented with 100 µl Pen/Strep (17-602F, Lonza); 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021, Sigma-Aldrich). COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe.

Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^{0}C) after criteria of Antosik et al. (2010) with minor modification: *Ist class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, *IInd class* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III^{d} class* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm).

The maturation culture medium was prepared in our laboratory after Parrish et al. (1986) protocol with slight modifications: TCM 199 HEPES modification media, (M2520) with 10% ECS and 15 µl FSH (F8174, Sigma-Aldrich) - *group M* (control), in experimental groups we added ascorbic acid (0.5 mM) – *group C* and rosmarinic acid (105 µM) - *group AR*. Pools of 8-10 COCs

were maturated in 400µl media in 4 well dishes (Nunc, Germany) covered with mineral oil at 38.5^0C in 5% CO_2 humidified air atmosphere for 44h. After 44h of culture, all COC were examined for maturation, signs as expansion and mucification of cumulus cells were observed and were cryopreserved by vitrification according to there experimental group (M, C, AR group) and their morphological class (M1, M2, M3, C1, C2, C3, AR1, AR2, AR3).

Vitrification steps were: 15 min in Freezing 1 media (750 µl DMSO, 750 µl EG, 850 µl ECS and 7.65 ml TCM99), 1 min in Freezing 2 media (1500 µl DMSO, 1500 µl EG, 1.71g sucrose, 520 µl ECS, 5.23 ml TCM199), aspiration in 0.5 ml straws, sealed with MRSIDUAL V3 device (IMV) and imersed into liquid N_2 container, where they staid for 6 days.

Thawing steps were: 1 min, at 37^0C in a water bath, 1 min into Thawing 1 media (3.42g sucrose, 5.28 ml TCM199 and 1.30 ml FCS), 3 min into Thawing 2 media (1.9g sucrose, 6.64ml TCM199 and 1.65 ml FCS).

COC's viability after vitrification was evaluated by 0.02% Trypan Blue (T646, Sigma) staining, for 2 minutes.

Results and discussions

The results of viability evaluation of 57 swine *in vitro* maturated COCs and after vitrification, done by Trypan blue staining methods are presented in figure 1 and 2.

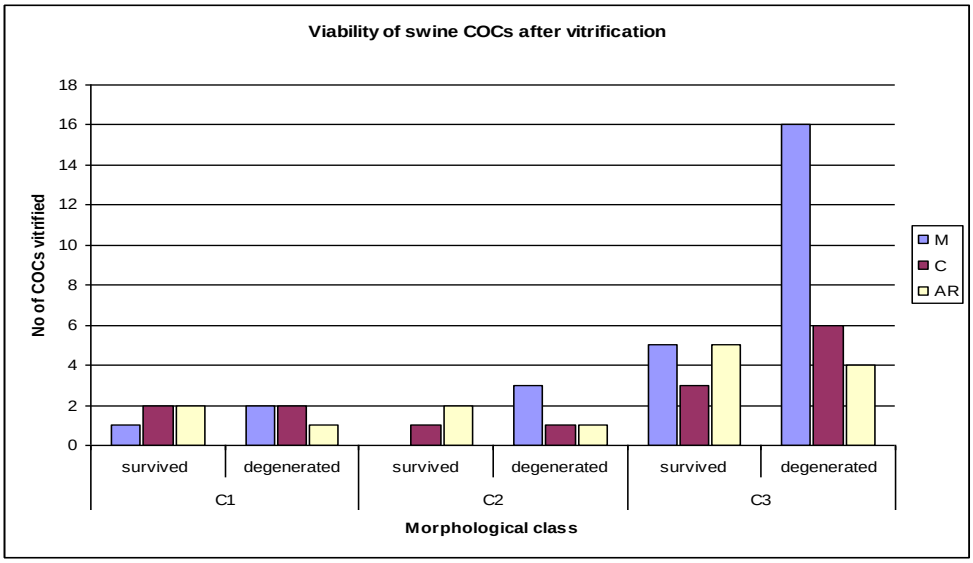


Figure 1. Classification of swine COC's after vitrification based on there viability

After thawing sow oocytes from the control group, 33.33% of the class I (M1) oocytes were viable and 66.66% non-viable, of the second class (M2) none was viable and in the third class oocytes (M3), 23.08% were viable and 71.19% were not viable.

Supplementation of the maturation media with vitamin C did not lead to an increase in the oocytes viability, regardless the morphological classification, thus class I (C1) and II (C2) 50% were viable and in the third class 33.33% (C3).

Choosing the vitamin C supplement as antioxidant for *in vitro* maturation media of swine COCs was based on other researcher's results. Comizzoli et al. (2003) observed that the compromised cat oocyte function during non-breeding season can be overridden by including supplemental FSH and antioxidants (0.5 mM vitamin C or cysteine) in maturation media. Kere et

al. (2013) testing various concentrations of vitamin C supplemented in IVM and IVC media of porcine oocytes and parthenotes and handmade cloned embryos observed that although nuclear maturation of oocytes was not affected by the addition of vitamin C, the intracellular glutathione levels were significantly increased and ROS reduced at 50 µg/ml vitamin C; added in IVC media, vitamin C improved blastocyst rates and total cell numbers and reduces apoptotic indices. In another study done on bovine oocytes, Sovernigo et al. (2017) suggest that antioxidants (vitamin C 50 µg/ml, quercetin 2µM, cyteamine 100µM, carnitine 0.5mg/ml or resveratrol 2µM) used during IVM may reduce oxidative stress which improved blastocyst development.

In COC's groups were we used rosmarinic acid as antioxidant we observed 66.66% of class I (AR1) and II (AR2) and 55.55% of COCs class III (AR3) survived after vitrification. Rosmarinic acid is used as an antioxidant in cryopreservation of semen. Olaciregui et al. (2017) proved for the first time that ovine spermatozoa freeze-drying in medium supplemented with 105 µM rosmarinic acid can be lyophilized effectively, stored at room temperature for long term, and even to starts embryo development after ICSI. Acid rosmarinic (105 µM) has beneficial effects also on boar semen cryopreservation were improves sperm parameters (total and progressive motility, viability, acrosome integrity) and sperm DNA integrity by reducing DNA oxidation rate (Luno et al., 2014; Luno et al., 2015). In swine reproduction, Somfai et al. (2014) reported the first successul piglet production from cryopreserved oocytes by vitrification.

Comparing groups C and AR with group M, relative to the number of vitrified oocytes, a slight increase in their viability is observed, with 16.67% (C1) and 33.33% (AR1), respectively.

The results obtained for the experimental groups (M, C and AR) require acceptance of the null hypothesis (Ho); the results does not support the hypothesis that treatment with antioxidants supports viability (Krusckal-Willis test, $p = 0.605$) or, on the contrary, causes mortality - unviability (Krusckal-Willis test, $p = 0.429$) of oocytes.

Regardless of the treatment applied, the oocyte class is associated with viability ($p = 0.048$) at statistically accepted thresholds. The classification of non-viable oocytes is close to the significance threshold (Krusckal-Willis test, $p = 0.059$).

Vitrification of oocytes can be done before or after *in vitro* maturation. Comparing these moments, Milovanov et al. (2016) concluded that cultivation of oocytes before vitrification brings more advantages for the meiotic resumption.

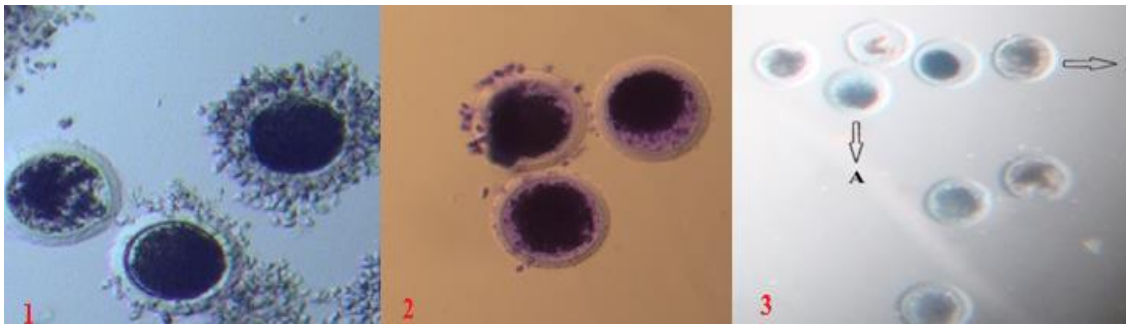


Figure.2 Live oocyte from group AR1 (1), dead oocytes from group M3 (2) and oocytes from group C3, A-dead oocyte, B-live oocyte (3)

Staining methods used in ART are very useful in order to see the quality of gametes and of embryos obtained. Some of them do not affect the viability of the cell, if are used correctly and for a short time (Brilliant Cresyl Blue, Hoechst 33342). Tripan blue (adiazo dye) is a supravital stain and an inexpensive marker that is used for studying cellular viability (membrane of live cell

is able to exclude the dye, whereas a nonviable cell will have a blue cytoplasm) (Melzer et al., 2016).

Testing the viability of the oocytes after cryopreservation is a mandatory step in order to see if the technique used had good results due to numerous factors implicated.

Further experiments are needed to clarify the effects of antioxidants on viability of the oocyte during vitrification and their capacity to sustain fertilization and developmental competence after thawing.

Conclusions

- Viability of oocytes depends on their class, while lack of viability is not necessarily associated with quality classes, so oocytes in C1 group had a higher viability of 16.67% and those in group AR by 33, 33% of the control group (M1).
- Supplementation of oocyte maturation media before vitrification with rosmarinic acid and ascorbic acid could determine a slight increase in viability, but this can not be supported statistically by our study.
- The use of Trypan blue to study oocytes viability is a quick, easy and efficient method

Aknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equipped through POSCCE 2669 program

References

21. ABEDI, A.; ROUHI, L., PIRBALOUTI, A.G. (2014) - *Effect of Gundelia Tournefortii leaves extract on in immature mouse oocytes,* Journal of Herbal Drug, 5, 2, 84-89.
22. AGARWAL, A., DURAIRAJANAYAGAM, D., PLESSIS, S. (2014) - *Utility of antioxidants during assisted reproductive techniques: an evidence based review*, Reproductive Biology and Endocrinology, 12, 112, https://doi.org/10.1186/1477-7827-12-112.
23. ANTOSIK P., KEMPISTY B., JACKOWSKA M., BUKOWSKA D., LIANERI M., BRUSSOW K.P., WOZNA P., JASKOWSKI J.M. (2010) - *The morphology of porcine oocytes is associated with zona pellucida glycoprotein 3 and integrin beta 2 protein levels*. Veterinarni Medicina, 55, (4): 154–162.
24. BEHESHTI, R., MOHAMMADI-ROSHANDEH,A., GHALEHKANDI GIASI, J., GHAEMMAGHAMI, S., HOUSHANGI, A.F. (2011) - *Effect of antioxidant supplements on in vitro maturation of bovine oocyte,* Advances in Environmental Biology, 5, 7, 473-1475.
25. BULGAKOV, V.P., INYUSHKINA, Y.V., FEDOREYEV, S.A. (2012) – *Rosmarinic acid and its derivates: biotechnology and applications*, Crit Rev Biotechnol, 32, 3, 203-217.
26. COMIZZOLI,P., WILDT,D.E. PUKAZHENTHI, B.S. (2003) - *Overcoming poor in vitro nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season*, Reproduction, 126, 809-816.
27. EL-SOKARY, M., KANDIEL, M.M.M., MAHMOUD, K.Gh.M., ABOUEL-ROOS, M.E.A., SOSA, G.A.M. (2013) – *Evaluation of viability and nuclear status in vitrified mature buffalo oocytes*, Global Veterinaria, 10, 3, 297-302.
28. FANAEI H, KHAYAT S, HALVAEI I, RAMEZANI V, AZIZI Y, KASAEIAN A, MARDANEH J, PARVIZI MR, AKRAMI M. (2014) - *Effects of ascorbic acid on sperm motility, viability, acrosome reaction and DNA integrity in teratozoospermic samples*, Iranian J Reprod Med., 12: 103-110.
29. KANG, J.T., MOON, J.H., CHOI, J.I., PARK, S.J., KIM, S.J., SAADELDIN, I.M., LEE, B.C. (2016) - *Effect of Antioxidant Flavonoids (Quercetin and Taxifolin) on In vitro Maturation of Porcine Oocytes,* Asian Australas. J.Anim.Sci., 29, 3, 352-358.
30. KERE, M., SIRIBOON, C., LO, N., NGUYEN, N., JU, J. (2013) – *Ascorbic* Acid *Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation,* Journal of Reproduction and Development, 59:78-84
31. KONC, J., KANYO, KATALIN, KRISTON, RITA, SOMOSKOI, B., CSEH, S. (2014) – *Cryopreservation of embryos and oocytes in human assisted reproduction*, BioMed Research International, article ID 307268, http://dx.doi.org/10.1155/2014/307268

32. LUÑO V, GIL L, OLACIREGUI M, GRANDIA, j., ANSO, T., DeBLAS, I (2015) - *Fertilisation rate obtained with frozen-thawed boar semen supplemented with rosmarinic acid using a single insemination timed according to vulvar skin temperature changes*, Acta Veterinaria Hungarica, 63, 1, 100-109.
33. LUÑO V, GIL L, OLACIREGUI M, GONZÁLEZ N, JEREZ RA, DE BLAS I. (2014) - *Rosmarinic acid improves function and in vitro fertilising ability of boar sperm after cryopreservation*, Cryobiology, 69, 1, 157-62.
34. MBEMYA, G.T., VIEIRA, L.A., CANAFISTULA, F.G., LOIOLA PESSOA, O.D., RIBEIRO RODRIGUES,A.P. (2017) – *Reports on in vivo and in vitro contribution of medical plants to improve the female reproductive function,* Reprod Clim, (http://dx.doi.org/10.1016/j.recli.2016.11.002)
35. MELZER, S., NUNES, C.S.M., ENDRINGER, D.C., ANDRADE, T.U., TARNOK, A., LENZ, D. (2016) – *Trypan blue as an affordable marker for automated live-dead cell analysis in image cytometry*, Scanning, 9999, 1-7.
36. MILOVANOV, CORNELIA, BERA-LUTESCU, EMANUELA, ZARCULA SIMONA, KELLER, T., OTAVA, G., TULCAN, CAMELIA, HUTU, I., BONCA, GH., AHMADI, M., MIRCU, C. (2016) – *Vitrification of germinal vesicle and metaphase II swine oocytes*, Lucr Stiin Med Vet USAMV Iasi, Ed."Ion Ionescu de la Brad", 59, 174-178.
37. OLACIREGUI,M., LUNO, VICTORIA, DOMINGO, PAULA., GONZALEZ, NOELIA, GIL, LYDIA (2017) – *In vitro developmental ability of ovine oocytes following intracytoplasmatic injection with freeze-dried spermatozoa*, Scientific reports, 7, 1096, DOI:10.1038/s41598-017-00583-0, www.nature.com/scientificreports.
38. *PARRISH J.J., SUSKO-PARRISH J.L., LEIBFRIED-RUTLEDGE M.L., CRITSER E.S., EYESTONE W.H., FIRST N.L***.** (1986) -Bovine in vitro fertilization with frozen-thawed semen, Theriogenology, 25, 591–600.
39. ROYCHOUDHUR, S., AGARWAL, A., VIRK, G., CHO, C-L**.,** (2017) - *Potential role of green tea catechins in the management of oxidative stress-associated infertility**,*** http://dx.doi.org/10.1016/j.rbmo.
40. SAADESH, E., SHAH, F., BALHARA, A.K., THIRUMARAN, S. M. K., YADAV, S., Yadav, P. S., (2014) **-** *Effect of growth factor and antioxidant on in vitro maturation of oocytes and cleavage rates of in vitro produced Indian buffalo (Bubalus bubalis) embryos*, Veterinarski Arhiv, 84 ,5, 459-474).
41. SALEK-ABDOLLAHIF, BAHARARA J, NEJADSHAHROKHABADI KH, NAMVAR F, AMINI E. (2015) - *Effect of Phoenix dactylifera pollen grain on maturation of preantral follicles in mice NMRI mice*. J HerbMed Pharmacol.,4, 3, 93-97.
42. SOMFAI, T., YOSHIOKA, K., TANIHARA, F., KANEKO, H., NOGUCHI, J., KASHIWAZAKI, N., NAGAI, T., KIKUCHI, K. (2014) – *Generation of live piglets from cryopreserved oocytes for the first time using a defined system for in vitro embryo production*, PLoS ONE 9(5): e97731. doi:10.1371/journal.pone.0097731.
43. SOVERNIGO,T.C., ADONA, P.R., MONZANI, P.S., GUEMRA, S., BARROS, F., LOPES, F.G., LEAL, C (2017) – *Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production*, Reproduction in Domestic Animals, 52, 4, 561-569.
44. SPRÍCIGO JFW, DIÓGENES MN, LEME LO, GUIMARÃES AL, MUTERLLE CV, SILVA BDM, ET AL. (2015) - *Effects of Different Maturation Systems on Bovine Oocyte Quality, Plasma Membrane Phospholipid Composition and Resistance to Vitrification and Warming*. PLOS ONE 10(6): e0130164. doi:10.1371/ journal.pone.0130164.
45. TATEMOTO, H., OOTAKI, K., SHIGETA, K., MUTO,N. (2001) – *Enhancement of developmental competence after in vitro fertilization of porcine oocytes by tratment with ascorbic acd 2-O-α-glucoside during in vitro maturation,* Biology of Reproduction, 65, 6, 1800-1806.
46. VARO-GHIURU,F., MICLEA ILEANA, HETTIG,A., LADOSI,I., MICLEA, V., EGERSZEGI,I., ZAHAN, M. (2015) - *Lutein, trolox, ascorbic acid and combination of trolox with ascorbic acid can improve boar semen quality during cryopreservation*, CryoLetters, 36, 1, 1-7.
47. YANG, B.C., IM, G.S., CHANG, W.K., LEE, Y.K., OH, S.J., JIN, D.I., IM,K.S., LEE, C.K. (2003) – *Survival and in vitro developemnt of immature bovine oocytes cryopreserved by vitrification*, Asian-Aust.J.Anim. Sci, 16, 1, 23-28.

Content of amino acids in blood serum in sows with idiopathic hipogalaxy

Viorica GURDIS
State Agrarian University of Moldova
wwwvictoria@mail.ru

Abstract

Hipogalaxy is the result of some pathological processes occurred in gestation which are manifested by hormonal deviation and deviation of some metabolic processes. A particular content of amino acids is necessary for a normal vital activity of the body and an adequate metabolism. By this experiment it was observed the influence of amino acids over the lactation. The results of amino acids screening in sows attest that their values in hypogalactic sows are different from those of the animals with a normal lactation by the reduced content of cysteine and tryptophan in the serum and the content of tyrosine significantly increased. The achieved results can be indicated for the precocious diagnosis of hypogalaxy.

Key words: *hipogalaxy, tryptophan, cysteine, tyrosine, postpartum, lactation.*

Introduction

From the physiological point of view the hipogalaxy represents the final result of some pathological processes during the gestation which are manifested by the hormonal deviation of hypothalamic-pituitary axis and of some metabolic processes. Keeping a particular balance of amino acids in the body represents the necessary condition which ensures both the metabolic balance and the optimal galactopoiesis. That is why keeping the functional stability of metabolic processes in the body represents the basis without which any complex therapy cannot bring positive results [5,7,8]

For a normal vital activity of the body and an adequate metabolism it is necessary a particular qualitative and quantitative content of amino acids. Amino acids in the body constitute the main layer which ensures the synthesis of proteins, enzymes, purines and pyrimidines, biologically active compounds of peptide origin and other compounds. If necessary, the amino acids can serve as the energy source by the oxidation of their carbonic components [9,10].

A special interest in the regulation of vital processes represents the tyrosine which is a semi-essential amino acid which forms by the hydroxylation of phenylalanine. From the tyrosine there are synthesized thyroid hormones, it can constitute the glucose source or fatty acids and melanin.

Cysteine, whose predecessor is methionine, participates to the formation of adrenocorticotropic hormone, insulin and glutathione. Recently it was found that from the cysteine there is formed cysteamine which has a protective role against the ionized radiation. At its turn, the cysteine is the precursor of oxytocin hormone which stimulates the contraction of myoepithelial cells of mammary glands increasing the pressure in the galactofore channels and in this way facilitates the elimination of milk.

Tryptophan represents an amino acid which serves as the predecessor of serotonin, nicotinic acid and melatonin. The tryptophan is in the composition of α – lactoalbumin which is in the cells of mammary glands and participate to the synthesis of lactose as a component (B Protein) of the enzymatic system which is specific for the lactogenic mammary glands. The insufficiency of tryptophan is manifested by anemia, female and male sterility and in young people affects the nervous system in the situation of hereditary disorders their metabolism [9,11].

Metabolic spectrum of amino acids is in direct connection with the functional condition of cells and it can fully characterize the balance. Our attention was focused on several amino acids which also influence the lactation.

Materials and methods

The researches were made in a farm of pigs where the incidence of hypogalaxy was of 4 %. In the experiment there were include 10 parturient sows (5 sows with normal lactation and 5 hypogalactic sows) in the 2nd and the 3rd lactation. The animals were classified in two equal groups: with normal lactation (I group) and hypogalactic sows (II group). The sows were kept in closed premises and they were fed with feed of complete value according to the technology of 3 phases. It was performed the clinical examination and there were taken blood samples for biochemical analyses for broadcasting, diagnosing and treatment of hypogalaxy which can have success only if the indicators of metabolism are investigated in sows with normal lactation and in hypogalactic sows.

The animals were fed three times a day, water was distributed through the automate trough, the food was in correspondence with the condition and physiological need but there were situations of technological non-compliance (lack of food, feeding with non-correspondent food etc.).

The zoo-hygienic conditions for keeping corresponded to the species and physiological condition of animals.

Results and discutions

Clinical examination provides complex data, aims for the examination of health condition of animals. According to the opinion of some researchers for finding the hypogalaxy syndrome in sows and monitoring the efficacy of the administrated treatment there can also be used successfully the routine clinical indicators which in direct way reflect multiple processes.

General conditions of sows with normal lactation and hypogalactic sows was satisfactory and it was confirmed by the normal values of clinical indicators (T, B, P) which varied within the reference limits, but at the same time in hypogalactic sows it was observed the reduction of appetite, reduced maternal instinct and no interest towards progeny.

Table 1. Clinical indicators

Groups	n	**T**(ºC)	**P** (contr./minute)	**B** (movements/min)
		M ± m	M ± m	M ± m
I Sows with normal lactation	10	38,2 ± 0,17	96,3 ± 1,32	26 ± 0,4
II Hypogalactic sows	65	38,5 ± 0,2	94,24 ± 0,92	25 ± 0,8

The values of body temperature, frequency of heart contractions and breath rate are represented in Chart no. 1. Animals which were under supervision, as it was mentioned above were in satisfactory general condition. Body temperature of sows with normal lactation and of hypogalactic sows in average was bigger with 0,3º C in comparison with those with normal lactation. We mention that the values of body temperature registered by us for the investigated sows correspond fully with the information from the literature (1,2).

It is well known the fact that the examination of heart contraction frequency and breath rate is very important in establishing health condition and body reactivity in different extreme conditions. In chart no. 1 it is also seen that the value of heart contraction frequency in both groups

was within the physiological limits. It was a small difference in the limits of calculation error. The heart frequency in researched animals of both groups corresponds to the limits established by other (2, 4).

Respiratory movements in sows of the Ist group were of mixt type, costo – abdominal, rhythmic, symmetric. The frequency of respiratory movements of these animals on the average, per group corresponded to the physiological limits and constituted 26 breaths per minute. The frequency of breath in hypogalactic sows was 25 breaths per minute and it corresponded to the reference limits. Figures shown in chart no. 1 and the indicators of clinical examination show that the hypogalaxy does not influence negatively the general condition of animals, values of body temperature, frequency of heart contractions and breath rate.

The mammary gland in hypogalactic sows had a different aspect. In some sows the mammary glands were rigid, congested, but with flaccid teat. In others, on the contrary they had normal sizes and consistence and were sensible, warm when palpated, skin of purple color. When suckled, pigs suckled actively producing sounds for a long period of time. Often they were trying repeatedly to suckle in more frequent intervals and after suckling they had not become silent. As the consequence of the effort to suckle the teats were traumatized. When milking only some milk drops with normal aspect could be obtained or nothing could be obtained. As the energetic reserves of pigs were decreasing their attempts to suckle also were decreasing and often they migrated to the warmer portions of the box. In comparison with hypogalactic sows, the mammary gland of sows with normal lactation was well developed, turgid condition, slightly sensitive to palpation and when milked there were obtained 1-2 ml of milk.

The average quantity of milk eliminated after a suckling in sows with normal lactation constituted on average 216,5 ml. Gravimetry made to hypogalactic sows show that these animals were secreting and eliminating milk after a suckling on average of 80,67 ml or 2,4 times less in comparison with sows with normal lactation. The quantity of milk which was eliminated by the sows with normal lactation during a suckling varied from 182 g to 240 g, and in hypogalactic sows from 54 g to119 g.

A special role have the biochemical investigations in appreciating health condition, in this context we established the goal to investigate the level of some amino acids in blood serum of the sows from those two groups. Data in chart no. 2 shows that in hypogalaxy the level of cysteine decreases significantly, being 2 times lower in comparison with the sows with a normal lactation (21,0 ±2,3 µmol/l in comparison with 42,7 ±3,4 µmol/l, P<0,001). In all appearances, in hypogalaxy normal processes of transforming methionine are disturbed and as a consequence the synthesis of cysteine is reduced and this fact reduces the synthesis of prolactin and respectively the lactogenesis decreases.

Table 2. Content of cysteine, tyrosine and tryptophan in sows' blood

Indicators	Sows with normal lactation	Hypogalactic sows	P
	M ± m/ *lim*	**M ± m**/ *lim*	
Cysteine (µmol/l)	**42,7 ± 3,4** (37 -48)	**21,0 ± 2,3** (19 – 25)	< 0,001
Tyrosine (µmol/l)	**52,8 ± 3,2** (46 – 63)	**80,3 ± 4,1** (73 – 86)	< 0,001
Tryptophan (µmol/l)	**39,8 ± 2,6** (36 – 46)	**24,5 ± 2,7** (10 – 50)	< 0,001

The content of tyrosine in blood (chart no. 2) in hypogalactic sows (group II) exceeds with 52 % the value registered in sows with normal lactation (group I). It results that in hypogalaxy the synthesis speed of thyroid hormones and dopamine who predecessor is tyrosine is significantly reduced.

A significant place in the nitrogen metabolism is occupied by the content of tryptophan. The data achieved after our investigations (chart no. 1) show that the level of tryptophan in blood of hypogalactic sows is with 15,3 µmol/l lower in comparison with those from the I group with a normal lactation. Thus, we find in hypogalactic sows the absence of correlation between the dynamics of the tyrosine and tryptophan level in blood, fact which eventually can lead to the reduction of prolactin secretion in anterior pituitary gland.

Conclusions

1. General condition of hypogalactic sows was apparently satisfactory but at the same time it was found the reduction of appetite, reduced maternal instinct and no interest towards the progeny.
2. The result of the screening of amino acids in sows show that their values in hypogalactic sows are different from those with a normal lactation by: the content of cysteine and tryptophan in serum is significantly reduced ($P < 0,05$) and the content of tyrosine is significantly increased ($P < 0,05$);
3. Screening of amino acids can be indicated for the precocious diagnosis of hypogalaxy.

Bibliography

1. Бобрик Д.И Распространение и ранняя диагностика синдрома метрит-мастит агалактия у свиноматок, Ученые записки УО ВГАВМ, т.53 вып.1 2017г
2. Бобрик Д.И., Разуванов С.А. Профилактик синдрома метрит - мастит- агалактия путем проведения коррекции родового акта у свиноматок, Ученые записки УО ВГАВМ, т.53 вып.1 2017г
3. Glock X. T., Bilkei G., The effect of postparturient urogenital diseases on the lifetime , 2005.
4. Гречухин, А. Н. Синдром метрит – мастит – агалактия у свиноматок, Ветеринария, 2009.–No 5.–С.12–14.
5. Krieter, J.; Presuhn, U., Genetic variation for MMA treatment. Züchtungskunde 81, 149-154., 2009.
6. Păcală N., Petroman I., Petroman C., Bencsik I., Dronca D., Nistor E., Cean A., Marin D., Pandur I., Observations on uterine infection frequency, during the puerperium period, in sows. Porc Res 2(1):19-22., 2012.
7. Perle Boyer, DVM; Glen W., Postpartum dysgalactia syndrome in sows Last full review/revision October 2014 by.
8. Preißler, R., Tetens, J., Reiners, K., Looft, H., Kemper, N., Biological pathway analysis for postpartum dysgalactia syindrome in sows via a genome-widw association study. Institute of Animal Breeding and Husbandry, Christian-Albrechts-University, Kiel, Germany., 2014.
9. Trottir N., Easter R. Dietary and plasma branched-chain aminoacids in relation to tryptophan: effect on voluntary fed intake and lactation metabolism in the primiparous sow// Journal of Animal Science.-1995. V.73.-N. 4.-p. 1086 – 1092.
10. Mavromichalis I, Parr TM, Gabert VM, Baker DH True ileal digestibility of amino acids in sow's milk for 17- day-old pigs. J Anim Sci. 2001 Mar;79(3):707-13.
11. Pozzi, P.S.1 and Alborali, G.L. Reproductive diseases in sows (Sus scrofa domestica): A Review2Israel Journal of Veterinary Medicine Vol. 67 (1) March 2012

Cell growth characteristics of equine synovial fluid stem cells

Emoke PALL[1,2], Klementina Katalin PALL[2], Cristian CRECAN[1], Simona CIUPE[1], Mihai CENARIU[1], Ioan GROZA [1]

[1]University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Cluj-Napoca, Mănăştur, no. 3-5, 400372, Cluj-Napoca, Romania

pallemoke@gmail.com

[2]University of Szent Istvan, Godollo, Hungary

Abstract

Equine mesenchymal stem cells (MSCs) have been isolated from various sources such as: peripheral blood, bone marrow, adipose tissue, umbilical cord, placenta, Wharton Jelly. Besides these synovial fluid and synovial membrane represents a promising source of mesenchymal stem cells, which can be harvested by minimally invasive methods. MSCs from these sources have the potential to self-renew and differentiate into multiple lineages such as chondrogenic, adipogenic and osteogenic. The aim of our study was to evaluate the growth characteristics of equine synovial liquid stem cells harvested from the tarsometatarsal joint during arthroscopic surgery. Samples were collected in a sterile syringe and were diluted and centrifuged at 1500rpm for 7 min. The supernatant were removed and the cells were resuspended in propagation medium: Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (Sigma-Aldrich). The medium was changed after 3 days. The proliferation ability, cell doubling number, cell doubling time, daily duplication rate and clonogenic efficacy was evaluated. Isolated cells exhibited plastic adherence capacity, monolayer growth, and fibroblast-like morphology, high growth and clonogenic capacity. Our study demonstrated the characteristics of equine synovial fluid derived stem cells, an ideal candidate for veterinary regenerative medicine.

Keywords*: stem cells, synovial fluid, horse, proliferation, cells growth*

Introduction

Mesenchymal stem cells (MSCs) are multipotent precursor cells with self-renewal and differentiation capacity (Dominici et al. 2006, Pall et al., 2015). MSCs are a promising therapeutic tool in veterinary medicine (Bahamondes et al., 2017) have been isolated from different animal species including rodents (Penny et al., 2012), dogs (Guercio et al., 2013, Bahamondes et al., 2017), horses (Barberini et al., 2014, Pall et al., 2016) and rabbits (Tan et al., 2013). According to the International Society for Cellular Therapy published in 2006, human MSCs are characterized by plastic adherence capacity, trilineage differentiation capacity and expression of surface markers such as CD105, CD90, CD44, CD73, CD79α and are negative for the expression of hematopoietic markers CD34, CD45 and CD19, CD14, human leukocyte antigen HLA-DR (Dominici et al., 2006). MSCs isolated from animals are not fully characterized. Equine mesenchymal stem cells (MSCs) have been isolated from various sources such as: peripheral blood, bone marrow, adipose tissue, umbilical cord, placenta, Wharton Jelly (Barberini et al., 2014, Tessier et al., 2015, Pall et al., 2016). Besides these synovial fluid and synovial membrane represents a promising source of mesenchymal stem cells, which can be harvested by minimally invasive methods. The aim of our study was to evaluate the growth characteristics of equine synovial liquid stem cells harvested from the tarsometatarsal joint during arthroscopic surgery.

Material and methods

Samples (n=5) were collected in a sterile syringe during atroscopic surgery. The samples were harvested after owner's agreement. The samples were diluted and centrifuged at 1500rpm for

7 min. The supernatant were removed and the cells were resuspended in propagation medium: Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (Sigma-Aldrich). Cultures were incubated at 37 °C in humidified atmosphere with 5 % CO_2. After 72 h, non-adherent cells were removed and the medium was replaced.

Proliferation ability of equine synovial fluid derived stem cells was determined using MTT colorimetric assay. A concentration of $1x10^4$cells/well was cultured in a 96-well plate. After 24 h 20 µl MTT (2 mg/ml, Sigma-Aldrich) was added to each well and cultures were incubated at 37 °C for 3 h. The fromazan were dissolved with 100 µl DMSO (dimethylsulfoxide, Sigma-Aldrich) and the absorbance was measured at 450 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). In order to evaluate the proliferation rate the population doubling time were assessed. A total number of $1x10^5$ cells/ well were seeded in 24-well cell culture plates. After 24 h (t24h) the non-adherent and the adherent cells (N0) were counted. 24 h later (t48h) the adherent cells from three wells were counted (N48h). The doubling time (tD) was calculated according to the formula: tD = (log 2 × t)/ (log N48h - log N0).

The clonal capacity of cells was evaluated using CFU-F assay. $5x10^2$cells/cm^2 was cultured in expansion medium. After 10 days the cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO) in 10% methanol for 20 minutes and were examined under phase contrast inverted light microscope (Nikon TS100, Nikon Instruments, Europe) and the colonies (> 50 cells) were counted. CFU-F efficiency was estimated according to the formula: CFU-F efficiency = (counted CFU-F/cells originally seeded) × 100.

Results and discussion

MSCs were isolated from harvested from equine tarsometatarsal joint during arthroscopic surgery. The isolated cells were characterized to confirm their caracteristics such as plastic adherence, expression of specific surface antigens and differentiation potential. Equine synovial fluid derived mesenchymal stem cells showed fibroblast-like morphology (fig 1.), adherence to plastic surface and express the specific surface markers (CD90, CD105, CD44, results not shown here). The proliferation capacities of cells were evaluated using MTT assay.

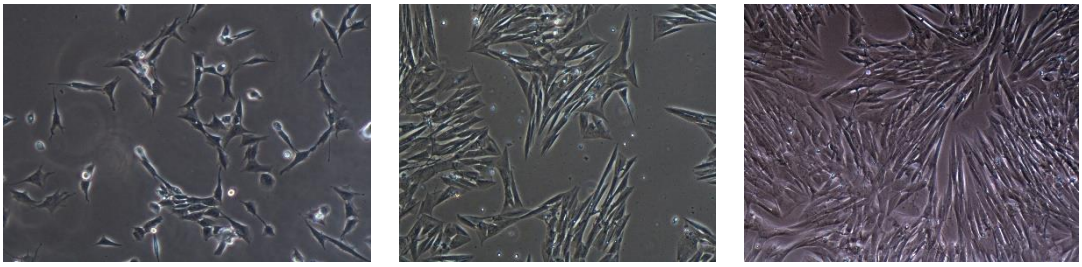


Figura 1. Equine synovial fluid derived cell morphology

The isolated cells lines exhibited similar proliferation potential in evaluated period (d1-D6). Optical density (*OD*) values at each time were evaluated (fig 2.).

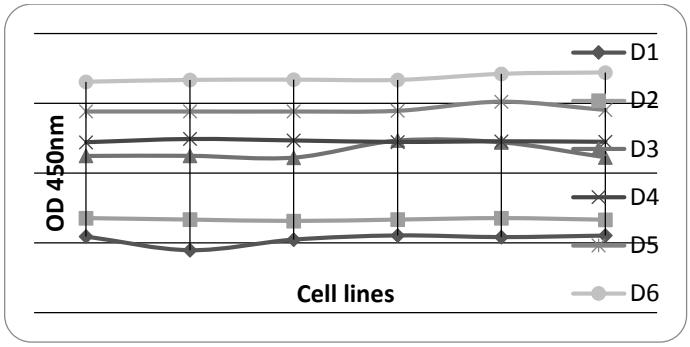


Figure 2. Proliferation potencial of equine synovial fluid derived mesenchymal stem cell

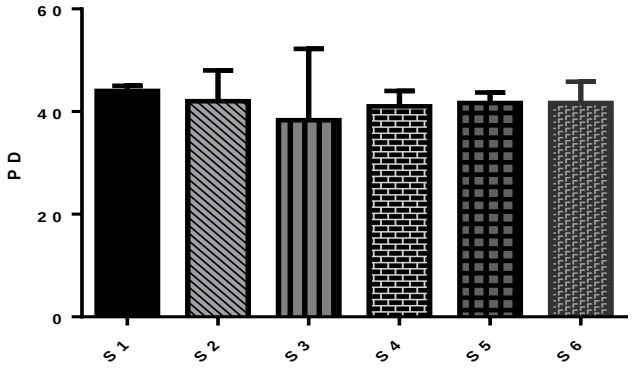


Figure 3. Population doubling time in equine synovial fluid derived mesenchymal stem cell lines

Individual assessment at each cell line can be concluded that for line S1 the population doubling time was 44±1.01h, 42±5.8 in S2, 38.33±13.86 h in S3, 41.00±3.00 in S4, 41.66±2.08 in S5 and 41.66±4.16 in S6 (fig. 3). The average of population-doubling time (PD) was 41.44 ± 1.83 h.

The clonogenic potential of MSCs was assessed by CFU-F assay. Isolated cells lines displayed colony-forming ability; the frequency of colony forming cells for S1 was 37.66±6.42 colonies/100 cm2, 36.66±6.42 colonies/100 cm^2 in S2, 36.33±7.57 colonies/100cm_2 in S3, 38.66±4.04 colonies/100cm2 in S4, 35±4.35 in S5 and 37±4.58 colonies /100cm2 in S6 (fig 4.).

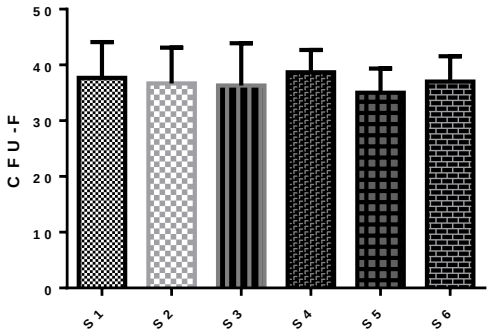


Figure 4. The average of colonigenic potential (CFU-F assay) of equine synovial fluid derived mesenchymal stem cell

Multipotent mesenchymal stem cells (MSCs) represent a promising source of cells for regenerative medicine therapeutic approaches in both human and veterinary medicine given their properties (Singer et al., 2011, Carrade et al., 2013, Clark et al., 2016, Pall et al., 2016). MSCs can be defined by their morphology, expression of a panel of cell surface markers and their tri-lineage differentiation (Dominici et al., 2006, Clark et al., 2016). In our study, cells from synovial fluid were evaluated as possible sources of MSCs, for equine regenerative medicine. The horse is a valuable species for evaluating the usefulness and efficiency of MSC treatment (Pezzanite et al., 2015). MSCs from different sources are used, but most often autologous bone marrow-derived MSCs are used to treat musculoskeletal disorders including tendonitis, osteoarthritis, cartilage damage, and meniscal injuries (Schnabel et al., 2009, Frisbie and Smith 2010, Caniglia et al., 2012, De Schauwer et al., 2013, Pezzanite et al., 2015).

MSCs also can modulate endogenous tissue and immune cells (Parekkadan et al., 2010) and can migrate to the site of injury after receiving specific signals (Chen et al., 2011). MSCs also can be subject to cryopreservation with minimal loss of potency (Lee et al., 2005), for future heterologous or autologous transplantation. Numerous studies and clinical trials have demonstrated the role of MSCs, but further studies are needed to elucidate the exact therapeutic mechanisms.

Conclusions

Our study showed that mesenchymal stem cells could be successfully isolated form synovial fluid. Characterization of these cells can be achieved based by their morphology, immunophenotype and differentiation potential. Results from our study demonstrate the proliferative and clonogenic potential of equine synovial fluid derived stem cells, and can serve as a potential source of mesenchymal stem cells for veterinary regenerative medicine.

References

1. Bahamondes Francisca, Estefania Flores, Gino Cattaneo, Flavia Bruna, Paulette Conget, Omental adipose tissue is a more suitable source of canine Mesenchymal stem cells, BMC Vet Res. 2017; 13: 166.
2. Barberini Danielle Jaqueta, Natália Pereira Paiva Freitas, Mariana Sartori Magnoni, Leandro Maia, Amanda Jerônimo Listoni, Marta Cristina Heckler, Mateus Jose Sudano, Marjorie Assis Golim, Fernanda da Cruz Landim-Alvarenga, Rogério Martins Amorim, Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: immunophenotypic characterization and differentiation potential, Stem Cell Res Ther. 2014; 5(1): 25.
3. Barberini DJ, Freitas NP, Magnoni MS, Maia L, Listoni AJ, Heckler MC, Sudano MJ, Golim MA, da Cruz Landim-Alvarenga F, Amorim RM. Equine mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord: immunophenotypic characterization and differentiation potential. Stem Cell Res Ther. 2014 Feb 21;5(1):25.
4. Caniglia, C.J., Schramme, M.C. and Smith, R.K. (2012) The effect of intralesional injection of bone marrow derived mesenchymal stem cells and bone marrow supernatant on collagen fibril size in a surgical model of equine superficial digital flexor tendonitis. Equine Vet. J. 44, 587-593.
5. Carrade DD, Borjesson DL. Immunomodulation by mesenchymal stem cells in veterinary species. Comparative Medicine. 2013; 63(3):207–217.
6. Chen F-M, Wu L-A, Zhang M, Zhang R, Sun H-H. Homing of endogenous stem/progenitor cells for in situ tissue regeneration: promises, strategies, and translational perspectives. Biomaterials. 2011; 32(12):3189–3209.
7. Clark Kaitlin C., Amir Kol, Salpi Shahbenderian, Jennifer L. Granick, Naomi J. Walker, Dori L. Borjesson Canine and Equine Mesenchymal Stem Cells Grown in Serum Free Media Have Altered Immunophenotype, Stem Cell Rev. 2016; 12: 245–256.
8. De Schauwer C, Van de Walle GR, Van Soom A, Meyer E. Mesenchymal stem cell therapy in horses: useful beyond orthopedic injuries? Vet Q. 2013; 33:234–41.

9. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006; 8:315–7.
10. Frisbie DD, Smith RK. Clinical update on the use of mesenchymal stem cells in equine orthopaedics. Equine Vet J. 2010; 42:86–9.
11. Guercio A, Di Bella S, Casella S, Di Marco P, Russo C, Piccione G., Canine mesenchymal stem cells (MSCs): characterization in relation to donor age and adipose tissue-harvesting site. Cell Biol Int. 2013 Aug; 37(8):789-98.
12. Lee MW, Yang MS, Park JS, Kim HC, Kim YJ, Choi J. Isolation of mesenchymal stem cells from cryopreserved human umbilical cord blood. Int J Hematol. 2005;81:126–30
13. Páll E, Florea A, Soriţău O, Cenariu M, Petruţiu AS, Roman A. Comparative Assessment of Oral Mesenchymal Stem Cells Isolated from Healthy and Diseased Tissues.Microsc Microanal. 2015 Oct; 21(5):1249-63.
14. Pall Emoke, Toma Segiu, Cristian Crecan, Mihai Cenariu, Groza Ioan, Isolation and Functional Characterization of Equine Adipos Tissue Derived Mesenchymal Stem Cells, Agriculture and Agricultural Science Procedia, Volume 10, 2016, Pages 412-416.
15. Parekkadan Biju, Milwid Jack M., Mesenchymal Stem Cells as Therapeutics, Annu Rev Biomed Eng. 2010 Aug 15; 12: 87–117.
16. Penny Jasmine, Pat Harris, Kevin Shakesheff, Ali Mobasheri The biology of equine mesenchymal stem cells: phenotypic characterization, cell surfacemarkers and multilineage differentiation, Front Biosci (Landmark Ed). 2012 Jan 1; 17:892-908.
17. Pezzanite Lynn M, Lisa A Fortier, Douglas F Antczak, Jennifer M Cassano, Margaret M Brosnahan, Donald Miller, Lauren V Schnabel, Equine allogeneic bone marrow-derived mesenchymal stromal cells elicit antibody responses in vivo, Stem Cell Res Ther. 2015; 6(1): 54.
18. Schnabel LV, Lynch ME, van der Meulen MC, Yeager AE, Kornatowski MA, Nixon AJ. Mesenchymal stem cells and insulin-like growth factor-I gene-enhanced mesenchymal stem cells improve structural aspects of healing in equine flexor digitorum superficialis tendons. J Orthop Res. 2009; 27:1392–8.
19. Singer NG, Caplan AI. Mesenchymal stem cells: mechanisms of inflammation. Annual Review of Pathology. 2011; 6:457–478.
20. Tan SL, Ahmad TS, Selvaratnam L, Kamarul T. Isolation, characterization and the multi-lineage differentiation potential of rabbit bone marrow-derived mesenchymal stem cells. J Anat. 2013 Apr; 222(4):437-50.
21. Tessier Laurence, Dorothee Bienzle, Lynn B. Williams, Thomas G. Koch, Phenotypic and Immunomodulatory Properties of Equine Cord Blood-Derived Mesenchymal Stromal Cells, PLoS One. 2015; 10(4): e0122954.

Epidemiology of atopic dermatitis and other allergic skin diseases in dogs and cats in Western Romania

Tiana SUICI, Gh. DARABUS, Narcisa MEDERLE, Mirela IMRE, C. SIRBU S. MORARIU
Facultatea de Medicină Veterinară Timișoara
sujic.tijana@yahoo.com

Abstract

Allergic skin diseases in dogs and cats are an emerging problem worldwide. In the past few years, the number of cases has greatly increased and a rising interest in their treatment and diagnosis exists among veterinarians. The aim of this study is to make a short description of the epidemiological situation of these skin problems in the western part of Romania. The study was conducted during a 3-year period in a total of 8 clinics from three different counties in western Romania: two clinics from the Mehedinti County, three clinics from Timis County and three clinics from Arad County. Reports were made regarding the number of patients, which were presented at the clinics and were diagnosed with atopic dermatitis, flea allergy dermatitis or other types of allergy (food allergy, contact allergy). This way we managed to obtain a preliminary ratio of allergic affections in dogs and cats in the western part of Romania. The average value for cats in all three counties was 10.74% of all cases presented at the clinics and 45.22% of the cases that presented dermatological symptoms. The average value for dogs in all three counties was 5.94% of all cases presented at the clinics and 42.49% of the cases that presented dermatological symptoms.

Keywords: *allergy, dogs, epidemiology, western Romania*

Introduction

Dogs, similar to humans, may develop a syndrome of spontaneous, inflammatory, pruritic dermatitis with characteristics such as a young age of onset, characteristic distribution of lesions and IgE sensitization to common environmental allergens generically called atopic dermatitis (AD)(9). The main clinical features are skin lesions distributed around the mouth, eyes, ears, limbs and ventral abdomen, pruritus and alopecia, erythema, conjunctivitis and recurrent otitis (8). The clinical signs of AD and of all allergies in general can sometimes easily be mistaken for diseases such as demodectic mange (6), *Malassezia* infections or skin diseases caused by *Microsporum.*

The diagnosis of canine AD is based either on the characteristic clinical features or on results from various tests such as intradermal skin testing, IgE serology and even blood tests (BDT or LTT tests) (5).

Epidemiological data help us understand the distribution, risk factors and causes of disease, having a major influence in establishing a successful control and prevention protocol (12). According to studies, AD and allergic skin diseases are one of the most common skin conditions in dogs and cats with a prevalence of 3-15% in the general dog population and 3-58% of dogs affected with skin diseases presented to veterinarians (8).

Materials and methods

The study was conducted over a three-year period (2014-2016) in a total of eight clinics from three counties in western Romania: Mehedinti, Timis and Arad. The clinics taken into study were as follows: *Vet Point Vest, Vetagrica* and *Bioanima* in Arad County, *Ilivet* and *Negrostar* in Mehedinti County and *Salvet, Dr. Ciolea Felician private practice* and the *Dermatology clinic of FVM* in Timisoara.

The information used in this paper comes from the consultation registers of the above-mentioned clinics. A total number of 13.254 dogs and 4.708 cats were registered in these three

years in the eight clinics. The overall number of animals with a dermatological diagnosis was 2971 of which 1852 were dogs and 1119 cats. Results in detail are illustrated in the graphics and tables that follow.

Table 1. Repartition of dogs presented in clinics from Timisoara, Arad and Mehedinti

Total cases of dogs (no.)			Total of dermatological cases (no.)			Total allergies (no.)		
Years			Years			Years		
2014	2015	2016	2014	2015	2016	2014	2015	2016
4098	4514	4642	582	634	636	229	289	269
General total= 13254			General total= 1852			General total= 787		

Table 2. Repartition of cats presented in clinics from Timisoara, Mehedinti and Arad

Total cases of cats (no.)			Total of dermatological cases (no.)			Total allergies (no.)		
Years			Years			Years		
2014	2015	2016	2014	2015	2016	2014	2015	2016
1461	1553	1694	339	397	383	143	178	185
General total= 4708			General total= 1119			General total= 506		

Table 3. Repartition of both cats and dogs in all three counties

Total cases (no.)			Total of dermatological cases (no.)			Total allergies (no.)		
2014	2015	2016	2014	2015	2016	2014	2015	2016
5559	6067	6336	921	1031	1019	372	467	254
General total= 17962			General total= 2971			General total= 1093		

In all three counties the highest rate of dermatological diseases in dogs and cats was recorded in 2015 when the rate was 16.99% of the total cases followed by 2014 with a rate of 16.57% and 2016 with a rate of 16.08% (figure 1).

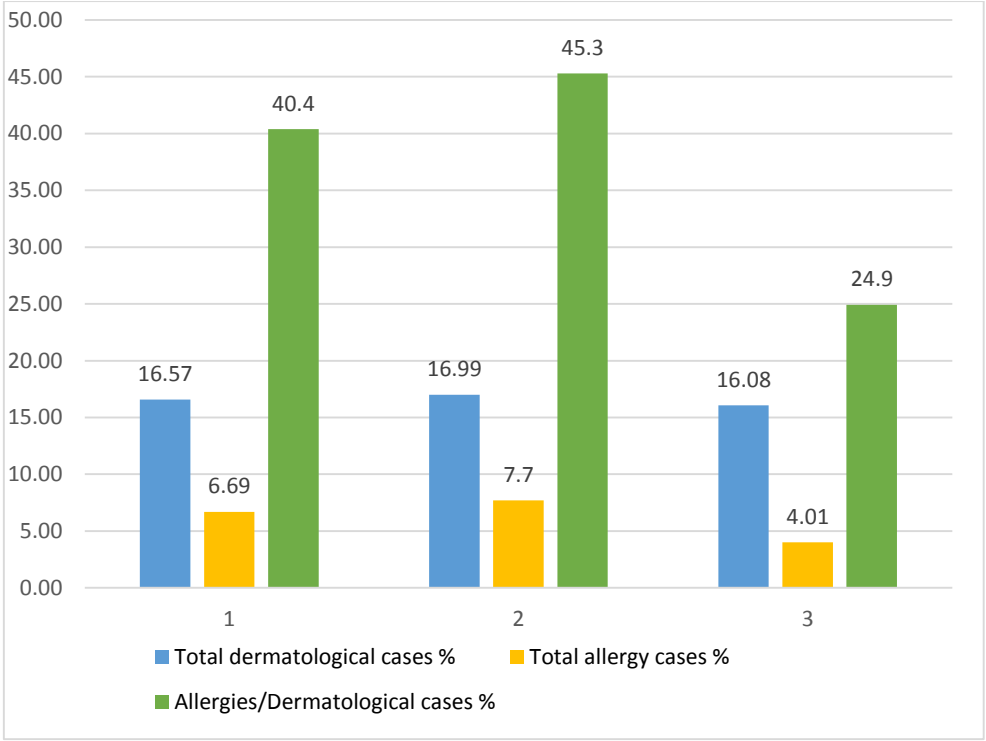


Fig.1- Percentage of dermatological cases and AD or other allergic cases

Allergies were present in a higher rate in the year 2015 when the percentage of AD and other allergic diseases was 7.70% of the total cases and 45.3% of the dermatological cases. Gradually followed the years 2014 with a rate of 6.69% of the total cases and 40.4 % of the dermatological cases and the year 2016 with a rate of 4.01% of the total respectively 24.9% of the dermatological cases.

The overall percent of allergic skin conditions including AD was 6.1% during the period 2014-2016 out of a total number of 17,962 animals.

According to earlier research, the prevalence of AD in the canine population was estimated at large to be 15% (1). More recently, scientific papers state estimates of 10 % (9). Unfortunately, the true prevalence and incidence of AD in the general dog population is very difficult to establish because of the slight unreliability of data caused mostly by the fact that mild cases are often successfully managed with symptomatic therapy without a specific diagnosis and some clinical manifestations of AD are difficult to recognize. Lund et al. (4) has conducted a study in the USA on 31,484 dogs examined in 52 private practices and reached an estimate of 8.7% dogs diagnosed with allergies. On the other hand, more recent studies also from the USA state a rate of 27% incidence of AD among dogs and cats (10). Canadian studies (11) stated that AD stood for 12.7% of the cases while in France, DeBoer's (2) survey reports a 27% rate of 11,373 dogs.

Conclusions

In the western part of Romania, the total prevalence of AD and other allergic skin diseases was highest in 2015 when the rate was 7.7%, AD or other allergic skin diseases representing 45.3% of dermatological cases.

The overall rate of allergic skin diseases in Western Romania during the years 2014-2016 was 6.1% out of 17.962 animals.

Acknowledgements

This study is published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project No. POSDRU/159/1.5/S/132765 and this research work was carried out with the support ofthe projectDezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinei veterinare şi tehnologiilor inovative pentru RO 05, cod SMIS-CSNR 266.

References

1. CHAMBERLAIN, K.,W., 1974-Atopic (allergic) dermatitis, Vet. Clin. N. Am. 4,29-39;
2. DEBOER, D., J., 1989-Survey of intradermal skin testing practices in NorthAmerica, J. Am. Vet. Med. Assoc. 195, 1357-1363.
3. HILLIER A., GRIFFIN, C.E. 2001-The ACVD task force on canine atopic dermatitis (I): incidence and prevalence; Veterinary Immunology and Immunopathology 81, 147-151;
4. LUND, E.M., ARMSTRONG, P.J., KIRK, C.A., KOLAR, L.M., KLAUSNER, J.S., 1999-Health status and population characteristics of dogs and cats examined at private veterinary practices in the United States, J. Am. Vet. Med. Assoc. 214, 1336-134;
5. MARSELLA, R., (2013)-Atopic disease, In: Miller,W.H.J., Griffin, C.E., Campbell, K.L. (Eds.), Muller & Kirk's Small Animal Dermatology, Seventh Ed. Elsevier Mosby, St. Louis,MO, USA, pp. 365–388
6. MEDERLE NARCISA, DARABUS GH., OPRESCU, I., MORARIU, S., ILIE, M., INDRE, D., MEDERLE, O., 2010- Diagnosis of canine demodicosis, Sci. Parasitol., 11 (1): 20-23
7. MILLER, W.H.J., GRIFFIN, C.E., CAMPBELL, K.L., 2013- Muller & Kirk's Small Animal Dermatology, Seventh Ed. Elsevier Mosby
8. OLIVRY, T., SARIDOMICHELAKIS, M.,N. 2016 -An update on the treatment of canine atopic dermatitis, The Veterinary Journal 207, 29-37;
9. PUCHEU-HASTON C., M. 2016- Atopic dermatitis in the domestic dog, Clinics in dermatology, vol. 34, 2, 299-303
10. SCOTT, D.W., MILLER, W.H., GRIFFIN, C.E., 2001-Small animal dermatology, 6th Edition, W.B. Saunders, 574-601.
11. SCOTT, D.W., PARADIS, M., 1990- A survey of canine and feline skin disorders seen in a university practice: small animal clinic, University of Montreal, Saint-Hyacinthe, Quebec, Can. Vet. J. 31, 830-835;
12. TORRENCE, M.E., 1997- Understanding Epidemiology, Mosby Year Book, 1-17;

Generating bovine embryos through ICSI

Thomas KELLER, Simona MARC, Horia CERNESCU, Camelia TULCAN, Ioan HUȚU, Gabriel OTAVA, Ana-Maria RAȚIU, Georgiana UNGUREANU, Călin MIRCU
Banat University of Agricultural Sciences and Veterinary Medicine Timisoara, Faculty of Veterinary Medicine, Calea Aradului, no.119, 300645 Timişoara, jud. Timiş, Romania
calinmircu@usab-tm.ro

Abstract

Through ICSI, competition between sperms and also sperm-oocyte interaction are avoided thus ICSI proving reliable when sperm is not suitable for IVF. In bovine, the limiting step is represented by low rate of sperm head decondensation subsequent ICSI. Intracytoplasmatic sperm injection allows avoiding many critical moments that may occur during normal or in vitro fertilization. Oocytes were obtained from ovaries from slaughtered cows. These were transported in 0.9% NaCl solution in isothermal bags at a temperature of 25-30 ° C. The ovaries were brought from the slaughterhouse within 2 hours. Harvesting of the oocytes was made through the aspiration method. After maturation, oocytes were fertilized using sperm that was prepared using Percoll method and then treated with TritonX. The volume of the TritonX solution that accompanies the sperma and which remains in the oocyte is extremely important given that by its action, TritonX removes the acrosome, thus releasing a rich enzyme content and facilitating the dehydration of the male pronucleus. Even though the number of 2 nucleus, 2 cells or 4 cells oocytes is inferior to the data found in the literature, compared to the results achieved last year in the assisted reproduction laboratory within CLC-HC Timisoara, it marks significant progress. At the 2 cells stage, there were several oocytes from group 1 (24.39% vs. 12.5%), while at the 4 cells stage there were 14.63% oocytes from group 1 and 25% group 2. The use of TritonX solution for sperm treatment as well as shortening the duration of ICSI execution allowed us to get encouraging results. The results obtained are inferior to those presented in the literature but are far superior to those we obtained last year when the ICSI technique was assembled. Achieving the two- and four-cell embryonic stages justifies us thinking that we are mastering the ICSI technique.

Keywords: *bovine, embryos, ICSI, TritonX*

Introduction

Intracytoplasmatic sperm injection allows avoiding many critical moments that may occur during fertilization. Applying this technique involves sophisticated equipment as well as detailed specialist knowledge. It is essential to have a good knowledge of working steps equal to the use of certain specific reagents of the highest quality (Godja et al., 2016).

The ICSI technique in cattle was assembled in CLCHC Assisted Reproduction Laboratory last year, and the results obtained determined us to continue its application. In the present paper, we attempted to demonstrate the implications of using TritonX for sperm treatment as well as reducing working times in order to shorten both the interval in which the sperm is in contact with the slowing solution of their movements and the period of oocytes outside the incubator.

In cattle, intracytoplasmic sperm injection (ICSI) has low efficacy (Canel et al., 2014; Zambrano et al., 2016). The content of acrosome may be considered responsible for this effect due to the large number of hydrolytic enzymes released in oocytes.

In order to eliminate the acrosome and destabilize sperm membranes, researchers at the University of Frontera (Zambrano et al., 2016) chose the use of Lizolecitin and Triton-X 100. The rate of oocyte development was assessed along with pronuclear formation and embryo quality. The use of Lizolecitin and Triton-X 100 (0.01%, 0.02%, 0.03%, and 0.04%) decreased the sperm viability based on the dose used. At the same time, an improvement in the acrosome reaction for all Lizolecitin and Triton-X 100 concentrations was observed, reaching already a concentration of 100% from 0.05% of both treatments.

A higher rate of cell division was observed in the intracytoplasmic injection of sperm treated with Triton-X 100 (66%) and Lizolecitin (65%) compared to the untreated sperm group (51%). At the same time, a significant increase in blastocyst formation was observed in the Lizolecitin treated group (29%) compared to the control group (21%). There was no difference in the formation of pronucleus and the number of embryos developed.

The researchers concluded that sperm treatment with Lizolecitin and Triton-X 100 improves the rate of embryo development without affecting the quality of embryos produced by using this technique.

Galli et al. (2003) studied the effect of various substances used for sperm pretreatment. In the study, the sperm was activated with heparin, D-penicillamine, hypotaurine and epinephrine before ICSI.

The effect of the use of dithiothreitol for pretreatment of spermatozoa or oocytes to be injected was then studied. Activation of sperm with heparin in combination with epinephrine did not improve the development of embryos following ICSI. Instead, the use of dithiothreitol for pre-treatment of oocytes improved cell division and blastocyst formation in the case of inactivated embryos and an acceleration of blastocyst development when embryos were activated. At the same time, sperm pretreatment with dithiothreitol followed by ICSI showed a significant increase in embryonic development rates over the first 7 days.

The study by Canel et al. (2014) aimed primarily at identifying how different treatments applied to semen, such as sperm pretreatment with heparin and L-glutathione or sexing of semen, can influence the development of embryos following intracytoplasmic sperm injection in cattle.

Cell division and blastocysts were assessed at 2 and 7 days after ICSI. The results of the study show a significant increase in the rate of cell division and blastocysts in the pretreated batch with heparin and L-glutathione group compared to the untreated group. The use of sperm semen has also improved the cell division rate, with no major differences in blastocyst counts.

Research has also been carried out on the quality of oocytes and semen (Ohlweiler et al., 2013). During the study, experiments were performed using better or worse quality oocytes and sperm.

The blastocyst formation rate was significantly higher in the case of good quality oocytes (23.3% versus 11.1% in the case of inferior quality oocytes), regardless of the characteristics of the semen. At the same time, there was no major difference in the rate of blastocyst formation using poor quality semen (regardless of oocyte quality). However, the use of good quality semen has been shown to have a major influence on the blastocyst formation rate (25.7% versus 9.2% in the case of poor quality oocytes).

The influence of oocytes and sperm quality on ICSI was subsequently evaluated. No major differences were observed in the blastocyst formation rate irrespective of the quality of oocytes or semen.

The conclusion of the researchers was that the use of intracytoplasmic sperm injection is an effective way to achieve the in vitro generation of bovine embryos irrespective of the quality of oocytes or sperm.

Materials and methods

Oocytes were obtained from ovaries from slaughtered cows. These were transported in 0.9% NaCl solution in isothermal bags at a temperature of 25-30 ° C (Mayes, 2002). The ovaries were brought from the Nojag slaughterhouse, which is 167 km (2h) from the Assisted Reproduction Laboratory of the CLCHC and from Macea, Arad County, which is 80.5 km (1h 9min) from the laboratory. For the harvesting of the oocytes, the aspiration method (Chung et al., 2000) was chosen

and cultivated for those of category I. After harvesting, COC, together with the follicular fluid, were placed in a 50 ml conical tube for sedimentation. After 5 minutes of rest, they were sucked from the bottom of the tube with the help of a pipette. This operation was repeated 4-5 times to make sure all oocytes were sucked together with the cumulus cells.

The next step was to wash the COC by passing successively into 2 PBS plates. Thus, they are prepared for maturing in 4-well plates. After being held in 400 µl of TCM 199 enriched with 10% ESC and coated with mineral oil to prevent oxygen action, for 22h for maturation in the incubator, they were removed from the PBS-washed incubator and then denuded in 0.4 ml of 0.1% hyaluronidase (Sigma) and washed in two steps of PBS.

The sperm strains stored in liquid nitrogen were thawed at 37 degrees for one minute (Rahman, 2010). Sperm was prepared using Percoll method. Through the density gradient (Percoll), dead, abnormal (low density) sperm and detritus (cell contamination) of ejaculate accumulate in the corresponding densities, while the density of the sperm (mobile, normal) crosses the gradient and is found on the bottom of the tube. To eliminate all traces of gradient used, sperm isolated by this method is washed in a culture medium. In order to centrifuge in the concentration gradient, 90% and 45% Percoll solutions were prepared by dilution with Earl 1x solution. The Percoll concentration gradient solution was kept on a water bath for 4 hours at 37 degrees before use. 200 µl of semen from each bull was used. After bringing the samples to 37 ° C, they were slowly added dropwise onto the walls of the centrifuge tube over the Percoll solution in a concentration gradient (90%-45%) pre-heated for 4 hours at 37 ° C. Then the mixture was centrifuged (Hettich 350R) for 20 minutes. After which the supernatant was removed and 2 ml of Earl 1X solution was added. The mixture was homogenized and analyzed for the determination of seminal material parameters. To facilitate manipulation of the sperm and to immobilize it, the use of Triton-X 100 (Sigma) was chosen. It is necessary to mix 50 µl of sperm suspension with an equal volume of TCM 199 and 0.1% Triton-X 100. The mixture is centrifuged at 2000xG for 3 minutes.

After removing the cumulus cells, the oocytes were placed in a drop containing 5 µl of IVF-TALP (in vitro fertilization-Tyrode's albumin pyruvate albumin). The sperm was transferred to 10 µl of the culture medium (Sp-TALP) containing 10 µl / ml heparin. ICSI was performed at a magnification x200 microscope in drops of 30µl of TCM199 + BSA 3mg / ml medium coated with silicone oil and kept at 37 ° C on the microscope warm plate. On the fertilization microplate were placed two drops of TCM199 + BSA medium into which the oocytes were introduced and the drop was covered with mineral oil. A drop containing spermatozoa treated with TritonX was placed on the same microplate, from which a single sperma was extracted (Figure 1 A). The oocytes were fixed in the micromanipulator pipette polar body at 6 o'clock, and the sperm was injected perpendicularly (approximately at 3 o'clock) (Figure 1 B).

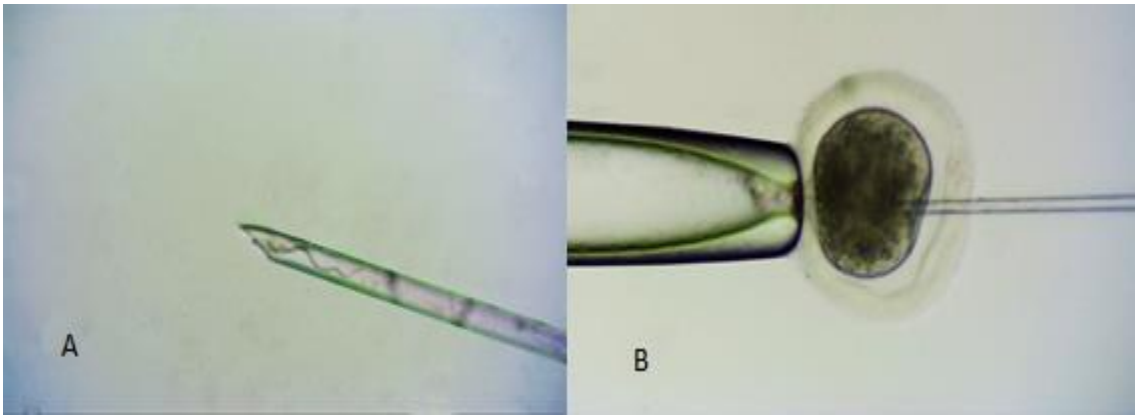


Fig.1. Sperm in micropipette (A) and injecting of sperm into oocyte (B)

After ICSI, the oocytes were transferred to the culture medium of TCM199 + 20% ESC coated with silicone oil in 60x15 mm culture plates (Falcon, Fischer Scientific) and placed in the incubator at 37 ° C, 100% humidity and 5% CO2. 18 hours after sperm injection, oocytes were examined for the presence of the pronucleus. After 72h of ICSI, some oocytes were found to have 2 cells (Figure 2 A) and at 96 hours ICSI had 4 cells (Figure 2 B).

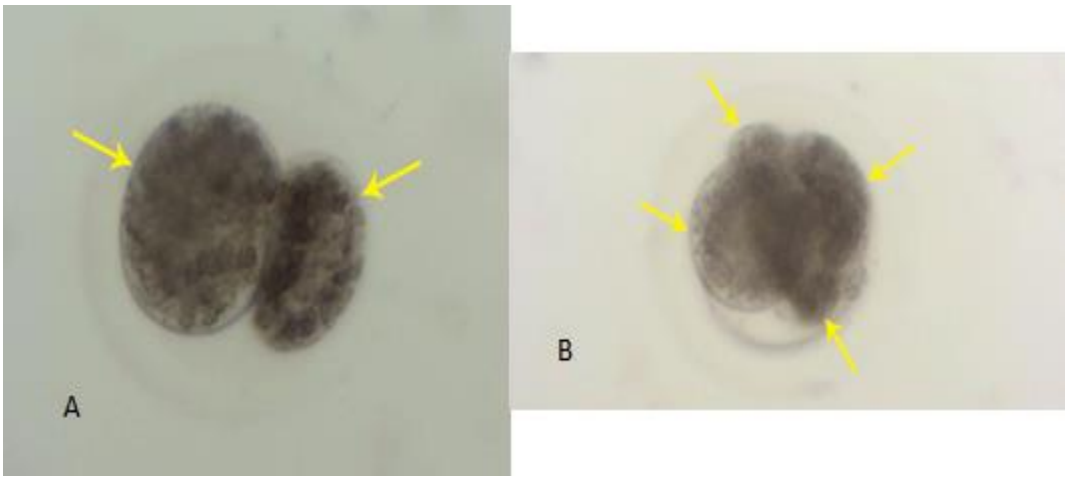


Fig.21. Oocytes with 2 cells (A) and 4 cells (B)

To highlight the division, oocyte staining with Hoechst 33342 (Thermofischer Scientific) was chosen. After they were removed from the incubator they were washed in a PBS step after which they were placed in Hoechst for 15 minutes in the incubator. After this period, they were examined in an immunofluorescence microscope to highlight the cell division (Figure 3).

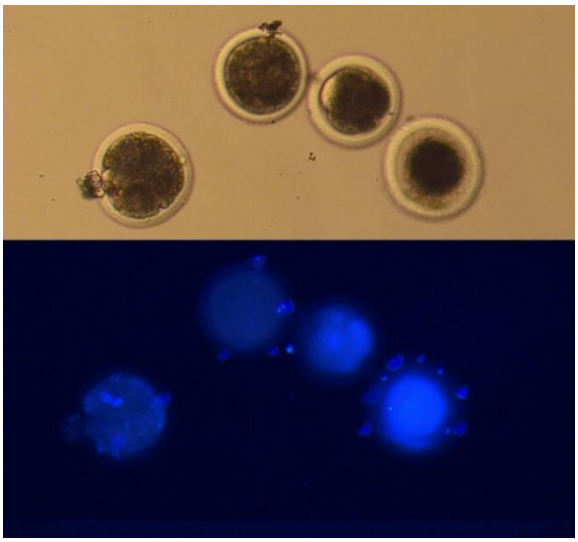


Fig.3. Before and after **using the Hoechst staining**, the presence of 4 cells is evidenced

Results and discussions

Depending on the length of ICSI's own workload (from sperm contacting to injection into oocytes), we divided the 73 oocytes injected into two groups (Table 1):

- Group 1, in which we included the 41 oocytes injected in less than 7 minutes;
- Group 2, containing 32 oocytes injected in more than 7 minutes.

We chose the 7 minute threshold taking into account both the recommendations in the literature and our values (the shortest time was 4 minutes and 3 seconds and the longest 17 minutes and 22 seconds), Values to which we calculated average and standard deviation. ICSI was performed in TCM supplemented with 20% ESC, the oocytes remaining in this medium for the first 24 hours. Subsequently, they were moved for another 24 hours in the maturation medium containing cumulus cells and 48 hours after ICSI time they were again moved to the TCM + ESC20% medium where they remained during the observation period. The 2N stage was also highlighted by the 24-hour examination from ICSI, 2 cells (2C) at 72 hours from ICSI and 4-cell (4C) at 96 hours from ICSI. The embryos remained in those stages for more than 24 hours, at which time we found degenerative processes and lack of further development.

Table 1. Results obtained from ICSI on cattle oocytes

Group	Number of OV x ICSI	Nonfertilized oocytes	Oocytes with one pronucleus	Oocytes with two pronucleus	Fertilized oocytes that have reached the stage of 2 cells	Fertilized oocytes reaching the 4-cell stage
Group 1	41	1	6	18	10	6
Group 2	32	4	4	12	4	8

Group 1, including oocytes subjected to ICSI in less than 7 minutes, had only unfertilized oocytes (2.45%). There were 6 oocytes with one pronucleus (14.63%) and 18 oocytes (43.90%) with two pronucleus. Of these 18, 10 fertilized oocytes (24.39%) reached the stage of 2 cells and 6 oocytes (14.63%) - the stage of 4 cells (Figure 4).

The percentages of fertilized oocytes (as evidenced by the presence of the two pronucleus) as well as those representing the 2 or 4 embryonic stages obtained at G1 are inferior to those presented in the literature but clearly superior to those we personally obtained (Godja et al., 2016).
Gali (2003) reported only poor results subsequent ICSI as cell division and blastocist development, despite bovine oocytes were activated and supposed to develop fertilization similarly with IVF.
Keskintepe and Brackett (2000) activated the oocyte at 30 minutes consequent sperm injection by incubation with A23187 ionophore for 5 minutes. Bull sperm was previously capacitated by incubation in a heparin-containing media. Authors reported 52,4% blastocyst division and 24,4% for blastocyst development.

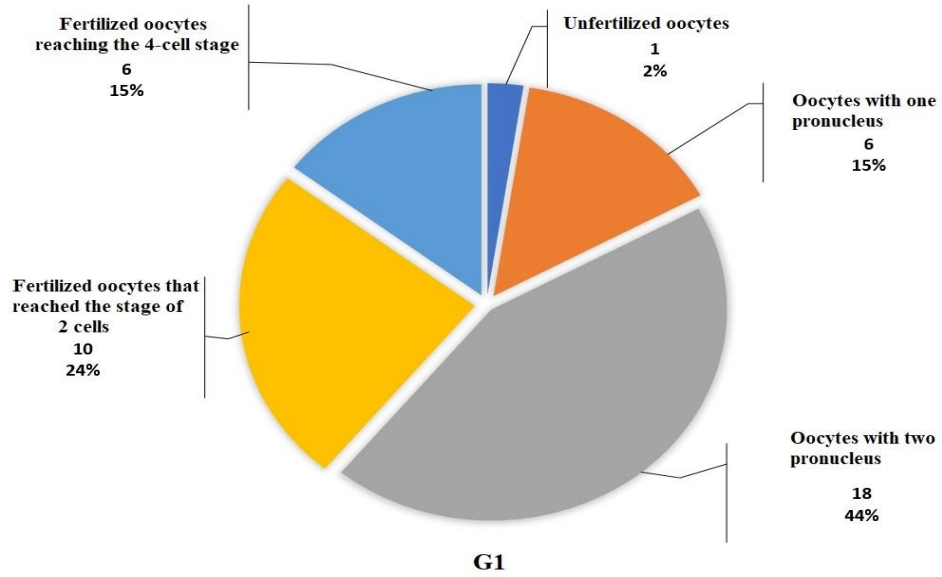


Fig. 2. Results from ICSI on cattle - Group 1

For group 1- containing oocytes at which ICSI occurred over a period of time longer than 7 minutes, there were 4 nonactive oocytes (12.5%) and 4 (12.5%) with a single pronucleus. Of the 12 oocytes (37.5%) who presented the stage of 2 pronucleus, 4 oocytes (12.5%) reached the stage of 2 cells and 8 oocytes (25%) - the embryonic stage of 4 cells (Figure 5).

Figure 1 shows eloquently the differences between the two groups. Even though the number of 2N, 2C or 4C oocytes is inferior to the data reported in the literature, compared to the results we achieved last year (11) marks significant progress. It should be noted that at the 2C stage there were several oocytes from G1 (24.39% vs. 12.5%), while at the 4C stage there were 14.63% oocytes from G1 and 25% G2 (Figure 6).

We consider that the volume of the TritonX solution that accompanies the sperm and which remains in the oocyte is extremely important given that by its action, TritonX removes the acrosome, thus releasing a rich enzyme content and facilitating the dehydration of the male pronucleus.

The results of the ICSI were also influenced by the running time of this work, and it is desirable that it be as short as possible. The experience gained by the ICSI person makes it possible to shorten this interval permanently, ensuring for oocytes spending as short a time as possible outside the incubator.

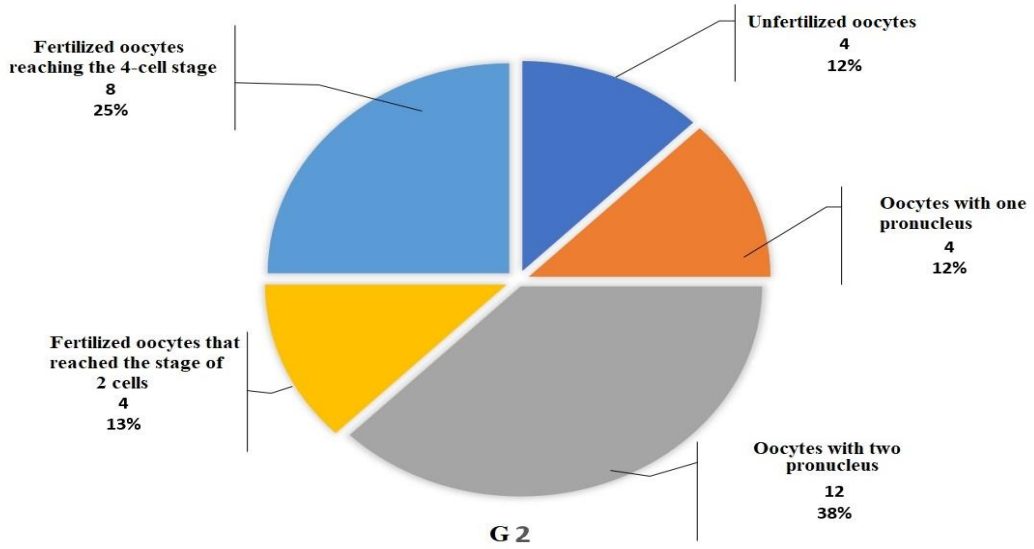


Fig. 3. Results from ICSI on cattle - Group 2

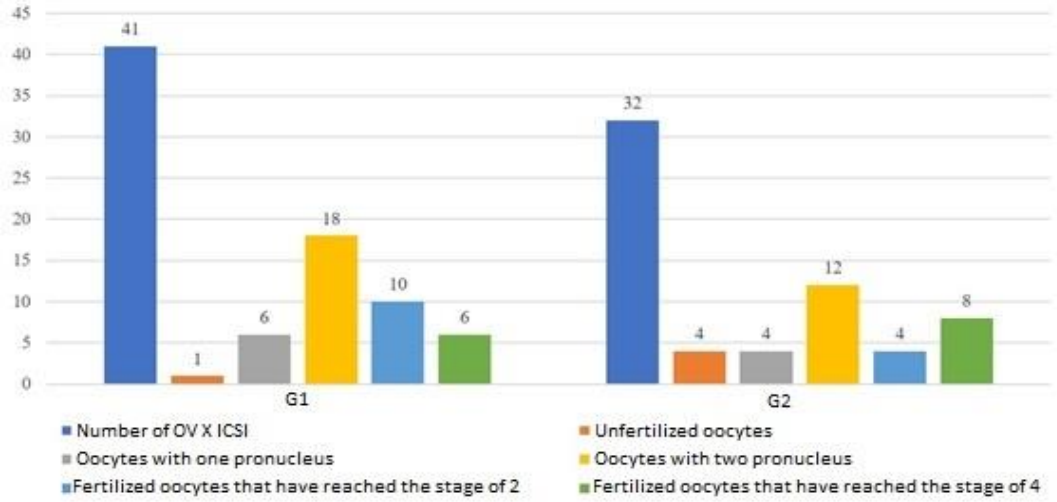


Fig. 4. Results obtained from ICSI on cattle oocytes

We consider important to keep oocytes subjected to ICSI in the culture medium containing cumulus cells, because this significantly improves oocyte metabolism by facilitating the exchange of nutrients.

To grasp any statistical differences, we used the chi test, and a statistically significant difference occurred at $p \leq 0.01$ between G1 and G2, which is of particular interest when we look at the results obtained with two or four cell embryos.

Stages C2 and C4 occurred 24 hours later than the accepted physiological time in bovine embryogenesis, which discusses both the moment of potential oocyte activation and subsequent survival.

The divergent opinions in the literature on whether or not the oocyte is activated following the simple ICSI can support the underlying assumptions regarding the installation of the delayed cell division and its closure after a short time as well as in the case of the 4C embryos the higher success rate registered at G2 compared to G1. In other words, according to our results, the longer duration of ICSI execution has enhanced cell division.

Conclusions

The use of TritonX solution for sperm treatment as well as shortening the duration of ICSI execution allowed us to get encouraging results.

The results obtained are inferior to those presented in the literature but are far superior to those we obtained last year when we assembled the ICSI technique.

Achieving the two- and four-cell embryonic stages justifies us thinking that we are mastering the ICSI technique.

Aknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory from the Horia Cernescu Research Unit equipped through POSCCE 2669 program

References

1. CANEL, N. G., BEVACQUA, R. J., HIRIART M. I., SALAMONE, D. (2014) - IVF vs. ICSI: efficiency and “bull fertility effect” using sex sorted or non sorted semen in bovine, Animal Reproduction, 11, 3, 301-301.
2. CHUNG, J.T., KEEFER, C.L., DOWNEY, B.R. (2000). Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI), Theriogenology, 53, 1273-1284
3. GALLI, C., VASSILIEV, I., LAGUTINA, I., GALLI, A., LAZZARI, G. (2003) Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol, Theriogenology, 60, 8, 1467-1480.
4. GODJA, G., RAȚIU A.M., UNGUREANU, G., KELLER, T., ZARCULA, S., CIOBOTA, A., OTAVĂ, G., TULCAN, C., HUȚU I., MIRCU, C. (2016) - Pronuclei formation subsequent to introcytoplasmic sperm injection in bovine, Lucrări științifice medicină veterinară – USAMV Ion Ionescu dela Brad, Iași, 59, 2, 159-166.
5. KESKINTEPE, L., BRACKETT, B.G. (2000) - Cryopreservation of bovine blastocysts obtained by intracytoplasmic sperm injection, Theriogenology, 53, 1041-1052
6. MAYES, M. - The meiotic arrest of bovine oocytes (Thèse présentée à la Faculté des études supérieures de l'Université Laval pour l’obtention du grade de Philosophiae Doctor), http://theses.ulaval.ca/archimede/fichiers/20201/20201.html (accesat în x).
7. OHLWEILER, L.U., BRUM, D.S., LEIVAS, F.G., MOYSES, A.B., RAMOS, R.S., KLEIN, N., MEZZALIRA, J.C., MEZZALIRA, A. (2013) - Intracytoplasmic sperm injection improves embryo production from poor quality bovine oocytes, Theriogenology, 79, 5, 778-783.
8. RAHMAN, A.N.M.A. (2010) - Intracytoplasmic sperm injection-revolution in human and animal assisted reproduction: A review, Biotechnology, 9, 392-410.
9. Thermofischer Scientific - Hoechst 33342 Solution (20 mM), https://www.thermofisher.com/order/catalog/product/62249 (accesat în 04.05.2017).
10. University of Wisconsin-Madison, Department of Animal Science (2003) - Percoll Separation Procedure, http://www.ansci.wisc.edu/jjp1/ansci_repro/lab/lab10/lab11_2003/percoll.html (accesat în 25.04.2017).
11. ZAMB RANO, F., AGUILA, L., ARIAS, M. E., SÁNCHEZ, R., FELMER, R. (2016) – Improved preimplantation development of bovine ICSI embryos generated with spermatozoa pretreated with membrane-destabilizing agents lysolecithin and Triton X-100, Theriogenology, 86, 6, 1489-1497.

The behaviour pattern of several gastrointestinal nematode genera in sheep and cattle from bethausen, Timis County

C. SÎRBU, Gh. DARABUS, M. ILIE, Mirela IMRE, Tiana SUICI, S. MORARIU
Facultatea de Medicină Veterinară Timișoara

Abstract

Parasitic infestations are one of the most important causes for animal disease and low productivity worldwide. Gastrointestinal nematodes (i.e. Trichostrongylus,) as well as trematodes (i.e. Fasciola spp. Paramphistomum spp.), cestodes (i.e. Echinococcus spp.) and protozoa (i.e. Eimeria spp.) are all in the category of most important parasitic diseases. Studies were conducted on cattle and sheep from Bethausen village, Timis County. In cattle, the following genera were identified : Trichostrongylus, Cooperia and Ostertagia while in sheep the Trichostrongylus, Ostertagia, Chabertia, Oesophagostomum and Haemonchus genera were noticed. The present study was based on following the dynamics of the output of parasitic elements from the April 2015 until March 2016. The best represented genus was Trichostrongylus both in cattle and sheep with a prevalence of 40%, followed by Chabertia – 33%, Ostertagia – 30%, Haemonchus – 26% and Cooperia – 15%. The maximum EPG was achieved in October and the minimum EPG was achieved in the months of January and February.

Introduction

Parasitic infestations are one of the main causes of animal sickness and low productivity worldwide.

Parasitism is currently at a high point in farms and households on a global level, despite many financial efforts directed towards control and prophylactic campaigns,. According to recent research, the most significant damage is caused by pulmonary and gastrointestinal helminthosis. However, it can be stated that these diseases ‘’don't kill the animal but destroy the farm”(5,6,23).

The fact that the parasites usually have a subclinical evolution (a phenomenon encountered especially in temperate areas) in any temporary and/or permanent system of pasturing leads to a decrease in the zootechnical performance.Several parasitic diseases can be mentioned in this context: cryptosporidiosis, neosporosis, hydatidosis, fasciolosis, paramphistomosis and trichostrongylosis. (4, 10, 11, 17, 18)

Thus, it causes considerable economical loss, due to the reduction of the growth rate, the reduction of food conversion rate, the reduction of the milk and meat production leading to the beginning of a subproductivity syndrome.The economic losses are due to expenses implied by treatments although for some of them the possibility of vaccination exists (19, 20).

The economic influence of a subclinical evolution of the parasitosis can be rightly appreciated only by taking into account all the elements, which are related to the pasture contamination and to the receptivity of the animals.

The aim of this research was to investigate the parasitic spectrum, especially the gastrointestinal nematodes, in a medium-sized village in Timis County, over a one-year period, starting in April 2015 and ending in March 2016.

Materials and methods

The research took place in Bethausen Village , Timis County. Bethausen Village is situated in the North-East of Timis County , on the right side of the Bega river, 26 km away from the town of Lugoj and 20 km away from the town Faget. Bethausen is situated in the centre and it is

surrounded by nearby villages as follows: Leucușești village in the East, Cutina Village in the West, Cliciova and Nevrincea villages in the South and Cladova village in the North.

The village has a surface of 9027 hectares, of which 4764 ha are tillable, 1265 ha are forests, 2066 ha pastures, 425 ha hays and orchards, 139 ha waters, 178 ha of roads and railways, 136 ha of country yards and buildings and 54 ha of non-productive fields. It is situated on both parts of Bega (26).

Cattle and sheep are frequently found on the pasture, which has its own source of water (a well with concrete gutters). The grass carpet had the following floristic composition: spontaneous species of perennial Gramineae *(Poa spp., Festuca spp., Dactylis spp., Bromus spp., Phleum pratense*) with small areas of leguminous plants (*Trifolium repens, Lotus corniculatus*) and other plants from the spontaneous flora.

Between 50 of 150 gr of freshly eliminated faeces were collected or taken directly from the rectum of the individuals .(4 cattle and 15 sheep)subjected to study. The samples were packed in plastic bags and refrigerated until processing.

The samples were transported to the Parasitic Diseases laboratory of FVM Timisoara and they were processed according to the following methods:

- Willis - to enhance de type of parasitism;
- McMaster - in order to find out the amount of parasites (EPG) in each individual from the experiment;
- Euzeby - to quantify the pulmonary parasites in ruminants(2,3,6,7).
- Larvae cultures.

Only three genera were noticed in cattle: *Ostertagia, Cooperia* and *Trichostrongylus*. The behaviour of the three-gastrointestinal nematodes was different according to season .

It can be seen that, during the spring months, the *Cooperia* genus was best represented in April-39.62% and May-37.14%. The weakest prevalence was registered during winter months: 16.12% in January and 14.58% in February. The *Ostertagia* genus had the highest prevalence in summer months.

The population peak was reached in July (51.45%) and the lowest numbers were recorded in December (26.92%).

On the other hand, the *Trichostrongylus* genus seems to feel better in the winter months when the rate is higher than 45%, peaking in December- 52.57%. The lowest values were observed in summertime in July (13.60%).

In Sweden, *Dimander* (8) highlights the presence of 14 species of gastrointestinal nematodes but the weight was held by 2 species: *Ostertagia ostertagi* and *Cooperia oncophora.*Five genders of gastrointestinal nematodes were identified in sheep after reading the larval cultures: *Haemonchus, Ostertagia, Trichostrongylus, Chabertia* şi *Oesophagostomum*.

It can easily be noticed that the *Haemonchus* genus has a relatively even distribution throughout the study period except for the winter months when its prevalence dropped, reaching values of zero in February. In addition, this genus has had the weakest infrapopulation representation.

In an increasing order followed the *Oesophagostomum* genus which had a slightly increasing trend throughout the entire period, especially during winter months with a peak (20%) in February.

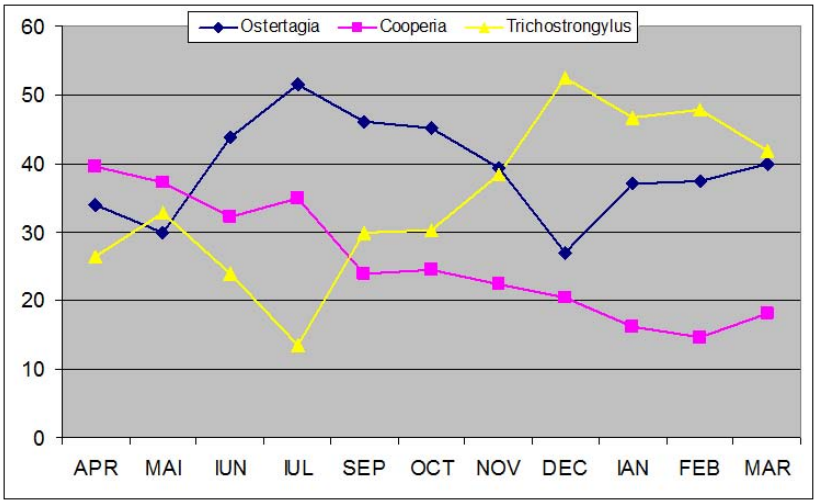


Fig.1 The behaviour of the Trichostrongylus genera in the bovine host according to seasons

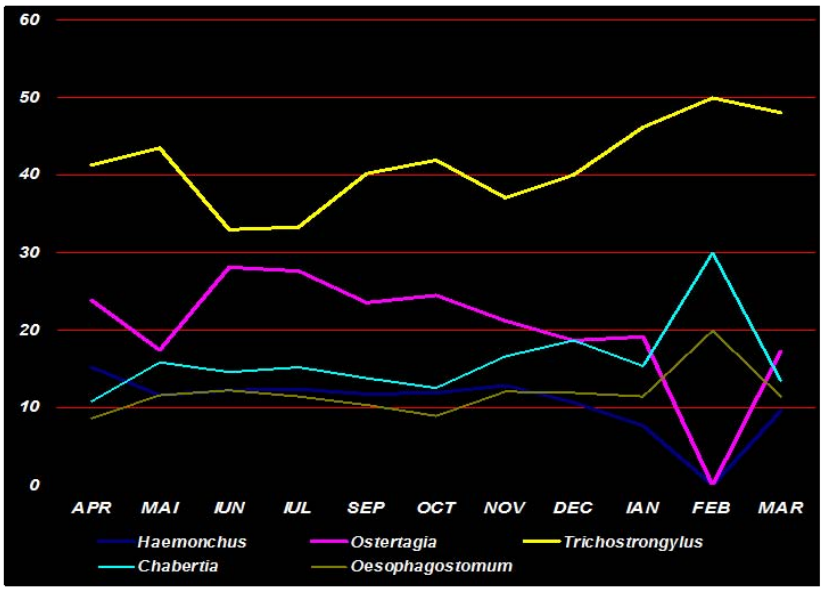


Fig. 2 The behaviour of gastrointestinal nematodes genera in the sheep host according to seasons

The genus *Chabertia* occupied third place. It had a behaviour similar to the genus *Oesophagostomum*, with a population peak in winter months when it reached 30%.

On the next position, we placed the genus *Ostertagia* that had its population peak in June-July. However, the population suffered a drastic decline up to 0% in February. Probably this drop of various *Trichostrongylus* infrapopulations is due to the hypobiosis phenomenon but also to aging of individuals that form the population. In addition, as a consequence of the "spring-rise" or "peri-parturient rise" phenomenon seen in the hypobiotic species (such as *Haemonchus* and *Ostertagia)*,

the populations recover in spring, thus contributing to the pollution of pastures with parasitic elements.

The most representative genus was *Trichostrongylus, which* had a relatively constant prevalence, often situated around 40%, regardless of the season. It has to be pointed out that in the case of species which do not use the hypobiosis phenomenon as a survival strategy, the infrapopulations were larger. However, this population rise is in fact due to the lowering of the individuals' number of the species which use hypobiosis.

The results of such coproscopic investigations are subjected to a number of variables such as: the moment of day in which the samples were collected (knowing that several species of helmints lay more eggs in the morning and others do this in the evening), the age of helmints (those who are elderly do not eliminate eggs), the abundance of female nematodes in the structure of the infrapopulation, their fertility, the pathogeny of the species, the quality of the host's immune response, the grazing seasons, the host's age, the consistency of faeces, etc. (9,15,22)

The study conducted by *Odoi* et all. (21) in Kenya has highlighted the presence of five genera of gastrointestinal nematodes: *Trichostrongylus* (42,0%), *Haemonchus* (35,8%), *Cooperia* (5,5%), *Strongyloides* (12,0%) and *Oesophagostomum* (4,7%). If in the case of *Trichostrongylus* the results were similar to ours, in the case of *Haemonchus* we noticed only 1/3 of the population reported in Kenya. Similar was the case of *Oesophagostomum.* In addition, we noted the presence of *Ostertagia* and *Chabertia* and the lack of *Strongyloides* and *Cooperia.*

However, in a study conducted in Venezuela by *Morales* et all. (16) they reported the presence of more gastrointestinal nematode genera, and the presence of more species: *Haemonchus, Trichostrongylus, Cooperia, Skrjabinema, Bunostomum, Oesophagostomum* and *Trichocephalus*.

In the American state of Nebraska, Colwell et all. (1) have observed that 99% of the gastrointestinal nematodes presents in lambs had only two representatives: *Ostertagia ostertagi* and *Nematodirus helvetianus*.

Theodoropoulos et al. (25) observed that in some arid regions of Greece, egg production through faeces increased rapidly in the summer months (June to August). In India, Shing et al. (24) had similar results to ours, with a low OPG in January-February and one that grew from July to peak in September

The research conducted in Pakistan on several sheep flocks by *Jan* et all. (14) has demonstrated a higher prevalence of parasitism with certain gastrointestinal nematodes in males compared to females: *Trichostrongylus spp*. – 12,5%/0%, *Haemonchus contortus* – 13,5%/11,5%, *Chabertia ovina* – 5,5%/1,5% while other nematodes were signaled only in females: *Ostertagia circumcincta* – 7,5%, *Oesophagostomum columbianum* – 10,5%.

In Romania, in the sheep from the western and Northwestern parts of the Timis County, *Indre* et all. (12) identified the following species of gastrointestinal nematodes, listed in a decreasing order: *Trichostrongylus* – 37%, *Chabertia* – 33%, *Ostertagia* – 30%, *Bunostomum* – 27%, *Haemonchus* – 26%, respectively *Cooperia* – 15%. The linear distribution of these parasites was as previously known with slight differences in what regards location: *Trichostrongylus colubriformis* – 75% in the duodenum, 87,5% in the jejunum and ileum, respectively 12,5% in the colon; *Nematodirus filicollis* – 42,85% in the duodenum and 85,71% in the jejunum and ileum or in the case of *Trichocephalus ovis* – 11,11% in the jejunum and ileum, 66,66% in the colon, respectively 88,88% in the cecum (13).

Conclusions

The coproscopic investigation made during cattle monitoring in Bethausen showed parasitism with only three genera of gastrointestinal nematodes: *Ostertagia, Trichostrongylus, Chabertia*.

Five parasitic genera were identified in sheep: *Haemonchus, Ostertagia, Trichostrongylus*, *Chabertia* and *Oesophagostomum.*

In both cattle and sheep, the biggest output of parasitic elements was seen in October.

Acknowledgements

This study is published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project No. POSDRU/159/1.5/S/132765 and this research work was carried out with the support ofthe projectDezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinei veterinare şi tehnologiilor inovative pentru RO 05, cod SMIS-CSNR 266.

References

1. COLWELL, D.D., GOATER, C.P., JACOBSON, K.M. (2002) - Prevalence and intensity of gastrointestinal nematodes in slaughter lambs from central Alberta. *Can. Vet. J*., 43, 775–777
2. CONSTANTIN, N. (coordonator) (2014) – Tratat de Medicină Veterinară, vol. VI. Editura Risoprint Cluj-Napoca.
3. COSOROABĂ, I., DĂRĂBUŞ, GH., OPRESCU, I. (1994) - Compendiu de parazitologie veterinară. Vol. I, Ed. Mirton, Timişoara.
4. DĂRĂBUȘ, GH., HOTEA, I., OPRESCU, I., MORARIU, S., BRUDIU, I., OLARIU, R.T., 2011 – Toxoplasmosis seroprevalence in cats and sheep from Western Romania. *Rev. Méd. Vét.*, 162 (6): 316-320.
5. DĂRĂBUŞ, GH., OPRESCU, I., MORARIU, S., MEDERLE, N. (2006) – Parazitologie şi boli parazitare. Ed. Mirton, Timişoara.
6. DĂRĂBUȘ, GH., OPRESCU, I., MORARIU, S., MEDERLE, N., ILIE, M. (2013) – Ghid practic în bolile parazitare, vol. 1. Editura Eurostampa Timișoara
7. DĂRĂBUȘ, GH., OPRESCU, I., MORARIU, S., MEDERLE, N., ILIE, M. (2014) – Ghid practic în bolile parazitare, vol. 2. Editura Eurostampa Timisoara
8. DIMANDER, S.O. (2003) – Epidemiology and control of gastrointestinal nematodes in first seazon grazing cattle in Sweden. Doctoral Thesis, Swedish University of Agricultural Sciences, Uppsala.
9. EYSKER, M., PLOEGER, H.W. (2000) – Value of present diagnostic methods for gastrointestinal nematode infections in ruminants. *Parasitology*, 120, 109-119.
10. IMRE, K., DĂRĂBUŞ, GH., MEDERLE, N., OPRESCU, I., MORARIU, S., ILIE, M., HOTEA, I., IMRE, M., INDRE, D., BALINT, A., SORESCU, D., 2010 - Intraspecific characterization of some *Cryptosporidium parvum* isolates from calves and lambs in Western Romania using molecular techniques. *Sci. Parasitol.*, 11 (1), 47-50.
11. IMRE, K., MORARIU, S., ILIE, M.S., IMRE, M., FERRARI, N., GENCHI, C., DĂRĂBUŞ, GH., 2012 – Serological survey of *Neospora caninum* infection in cattle herds from western Romania. *J. Parasitol.*, 98 (3): 683-685.
12. INDRE D., DĂRĂBUŞ GH., OPRESCU I., MORARIU S., MEDERLE N., ILIE M.S., HOTEA I., IMRE K., BALINT A. (2009) - Lineary distribution of nematoda into the gastrointestinal tract in sheep. *Lucr. Șt. Med. Vet. Iași*, 52, 1, 620-624
13. INDRE D., DĂRĂBUŞ GH., OPRESCU I., MORARIU S., MEDERLE N., ILIE M.S., MĂNDIŢĂ D.N. (2009) - The identification of gastrointestinal nematodes species in sheep in five localities from Timiș County. *Lucr. Șt. Med. Vet. Timișoara*, 42, 1, 94-100.
14. JAN, A., SHAH, H., AHMAD, I., YOUNAS, M., ULLAH, R., HAROON (2015) - Prevalence and comparison of ovine gastrointestinal helminthes parasites in domesticated and farmed, male and female sheep at University Town Peshawar, Pakistan. *J. Entomol. Zool. Stud*., 3, 3, 350-353.
15. KLOOSTERMAN, A., PLOEGER, H.W., FRANKENA, K. (1991) - Age resistance in calves to *Ostertagia ostertagi* and *Cooperia oncophora*. *Vet. Parasitol*., 39, 101-113.

16. MORALES, G., PINO, L.A., SANDOVAL, E., MORENO, L. (2001) - Gastrointestinal nematode infection in ewes raised in an arid zone of Venezuela. *Parasitol. Día*, 25, 1-2, 36-39.
17. MORARIU S., BULACU A., DĂRĂBUȘ GH., MEDERLE N., MIHĂILĂ C., NECHITI R., BRÎNCOVEANU A., BADEA C. (2014) - Prevalence of internal parasites in wisents (*Bison bonasus bonasus*) from two Romanian reservations - preliminary results. *Lucr. Șt. Med. Vet. Iași*, 57, 3-4, 170-173.
18. MORARIU, S., BART, J.M., COSOROABĂ, I., MORARIU, F., ILIE, M., DĂRĂBUŞ, GH., 2011 - Prevalence of cystic echinococcosis in sheep, cattle and swine from the western Romania. *Sci. Parasitol.*, 12 (1): 47-51
19. MORARIU, S., LIGHTOWLERS, M.W., COSOROABĂ, I., DĂRĂBUŞ, GH., BART, J.M., ILIE, M., MORARIU, F., MORAR, D., OPRESCU, I., MEDERLE, N., IMRE, K., BELEAN, M., 2010 - Utilization of EG95 vaccine for sheep immunization against cystic echinococcosis in Romania. *Sci. Parasitol.*, 11 (1): 29-34.
20. MORARIU, S., LIGHTOWLERS, M.W., COSOROABĂ, I., MORARIU, F., DĂRĂBUŞ, G., ILIE, M., BELEAN, M., 2005 - The first use in Romania of Eg95 vaccine to protect sheep against hydatidosis.*Rev. Rom. Med. Vet.*, 15 (2), 97-104.
21. ODOI, A., GATHUMA, J.M., GACHUIRI, C.K., OMORE, A. (2007) – Risk factors of gastrointestinal nematode parasite infections in small ruminants kept smallholder mixed farms in Kenya. *BMC Vet. Res*., 3, 6, 1-11.
22. PLOEGER, H.W., KLOOSTERMAN, A., RIETVELD, F.W., BERGHEN, P., HILDERSON, H., HOLLANDERS, W. (1994) - Quantitative estimation of the level of exposure to gastrointestinal nematode infection in first-year calves. *Vet. Parasitol*., 55, 287-315.
23. REINECKE, R.K. (1994) - Parasitic control in intensive vs. non-intensive system ruminants. *Vet. Parasitol*., 54, 1-3, 49-67.
24. SHING, D., SWARNKAR, C.P., KHAN, F.A., SRIVASTAVA, C.P., BHAGWAN, P.S.K. (1997) - Epidemiology of ovine gastrointestinal nematodes at an organised farm in Rajasthan, India. *Small Rum. Res*., 26, 31–37.
25. THEODOROPOULOS, G., KOUTSOTOLIS, K., NIKOLAOU, E., KALOGIANNIS, D., PETRAKOS, G. (1998) - Seasonal variation of gastrointestinal nematodes of sheep in the region of Joannina, Greece. *Int. J. Parasitol*., 28, 1287–1292.
26. *** - http://www.e-primarii.ro/primaria-bethausen, accesat la data de 24.05.2016

Preliminary research regarding the prevalence of digestive and respiratory parasitosis in meat cattle from the Hârtibaci Valley, Sibiu County

Radu NECHITI[1], Gheorghe DĂRĂBUȘ[2], Sorin MORARIU[2]
[1] Sanitary and Veterinary Circumscription Nocrich, jud. Sibiu;
[2] Faculty of Veterinary Medicine Timișoara
niky_dok@yahoo.com

Abstract

In Romania, the data regarding gastrointestinal and respiratory parasitism in cattle are scarce and incomplete. This study was undertaken on Angus breed cattle from an intensive-type exploitation in Nochrich, Sibiu County. The faeces samples were examined both through qualitative and quantitative methods. The most widespread type of parasitism was the one caused by the ciliate Balantidium coli (51.61%), followed in equal manner by Eimeria (32.25%) and Fasciola/Paramphistomum. The digestive strongyles (22.58%) were less representative. These were followed in a decreasing order by the infestation with Dictyocaulus viviparus (16.13%) and by Strongyloides spp. (12.90%).

Key words*: Angus breed cattle, internal parasites, prevalence, Nocrich.*

Introduction

In most cases, cattle are raised for milk, meat and skins but, more seldom, they can be used to keep a good maintenance of pastures, various sporting activities (rodeo, corrida) and for participation in contests in the agricultural field. Thus, they produce up to 90% of the total quantity of milk consumed worldwide, 30% of the meat quantity and 90% of the skins used in the leather industry (Acatincai, 2004).

The production forcing, along with the impact of dejections on the environment are deficiencies in the management of cattle. The prejudice brought to the state of health (and implicitly to production) by the presence of internal and external parasites should also be added. Although cows can host more species of parasites in the digestive and respiratory tracts, only some of them have a clinical or economic significance. We can list the species of the *Ostertagia* genus, located in the abomasum, species of the *Eimeria* genus especially *E. bovis* and *E. zuernii* but also the pulmonary nematode *Dictyocaulus viviparus.* Several species of trematodes like *Fasciola hepatica* and those of the genus *Paramphistomum* to which you can add cryptosporidia, *Neospora caninum* or *Echinococcus granulosus* as well as others can be mentioned (Darabus et al., 2011, Imre et al., 2010, 2012, Morariu et al., 2011). Their transmission is achieved mostly through a digestive path but it can also be realised transcutaneous, at pasture. In addition, the economic losses are owed to the expenses of antiparasitic treatments though more alternatives that are efficient are available like vaccines against some parasitic diseases (Morariu et al., 2005, 2010).

Bibliographic studies indicate a high incidence of parasitic diseases in cattle on an international level, especially in the case of those raised in a free-range system, on grazing lands (Darabus et al., 2006, Jager et al. 2005, Pilarczyk et al., 2009). This is the reason why the present paper aims to establish the epidemiological situation regarding the internal parasitism in cattle raised in an extensive system, especially of Angus breed cattle from the Hârtibaci Valley, Sibiu County.

Materials and methods

The Hârtibaci Valley is found in the central-Eastern part of the Sibiu County and it is part of the Hârtibaci Plateau. This area spreads on a surface of 237.515 ha, distributed as follows: 51% in the Sibiu County, 35% in the Brașov County and 14% in the Mureș County (Figure 1).

Fig. 1-Etnographic areas of the Sibiu County
(after http://bjastrasibiu.ro/biblioteci-din-judet/)

During the year 2016, a number of Angus cattle were subjected to coproparasitic examinations. They belonged to Karpaten Meat (KM) beef cattle exploitations in the Nochrich area, Sibiu County (Table 1). Both quantitative and qualitative coproscopic methods were used during examinations.

Results and discussions

From a total of 31 examined samples, using the above-mentioned methods, three were negative (9.67%), nine were positive with a single parasite species - monoparasitism (29.03%) and most of them presented polyparasitism, harbouring two or more species of parasites (61.29%).

The most widespread parasite was the ciliate *Balantidium coli* (51.61%), followed by *Eimeria* (32.25%) and in equal measure, by *Fasciola/Paramphistomum.* Surprisingly, the digestive strongyles were less representative (22.58%). These were followed, in a decreasing order, by *Dictyocaulus viviparus* (16.13%) and by *Strongyloides* spp. (12.90%). The data are presented in Figure 2.

The massive presence of *B. coli* denotes feeding deficiencies, which permitted an excessive multiplication of the ciliate and its transmission in the cattle herd.

Eimeriosis is largely spread, both in Europe and on the other continents were cattle are bred as well (Davoudi et al. 2011). The registered prevalence varied from country to country and from region to region (Bangoura et al. 2012) from 8.25% in Iran (Heidari et al., 2014) and 93% in Poland (Pilarczyk et al., 2009).

Table 1. The results obtained after the examination of faeces from Angus cattle

No.	Owner	Registrationno.	Sex	Age (years)	Observations
1.	Karpaten Meat	27541	F	6	Digestive strongyles, Eimeria, Balantidium
2.		31642	F	5	Eimeria, Balantidium
3.		18666	F	6	Eimeria, Balantidium
4.		8488	F	1	Negative
5.		9203	F	1,5	Digestive strongyles, Eimeria, Balantidium
6.		8978	F	1,5	Negative
7.		2385	M	1,5	Digestive strongyles, Eimeria
8.		2389	M	8 months	Digestive strongyles, Eimeria
9.		9306	F	8 months	Digestive strongyles, Eimeria
10.		9256	M	2	Dictyocaulus
11.		9264	M	2	Dictyocaulus
12.		9250	M	2	Negative
13.		9254	M	2	Balantidium
14.		8021	F	10 months	Balantidium
15.		8233	F	10 months	Fasciola/Paramphistomum
16.		8078	F	10 months	Fasciola/Paramphistomum
17.		8164	F	10 months	Fasciola/Paramphistomum
18.		8369	F	4	Fasciola/Paramphistomum
19.		8301	F	4	Fasciola/Paramphistomum, Balantidium, Strongyloides
20.		8256	F	4	Fasciola/Paramphistomum, Balantidium, Strongyloides, Digestive strongyles, Dictyocaulus
21.		8266	F	4	Fasciola/Paramphistomum, Balantidium, Strongyloides
22.		1572	F	6	Fasciola/Paramphistomum
23.		4269	F	5	Fasciola/Paramphistomum, Eimeria
24.		9527	F	6	Fasciola/Paramphistomum, Digestive strongyles, Dictyocaulus
25.		1637	F	6	Balantidium, Eimeria
26.		9526	F	7	Balantidium
27.		1634	F	7	Balantidium
28.		8104	F	2	Balantidium
29.		8138	F	2	Balantidium
30.		3400	F	4	Balantidium, Eimeria, Strongyloides, Dictyocaulus
31.		3397	F	4	Balantidium

However, cryptosporidiosis has a different distribution and even in the neighbouring areas, higher variations in values may be noticed: 5% in Sweden (Bjorkman et al., 2003) and 86.7% in Tunis (Soltane et al., 2007).

Fasciolosis has affected 0.5% cattle in Turkey (Sariozkan and Yalcin, 2011) and 90.7% of the Ethiopian herds (Behre et al., 2009) while *Paramphistomum* was present in 4.25% of the

cattle from Pakistan (Khan et al., 2008) and 53.4% of the Irish cattle population (Toolan et al. 2015).

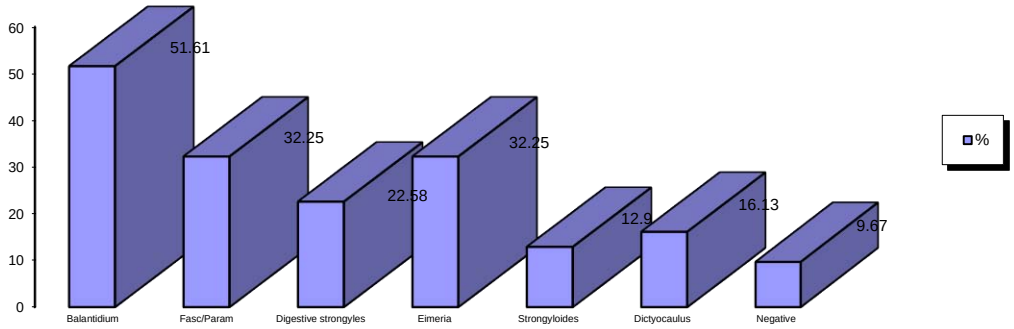


Fig.2- The percentual repartition of types of parasites identified in Angus cattle

Dictyocaulus viviparus was diagnosed in 1.8% of the calves investigated in Costa Rica (Jimenez et al. 2007) and 50% of the cattle investigated from a farm in Canada after the introduction of carrier calves (Wapenaar et al., 2007).

Regarding the trichostrongyles, data are variable according to the implied genus and according to the region. Thus, the prevalence ranges from 0.3% in Italy (Forbes et al., 2008) of serum samples and 81.4% in Costa Rica (Jimenez et al., 2007).

For Romania, the data regarding gastrointestinal nematodes is abundant for sheep and goats in the detriment of cattle. The research from the past few years has been addressed especially to parasitic diseases in sheep. The most recent detailed research in this matter was conducted by Avram (2003), who studied the situation of the internal parasitism in cattle from Satu-Mare County. It was observed that the prevalence of parasitism with gastrointestinal nematodes ranged according to region, having total values comprised between 38.6% and 66.2%. The most important genera implied in the parasitic pathology of cattle from Satu-Mare County were in decreasing order: *Trichostrongylus, Cooperia, Ostertagia, Haemonchus* and *Nematodirus.*

Conclusions

In the Karpaten Meat exploitation the infestation with various genera of parasites was reported.

Parasites from three classes were identified: Protozoa, Trematoda and Nematoda.

The most widespread parasites were: *Balantidium coli* (51.61%), *Eimeria* and *Fasciola/Paramphistomum* (32.25%), and less noticed were: *Dictyocaulus viviparus* (16.13%) respectively *Strongyloides* spp (12.90%).

Acknowledgements

This study is published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project No. POSDRU/159/1.5/S/132765 and this research work was carried out with the support ofthe projectDezvoltarea infrastructurii de cercetare, educaţie şi servicii în domeniile medicinei veterinare şi tehnologiilor inovative pentru RO 05, cod SMIS-CSNR 266.

References

1. Acatincăi, S., 2004 – Producțiile bovinelor, ediția a 2-a. Editura Eurobit, Timișoara.
2. Avram, E., 2003 - Supravegherea parazitologică a nematodozelor gastrointestinale (trichostrongilidoze) ale bovinelor din județul Satu Mare. Teză de doctorat, USAMVB Timișoara.
3. Bangoura, B., Mundt, H.C., Schmaschke, R., Westphal, B., Daugschies, A., 2012 - Prevalence of *Eimeria bovis* and *Eimeria zuernii* in German cattle herds and factors influencing oocyst excretion. *Parasitol. Res*., 110, 875–881.
4. Behre, G., Berhane, K., Tadesse, G., 2009 – Prevalence and economic significance of fasciolosis in cattle in Mekelle Area of Ethiopia.*Trop. Anim. Health Prod*., 41, 1503–1504.
5. Björkman, C., Svensson, C., Christensson, B., de Verdier, K. 2003 – *Cryptosporidium parvum* and *Giardia intestinalis* in calf diarrhoea in Sweden. *Acta Vet. Scand*., 44, 145-152.
6. Davoudi, Y., Gharedaghi, Y., Nourmohammadzade, E., Eftekhari, Z.S., Safarmashaei, S., 2011 - Study on prevalence rate of coccidiosis in diarrheic calves in East-Azarbaijan province. *Adv. Environ. Biol*., 5, 1563–1562.
7. Dărăbuș, Gh., Hotea, I., Oprescu, I., Morariu, S., Brudiu, I., Olariu, R.T., 2011 – Toxoplasmosis seroprevalence in cats and sheep from Western Romania. *Rev. Méd. Vét.*, 162 (6): 316-320.
8. Dărăbuș, Gh., Oprescu, I., Morariu, S., Mederle, N., 2006 - Parazitologie și boli parazitare. Editura Mirton, Timișoara.
9. Forbes, A.B., Vercruysse, J., Charlier, J., 2008 - A survey of the exposure to *Ostertagia ostertagi* in dairy cow herds in Europe through the measurement of antibodies in milk samples from the bulk tank. *Vet. Parasitol*., 157, 1, 100-107.
10. Heidari, H., Sadeghi-Dehkordi, Z., Moayedi, R., Gharekhani, J., 2014 – Occurrence and diversity of *Eimeria* species in cattle in Hamedan province, Iran. *Vet. Med.*, 59, 6, 271–275.
11. Imre, K., Dărăbuş, Gh., Mederle, N., Oprescu, I., Morariu, S., Ilie, M., Hotea, I., Imre, M., Indre, D., Balint, A., Sorescu, D., 2010 - Intraspecific characterization of some *Cryptosporidium parvum* isolates from calves and lambs in Western Romania using molecular techniques. *Sci. Parasitol.*, 11 (1), 47-50.
12. Imre, K., Morariu, S., Ilie, M.S., Imre, M., Ferrari, N., Genchi, C., Dărăbuş, Gh., 2012 – Serological survey of *Neospora caninum* infection in cattle herds from western Romania. *J. Parasitol.*, 98 (3): 683-685.
13. Jäger, M., Gauly, M., Bauer, C., Failing, K., Erhardt, G., Zahner, H., 2005 - Endoparasites in calves of beef cattle herds: management systems dependent and genetic influences. *Vet. Parasitol*., 31, 3, 173-191.
14. Jiménez, A.E., Montenegro, V.M., Hernández, J., Dolz, G., Maranda, L., Galindo, J., Epe, C., Schnieder, T., 2007 - Dynamics of infections with gastrointestinal parasites and *Dictyocaulus viviparus* in dairy and beef cattle from Costa Rica. *Vet. Parasitol*., 148, 3-4, 262–271.
15. Khan, U.J., Tanveer, A., Maqbool, A., Masood, S., 2008 – Epidemiological studies of paramphistomosis in cattle. *Vet. Arhiv*, 78, 3, 243-251.
16. Morariu, S., Bart, J.M., Cosoroabă, I., Morariu, F., Ilie, M., Dărăbuş, Gh., 2011 - Prevalence of cystic echinococcosis in sheep, cattle and swine from the western Romania. *Sci. Parasitol.*, 12 (1): 47-51
17. Morariu, S., Lightowlers, M.W., Cosoroabă, I., Dărăbuş, Gh., Bart, J.M., Ilie, M., Morariu, F., Morar, D., Oprescu, I., Mederle, N., Imre, K., Belean, M., 2010 - Utilization of EG95 vaccine for sheep immunization against cystic echinococcosis in Romania. *Sci. Parasitol.*, 11 (1): 29-34.
18. Morariu, S., Lightowlers, M.W., Cosoroabă, I., Morariu, F., Dărăbuş, G., Ilie, M., Belean, M., 2005 - The first use in Romania of Eg95 vaccine to protect sheep against hydatidosis. *Rev. Rom. Med. Vet.*, 15 (2), 97-104.
19. Pilarczyk, B., Balicka-Ramisz, A., Kozak, W., Ramisz, A., 2009 - Occurrence of endoparasites in heifers imported to Poland from the Netherlands. *Arch. Tierzucht*, 52, 265–271.
20. Sariözkan, S., Yalçin, C., 2011 - Estimating the total cost of bovine fasciolosis in Turkey. *Ann. Trop. Med. Parasitol*., 105, 6, 439–444.
21. Soltane, R., Guyot, K., Dei-Cas, E., Ayadi, A., 2007 – *Cryptosporidium parvum* (Eucoccidiorida: Cryptosporiidae) in calves: results of a longitudinal study in a dairy farm in Sfax, Tunisia. *Parasite*, 14, 4, 309–312.
22. Toolan, D.P., Mitchell, G., Searle, K., Sheehan, M., Skuce, P.J., Zadoks, R.N., 2015 - Bovine and ovine rumen fluke in Ireland - prevalence, risk factors and species identity based on passive veterinary surveillance and abattoir findings. *Vet. Parasitol.*, 212, 3-4, 168-174.
23. Wapenaar, W., Barkema, H.W., Eysker, M., O'Handley, R.M., 2010 - An outbreak of dictyocaulosis in lactating cows on a dairy farm. *JAVMA*, 231, 11, 1715-1718.
24. *** - http://bjastrasibiu.ro/biblioteci-din-judet/

Research on metabolic status in periparturient cows

[1]Sorin D. SORESCU, [1]Carmen IONIȚĂ, [2]Alice GRIGORE, [1]Emilia BALINT, [1]Cosmin ȘONEA [1]Ana Maria GOANȚĂ, [1]Roxana ȚÎMPĂU, Lucian IONIȚĂ

[1]FMVB Facultatea de Medicină Veterinară București, 105 Splaiul Independenței, Bucharest

[2]National Institute for Chemical andPharmaceutical Research and Development (ICCF), 112 Calea Vitan, Bucharest,

ionitacarmen63@yahoo.com; alicearmatu@yahoo.com; emilia_balint@yahoo.com ana_mv@yahoo.com; roxana_mariana_12@yahoo.ro; ionital@yahoo.com

Abstract

In the experiment, hematological and blood biochemical parameters were determined in a batch of 5 cows in the last week of gestation (Group 1) and 5 cows in the first week after calving (Group 2). Cows are clinically healthy and come from a farm where the milk production per fed animal is about 30 liters/day, cows being milked 3 times per day. Hematologic parameters were found within physiological limits, but in both groups the monocytes were found to be low, and in group 1, mild lymphopenia was detected. Investigated blood biochemical parameters allowed to assert that in cows in the last week of gestation, bilirubin was found to be significantly increased when recently-bred cows were within normal limits. In both lots, LDH was found to be significantly increased. Metabolic status also determined the protein fractions by means of electrophoresis: 10 samples were analyzed (Group 3 consisting of 5 cows in the last week of gestation and Group 4 consisting of 5 cows in the first week after calving). The values of the protein fractions were within the physiological limits and the Albumin/Globulin Ratio was found within physiological limits in group 3 and lower in group 4, which confirms gamma globulin reactivation immediately after calving.

Key words*: metabolism, parturition, protein fractions, immunoglobulins.*

Introduction

It is known that a lot of changes occur in the dairy cow during the transition period (21 days before parturition and 21 days after parturition). In this study we tried to obtain a more detailed table of the matabolic status of the periparturient cows closer to the moment of parturition (7 days before parturion and 7 days after parturition) (5, 9). Dairy cattle, like many other species, often consume less feed in the week prior to parturition (Grummer et al., 2004), and it can take up to a week post-calving before dry matter intake (DMI) exceeds what the cow was consuming in late gestation (6). The fatty liver present at one day after calving is negatively corelated whith feed intake one day prepartum (3).

Metabolic disorders are a key problem in the transition period of dairy cows and often appear before the onset of further health problems. Problems derive from the difficulty of the animals to adapt to large variations and disturbances occurring outside and inside the organism. (4) Oxidative stress is also known to be an important factor of the metabolic dysfunctions during this period. (Miller et al., 1993; Sordillo and Aitken, 2009). A lack of success in solving these issues may be due to predominant approaches in farm management and agricultural science. Instead, a successful adaptation of animals to their living conditions should be seen as an important end in itself. Both farm management and agricultural sciences should support animals in their ability to cope with nutritional and metabolic challenges by employing a functional and result driven approach (9). Techniques of modern hematology and biochemistry promise to further our understanding of the mechanisms of metabolic adaptation during the peripartal period, and to quantify the effects of nutrition and environment during pre-and postpartum periods on hepatic glucose and lipid metabolism (1,2,7).

Another important aspect of the blood and its constituents is the fact that is very dependable on the medium (temperature, way of collecting, stresss of collecting the blood) for evaluating

physiological changes in the physical and health status of an animal (Egbe-Nwiyi et al., 2000; Žvorc et al., 2006; Njidda et al., 2014).

Materials and methods

In this paper we aim to achieve a metabolic monitoring of the main biomacromolecules (proteins, lipids, carbohydrates) and enzyme and mineral status to prevent possible dismetabolites, which once detected could be rectified so that even the worst period of gestation , the transition period provides optimal comfort for completing gestation and obtaining healthy newborns.

To complete the research, we chose as location a cow farm near the capital, with a tradition of raising dairy cows. At present, the farm hosts 574 cows, of which 309 cows, 65 heifers, 172 calves over 6 weeks, 18 calves above 6 weeks and 10 other categories. Cows are kept in free standing on straw bedding. As experimental protocol, we made 4 groups, each one consisting of 5 cows, as follows:

Group 1: 5 cows in the last week of gestation and Group 2: 5 cows in the first week after calving, from which we collected blood samples in order to compare the biochemical and haematological blood parameters between the 2 groups;

Group 3 and group 4 with a similar consitency, but in this case, we collected blood samples for determination of the protein fractions using a technique of electrophoresis; all paraclinical examinations were performed in our discipline laboratory.

Results and discussion

As presenting in the table below, the hematological exam in cows during the gestation week reveal a monocytopenia present in all cows from group A and also a lymphocitopenia present at four of the cows from this group. The other parameters were found within physiological limits.

Table 1. The Results of the Hematological Exam at Cows in the Last Week of Gestation (Group1)

PARAMETER	U/M	Physiolog. limits	Cow 71055	Cow 27829	Cow 53538	Cow 65317	Cow 8126
WBC	$10^{-9}/mm^3$	4-12	7,72	6,60	8,75	**13,22**	8,82
LYM	$10^{-9}/mm^3$	2,5-7,5	2,56	3,64	3,76	5,61	3,30
MON	$10^{-9}/mm^3$	0-1	0,08	0,06	0	0,12	0,13
NEU	$10^{-9}/mm^3$	0,6-7,6	4,88	2,01	4,75	7,18	5,19
EOS	$10^{-9}/mm^3$	0,1-1	0,19	0,10	0,16	0,30	0,20
BAS	$10^{-9}/mm^3$	0-0,5	0	0	0,01	0,01	0,01
LYM	%	45-75	**33,2**	55,1	**42,9**	**42,4**	**37,4**
MON	%	2-7	**1,0**	**0,9**	**0,9**	**0,9**	**1,5**
NEU	%	15-65	63,3	42,5	54,3	54,3	58,8
EOS	%	1-8	2,5	1,5	1,9	2,3	2,2
BAS	%	0-3	0,1	0	0,1	0,1	0,1
RBC	$10^{-12}/mm^3$	5-10	6,65	6,96	7,34	8,18	7,29
HGB	g/dl	8-15	10,8	9,6	11,3	11,9	11,5
HCT	%	24-46	33,43	29,44	33,51	37,55	34,59
MCV	fl	40-60	50	42	46	46	48
MCH	pg	11-17	16,2	13,8	15,3	14,5	15,7
MCHC	g/dl	30-36	32,2	32,6	33,6	31,7	33,1
PLT	$10^{-9}/mm^3$	100-800	250	362	392	340	425

Monocytopenia is known to appear as a result of aplastic anemy, pancytopenia and also after using medication like: prednisolon, alprazolam, triazolam, but in this case we will strictly corelate it with the advanced stage of getation. Monocytes are the largest cells in the blood; are released into the blood and after a short while in circulation, migrate into different tissues, incidentally or specifically, in response to various chemotactic factors. In tissues, in response to different soluble factors, they differentiate into tissue macrophages with characteristic morphological and functional qualities, a process that has been called "activation" and which is reversible ("deactivation"). The cells of the phagocytic mononuclear system are very primitive phylogenetic, and no animal can live without them. They perform a wide variety of important functions in the body, including removal of foreign particles and senescent cells, dead or altered, regulation of other cell functions, processing and presentation of antigens in immune reactions, participation in various inflammatory reactions, destruction of bacteria and tumor cells.

Table 2. The Results of the Hematological Exam at cows in the first week after calving (Group 2)

PARAMETER	U/M	Physiolog. limits	78178	80501	23747	8096	8181
WBC	$10^{-9}/mm^3$	4-12	10,43	8,44	8,72	9,54	9,43
LYM	$10^{-9}/mm^3$	2,5-7,5	4,59	3,09	2,92	5,42	5,37
MON	$10^{-9}/mm^3$	0-1	**0,11**	0,04	0,85	0,09	0,16
NEU	$10^{-9}/mm^3$	0,6-7,6	5,41	4,02	4,64	3,70	3,69
EOS	$10^{-9}/mm^3$	0,1-1	0,32	0,47	0,30	0,32	0,21
BAS	$10^{-9}/mm^3$	0-0,5	0,01	0,01	0,01	0,01	0,01
LYM	%	45-75	**44**	46,1	**33,5**	56,8	57,0
MON	%	2-7	**1,0**	**0,5**	**9,7**	**0,9**	**1,7**
NEU	%	15-65	51,9	47,7	53,2	38,7	39,1
EOS	%	1-8	3,1	5,6	3,5	3,4	2,2
BAS	%	0-3	0,1	0,1	0,1	0,1	0,1
RBC	$10^{-12}/mm^3$	5-10	7,3	5,51	6,39	6,65	8,00
HGB	g/dl	8-15	11,2	9,0	10,7	9,7	11,3
HCT	%	24-46	35,65	28,36	32,14	30,91	34,57
MCV	fl	40-60	49	51	50	46	43
MCH	pg	11-17	15,4	16,4	16,8	14,6	14,2
MCHC	g/dl	30-36	31,5	31,9	33,3	31,4	32,7
PLT	$10^{-9}/mm^3$	100-800	242	257	294	379	297

Regarding Group 2, the results of hematological exam are very similar to those from group 1, the values are very little semnificative modified according to the physiological vallues.

At the biochemical examination of the blood, it was noticed an increasing of Lactat Dehydrogenase (LDH) and Total Bilirubin (T-bil), which is directly corelated with the fiziological status of the cows from Group 1. There are also variations of he other parameters: T-Cho, Creatinine and Uric acid.

Tabel 3. The Results of the Biochemical Exam at Cows in the Last Week of Gestation (Group 1)

Parameter	U/M	Physiolog. limits	71055	27829	53538	65317	8126
T- Pro	g/dl	5,8-8,5	6,9	6,5	7,1	6,8	6,3
Albumin	g/dl	2,5-3,7	2,9	2,7	2,7	3,3	3,5
Globulin	g/dl	3,3-4,8	4,0	3,8	4,4	3,5	**2,8**
BUN	mg/dl	10-25	10	11	**9**	11	13
UA	mg/dl	1,0-2,1	**0,9**	**0,8**	1,0	1,1	**0,9**
Cre	mg/dl	0,4-1,0	**1,4**	1,0	1,0	**1,2**	**1,1**
T-Cho	mg/dl	70-280	**59**	**50**	75	85	71
GOT	IU/l	78-132	88	89	68	91	92
LDH	IU/l	692	**1445**	**1348**	**1422**	**1654**	**1637**
T-Bil	mg/dl	0-0,3	**1,0**	**0,6**	**0,4**	**0,7**	**0,5**
GPT	IU/l	0-82	8	7	9	12	7
ALP	IU/l	0-80	118	113	110	135	69

Table 4. The Results of the Biochemical Exam at cows in the first week after calving (Group 2)

Parameter	U/M	Physiolog. limits	78178	80501	23747	8096	8181
T- Pro	g/dl	5,8-8,5	6,4	6,6	6,2	7,1	**5,4**
Albumin	g/dl	2,5-3,7	3,2	3,4	3,3	3,3	3,2
Globulin	g/dl	3,3-4,8	3,2	3,2	**2,9**	3,8	**2,2**
BUN	mg/dl	10-25	**9**	13	13	**7**	**9**
UA	mg/dl	1,0-2,1	**0,9**	**0,7**	**0,7**	**0,8**	**0,7**
Crea	mg/dl	0,4-1,0	**1,1**	**1,2**	**1,5**	1,0	**1,2**
T-Cho	mg/dl	70-280	98	66	98	92	77
GOT	IU/l	78-132	113	51	48	42	76
LDH	IU/l	692-1445	**2343**	**1973**	**1561**	**1588**	**2256**
T-Bil	mg/dl	0-0,3	**0,5**	0,3	**0,4**	0,3	0,3
GPT	IU/l	0-82	10	7	7	7	10

At the biochemical examination of the blood collected from group B, it is noticed that the values of LDH and T-Bil continue to be increased and also, Creatinine reveals an increasing. Uric Acid seems to be decreased at all the cows from this group.

Table 5. Determination of protein fractions by electrophoresis in Group 3

Fraction	U/M	Physiolog. limits	8181	8096	23746	78178	80501
Total Protein	g/dl	5,8-8,5	**5,40**	7,10	6,20	6,40	6,60
Albumin	g/dl	1,3-2,47	1,81	1,47	1,51	1,87	1,84
α_1	g/dl	0,19-0,78	0,27	0,30	0,37	0,31	0,33
α_2	g/dl	0,19-0,78	0,50	0.63	0,63	0,67	0,62
β_1	g/dl	0,32-0,84	0,80	**0,95**	0,79	**0,93**	0,77
β_2	gd/l	0,32-0,84	0,49	0,61	0,61	0,53	0,47
γ	g/dl	1,75-2,72	**1,54**	**3,14**	2,30	2,09	2,56
Alb/Glob	/	0,45-1,31	0,50	**0,26**	**0,32**	**0,41**	**0,39**

Determination of protein fractions reveals insignificant changes according to the physiological limits for the cows in the last week of gestation. The decrease Albumin/Globulin ratio is associated with the physiological status of the cows.

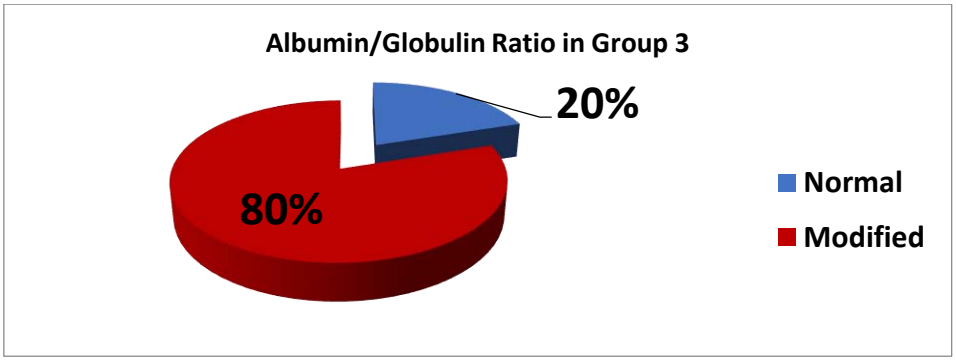


Fig. 1 Distribution of Albumin/Globulin Ratio in Group 3

Table 6. Determination of protein fractions by electrophoresis in Group 4

Fraction	U/M	Physiolog. limits	27189	53538	65317	71055	8126
Total Protein	g/dl	5,8-8,5	6,50	7,10	6,80	6,90	6,30
Albumin	g/dl	1,3-2,47	1,93	2,08	2,20	**2,62**	**2,76**
α_1	g/dl	0,19-0,78	0,42	0,35	**0,10**	0,41	0,30
α_2	g/dl	0,19-0,78	0,60	0,79	0,78	0,49	0,40
β_1	g/dl	0,32-0,84	**1,00**	0,75	0,97	0,69	0,64
β_2	gd/l	0,32-0,84	0,34	0,57	0,76	0,52	0.35
γ	g/dl	1,75-2,72	2,21	2,57	1,99	2,17	1,86
Alb/Glob	/	0,45-1,31	**0,42**	**0,41**	0,48	0,61	0,78

At one week after calving, the results of the elecrophoresis reveals very unsemnificative changes according to the physiological limits, as follows: 2 of the cows from this group had a decreased Albumin/Globulin Ratio and other two cows had an increased value of the albumin vallue. This fact might be associated with the feed intake and also with some interferences.

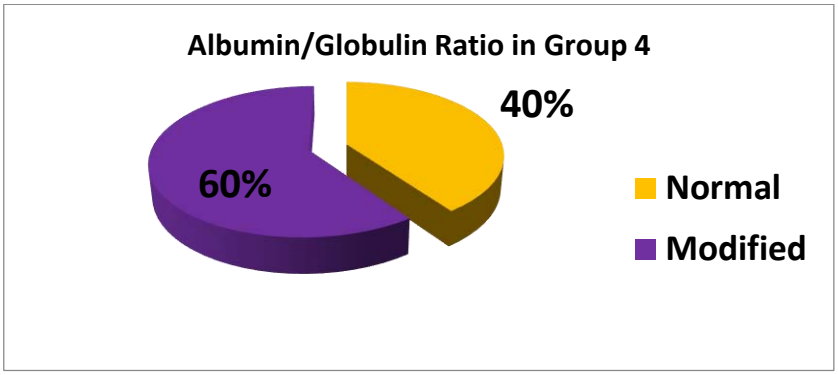


Fig. 2 Distribution of Albumin/Globulin Ratio in Group 4

Conclusions

Clnically, all the cows taken in our study are clinically healthy, but at a routine blood exam reveals a lot of changes in their metabolic status, as follows: monocytopenia, lymphocitopenia and increased values of Total Bilirubin and Lactat Dehidrogenasys in the first two groups of cows.

There are no major diferences between the cows one week prior parturition and the cows after parturition, due to the period taken into account for this study, but regarding albumin/ globulin ratio it was noticed that in the cows before parturition is more significant modified than in the cows after parturition, which confirms gamma globulin reactivation immediately after calving.

By correlating the results obtained, it can be argued that routine explorations are an important tool in the diagnostic of some problems with the management of the farm and also, of the animals like: alimentary disorders, deficiency of food intake, oxidative stress.

References

1. Calamari L., Ferrari Annarita, Minuti A., Trevisi E. (2016), *Assessment of the main plasma parameters included in a metabolic profile of dairy cow based on Fourier Transform mid-infrared spectroscopy: preliminary result,* BMC Vet Res. 2016; 12:
2. Fischbach F. (2009), *Manual of Laboratory and Diagnostic Tests Lippincott*, Williams & Wilkins, USA, 8 Ed., 2009, 199-210
3. Grummer R.(1993), *Etiology of Lipid-Related Metabolic Disorders in Periparturient Dairy Cow,* J. Dairy Sci 76:3882-3896
4. Ioniţă L. (2008). *Patologie şi clinică medicală veterinară.* Vol I, Editura Sitech, Bucureşti
5. James K.Drackley, Thomas R.Overton, G. Neil Douglas (2010*). Adaptations of Glucose and Long-Chain Fatty Acid Metabolism in Liver of Dairy Cows during the Periparturient Period*, Veterinary Immunology and Immunopathology
6. Lorraine M. Sordillo*, G. A. Contreras and Stacey L. Aitken (2009). *Metabolic factors affecting the inflammatory response of periparturient dairy cows,* Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA
7. Roland L., Drillich M, Iwersen M., (2014), *Hematology as a diagnostic tool in bovine medicine,* Journal of Veterinary Diagnostic Investigation, Vol. 26(5) 592 –59
8. Sundrum A., Clive J. C. Phillips (2009), *Metabolic Disorders in the Transition Period Indicate that the Dairy Cows' Ability to Adapt is Overstressed,* BMC Vet Res. 2009
9. Weary D., Keyserlingk G., Rushen J., (2009), *The welfare of dairy cattle - Key concepts and the role of science,* J. Dairy Sci. 92 :4101–4111, American Dairy Science Association.

New Zealand Crossbred male rabbitproduction performance fed with fructooligosacharide prebiotic isolated from banana peel

Suraya KAFFI SYAHPURA[1)], Kusmajadi SURADI[2)], Husmy YURMIATI[2)], Diding LATIFUDIN[2)]
[1)] Department of Animal Husbandry, Polytechnic Lampung,
Jl. Soekarno – Hatta no. 10, Rajabasa, Bandar Lampung 35144, Indonesia
[2)] Faculty of Animal Husbandry, Universitas Padjadjaran,
Jl. Raya Bandung – Sumedang Km. 21, Jatinangor - Sumedang 45363, Indonesia
Email : ivisoraya@polinela.ac.id

Abstract

The study was conducted to determine the production performance (growth performance and carcass production) of New Zealand White Crossbred (NZWC) male rabbits given FOS (fructooligosaccharide) from the isolation of Ambon banana peel (Musa paradisiaca var. Sapientum (L.) Kunt. Rabbit age of eight weeks, body weight average 496.12 ± 10.61 g, placed in individual cage, divided randomly into five treatments, and each treatment was repeated five times, each repetition consisted of two rabbits The treatment used was P0 (without FOS), P1 (FOS fed for 7 days), P2 (FOS fed for 14 days), P3 (FOS fed for 21 days), and P4 (FOS fed for 28 days). FOS treatment is 3% concentration, 2 mg / kg of body weight given orally, after 7 days adaptation. Drinking water is given ad libitum. The feed used is commercial feed, given two times a day (in the morning and afternoon). The data were analyzed with analysis of variance. The similarity of variance was tested using Barlett test and orthogonal polynomial test. Results indicated that there were interaction between without FOS fed and FOS fed, and has effect on the growth performance (feed intake, daily gain, slaughter weight, and feed conversion) are quadratic at 1% level, and carcass production (carcass weight, carcass percentage, meat weight, bone weight, and fat weight) are linear at 1% level.

Keywords: *banana peel, fructooligosaccharide, performance, rabbit*

Introduction

The New Zealand White Crossbred (NZWC) rabbit has been selectively developed in the meat production unit, primarily for its propagation properties, namely prolification, performance, rapid growth and rapid sexual development, size and uniform litter size and mothering ability and high milk production (Lukefahr, 1983, Lebas *et.al*., 1996; Sarwono, 2002; Raharjo, 2005). The demand for rabbit meat has been increase because of the various preparations that have been circulating in the community. Efforts that can be made to meet the demand for rabbit meat is to increase livestock populations with the consequent increase in land and feed requirements. Moreover, it can be done by increasing the individual production capability. Efforts to increase production capability can be achieved such as growth manipulation, by improving the quality of rabbit rations. The given rations should meet the needs of the rabbit and contain balanced nutrition that can support towards the achievement of optimal growth so achieves optimal production. One of the efforts to achieve the above objectives is giving FOS (Fructooligosaccharide) from the banana peel of industrial waste that still has nutritional value that serves as a prebiotic. Based on a number of studies revealed contains vitamin C, vitamin B, calcium, protein, carbohydrates and fiber are good for the body; so the banana peel still have the benefit to be used as animal feed, and the benefits will be better through the extraction and isolation that can produce FOS (Fructooligosaccharide), according patented number P00201406459 of the method of making FOS from banana peel (Musita, 2008, Kaffi, *et al.* 2014). FOS is an alternative solution for control of colibasilosis as an antimicrobial substance that has the ability to eliminate pathogenic bacteria in the digestive tract and as immunostimulant (to stimulate the immune system). The fructans

contained in FOS produced by hydrolysis of chicory plant inulin have been widely reported, but the production of FOS derived from banana peel waste has not been widely published.

Fruktooligosaccharide (FOS) in banana peel is potential to be developed as a prebiotic that can reduce the number of pathogenic bacteria, and improve the morphology of the intestine (colon) and also the thickness of the mucus layer. Fruktooligosaccharide (FOS) has an affinity to binding the bacterial cells in intestinal epithelium. Bacteria that have binding to FOS can not stick and colonize the intestinal wall, and finally carried out through the feces (Murniasih, 2010), besides FOS banana peel also contains fructan sugar β (2-1) which is a prebiotic oligosaccharide. Prebiotics are material /components that can be useful for the development of microflora in the intestines, to be fermented by lactic acid bacteria especially *Bifidobacteria* and *Lactobacillus;* will also produce short-chain fatty acids which can be used in the body as a source of energy. The implementation to meet the meat quantity and quality, if problems to achieve the current economic conditions, it can be done by diversifying the supply of animal protein, as the target of sustainable development goals (SDGs), namely the achievement of people's welfare. One alternative to meet the needs of meat/animal protein is from rabbit meat, because of prolific livestock, rabbits also produce good quality and quantity of meat. On the other side, needs special attention in the maintenance, because of the high mortality, so the utilization of prebiotic FOS from banana peel is expected to increase the intestinal microflora and rabbit immune system, which is expected to increase the growth and better meat quantity and quality. Many advantages derived from eating rabbit meat, namely high protein content and low cholesterol, so the rabbit meat can be promoted as healthy meat, and also the skin and the manure still have economic value (Yurmiati, 2006). The development of rabbit meat as a provider to date still encountered many obstacles because the rabbit meat has not been popular by some people making difficulties in rabbit meat marketing.

Materials and methods

Fifty male NZWC rabbits age eight weeks with the average body weight 496.12 ± 10.61 g divided randomly into five treatments. Each treatment consists of five replications and two rabbits in each repetition. The treatment are (P0) as the control treatment without giving FOS (Fructooligosaccharide), (P1) given FOS for 7 (seven) days, (P2) given FOS for 14 days, (P3) given FOS for 21 days, and (P4) which is giving FOS for 28 days. The FOS is derived from the isolation of Ambon banana peel (*Musa paradisiaca var. Sapientum (L.)* Kunt derived from banana chips industry using FOS isolation method from banana skin, patented number P00201406459 from Indonesian Law and Human Rights Department. 100 kg of banana peel which had been chipped in 2-3 cm sizes, soaked in 30 L ethanol 70% for 14 days. During the daily immersion, stirring for 10 minutes, and the filtrate was filtered using a filter cloth and evaporated with a vacuum evaporator up to 1 L. The concentrated filtrate is then extracted with ethyl acetate (EtOAc) to obtain a water fraction and EtOAc. The water fraction was evaporated to dryness and then incorporated in a LH-20 diaion chromatographic column then eluted with 3 liter H2O and 3 liter MeOH-H2O (3:7), also with 3 liter MeOH-H2O (7: 3), and 3 liter MeOH, respectively. Each fraction was qualitatively tested with TLC to determine the presence of FOS compounds. The fraction containing FOS, then further purified by using purification techniques such as chromatography column, Preparative Thin Layer Chromatography (PTLC), or crystallization. The FOS compounds obtained were then analyzed by spectroscopy. The presence of compounds present in the solution can be qualitatively and quantitatively identified with HPLC instruments based on standard solutions, to use as FOS standard solution. The peak emerging from the solutions is a specific retention time of each compound. Each fraction obtained was tested by TLC (Thin Layer Chromatography) method by dripping on plate. The plate was developed with a combination of methanol-water solvent to get

the spot. Tests were also performed by comparing the retention time of standard FOS compounds by using high performance liquid chromatography (HPLC) method using ODS (C-18) columns and mobile phase used Methanol:water with ratio of 20:80. The detector used is UV-vis with a range of 460-600 nm, with a 20 μm injection volume over 10-20 minutes (Kaffi, et al, 2014).

The data of FOS was performed after the rabbit was kept for 7 days for environmental adaptation, giving orally according to each treatment; once daily in the morning between 07- 08 a.m with 3% concentration,doses of 2 mg / kg body weight (Prata and Mussato, 2010), , and drinking water is given ad libitum. The rabbit is placed in an individual rabbit cage (50 x 40 x 35 cm) with eating and drinking apparatus of 50 enclosures, which are kept for eight weeks and also was given the Indofeed commercial rabbit feed. The second stage (between the 8th day until 28th day of maintenance) is collecting the rabbit production performance data. The data collected are the measurement of the observed variables: the production/growth performance (feed consumption, daily gain, feed conversion) and carcass quality (slaughter weight, carcass weight, meat weight, bone weight, and fat weight). After the data retrieval phase, all rabbits are fausted for 12 hours, then halally slaughtered by cutting the four channels, (the carotid artery, jugular vein, trachea, and esophagus). After slaughter, the internal organs was remove off, to obtain the carcass. The data were analyzed with analysis of variance. The similarity was tested using the Barlett test and the effect of the treatment was using orthogonal polynomial test.

Results and discussion

Growth Performance

Parameters used in the observation of rabbit growth include feed intake, feed conversion, and daily gain. Consumption is a basic factor for living and determining production. The results of the FOS analysis of isolated banana peel (*Musa paradisiaca var. Sapientum (L.)* Kunt. on New Zealand White Crossbreed male rabbits in the Table1.

Table 1. The Effect of Treatment on Feed Intake , Daily Gain, and Feed Conversions of New Zealand White Crossbred Male Rabbit

No	Variable	Treatments				
		P0	P1	P2	P3	P4
1.	Feed Intake (g/rabbit/day)	84.61**	91.15**	104.76**	108.81**	109.41**
2.	Daily gain (g/rabbit/day)	31.94**	46.30**	52.22**	59.23**	65.20**
3.	Feed Conversion	2.67**	1.98**	2.01**	1.84**	1.68**

Note: ** significant difference on level 1%

In the Table 1 shows that the highest average feed intake was achieved by New Zealand White Crossbred male rabbit receiving P4 treatment (FOS for 28 days), 109.41 g and the lowest was shown by P0 treatment (without FOS) 84.61 g. The highest daily gain in treatment P4 (65.20 g/rabbit/day) and the lowest on treatment P0 (31.94 g/rabbit/day). While the highest feed conversion at treatment P0 (2.67) and lowest at treatment of P4 (1.68). The means of ration consumption 99.75 g was lower than that recommended by Lebas *et al*. (1996); 110-130 grams for NZWC rabbits aged 4-11 weeks which were fed balanced diet. Factors affected the ration consumption level of rabbits, were environmental temperature, health, the feed physical form, food balance, body weight and growth rate (NRC 1977). The consumption increased, due to the rabbit trying to meet the energy needs, because the coarse fiber will lowered the energy digestibility coefficient, thus requiring high energy (Evans, 1981).

Lack of crude fiber in rabbit feed can decreased the digestive function, as enteritis (Cheeke and Patton, 1981), even rabbits can be fed once, twice or three times in a day. Usually rabbit enough to be fed once a day, and usually should give in the afternoon, because rabbits eat more at night (Ensminger, 1991). Based on the analysis result, it is known that the treatment has an effect on the feed intake. A further test using orthogonal polynomial is known that the treatment gives a quadratic effect on the 1% level, as shown in Figure 1 with the quadratic equation of y = 0.0313x2 + 1.8358x +83,233. The optimum point was obtained on the 36th day of observation with feed intake of 110.15 g/rabbit/day. And the determinant correlation (R^2) between the influence of the former feed intake of FOS (98%).

The rate of livestock growth is influenced by the amount, quality of the ration and by the environmental temperature. The growth pattern will depend on the management system, the nutritional level of available feeding health and climate (Templeton, 1968). Growth is a change of elements that includes changes in life weight, shape, linear dimension and body composition. Also changes the body components such as muscles, fats, bones and organs; and carcasses chemical components, especially water, fat, protein and ash. The normal growth pattern is a combination of the growth patterns of all constituent components. Under ideal environmental conditions, the shape of the post-natal growth curve for all livestock species is similar, following the sigmoid growth curve pattern. In accordance with the carcass component growth pattern that begins with rapid bone growth, then after reaching puberty, the rate of muscle growth decreases and the fat deposition increases.

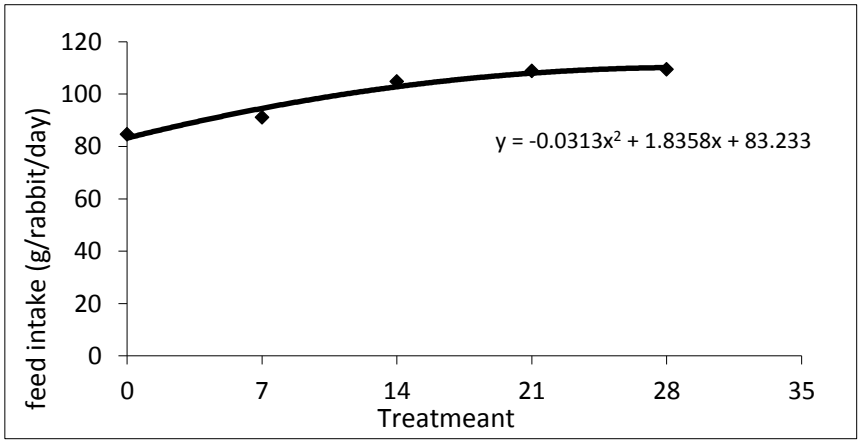


Fig. 1. Feed intake of rabbit rations of various treatments giving FOS (Fructooligosaccharide) from banana peel

Sexual puberty is achieved when the reproductive organs have developed and well function (Blakely and Bade, 1994). During the growth process, livestock is affected by several factors including genetic, feeding, temperature, adaptability and environment. Sex does not affect carcass or meat quality but the age (Parigi-Bini *et al*. (1992). Growth is the process of weight gain and change of body shape and composition, because of the different growth rates of each component. The growth speed of a young rabbit, are twice the weight of his body every week, so at the age of three weeks can reach 0.45 kg body weight. After consume solid feed, the growth rate can reach 30 to 50 g/day from the age of three to eight weeks. When breast feeding is the only food consumed, the growth rate in that period is only 10 to 20 g/day (Spreadbury, 1978), after 10 to 12 weeks of horizontal growth curve (Lang, 1981); and the results of research obtained is in accordance with,

that the average weight gain 50.98 g/rabbit/day. Further test using orthogonal polynomials, the treatment gives a quadratic effect at 1% level (Fig. 2).

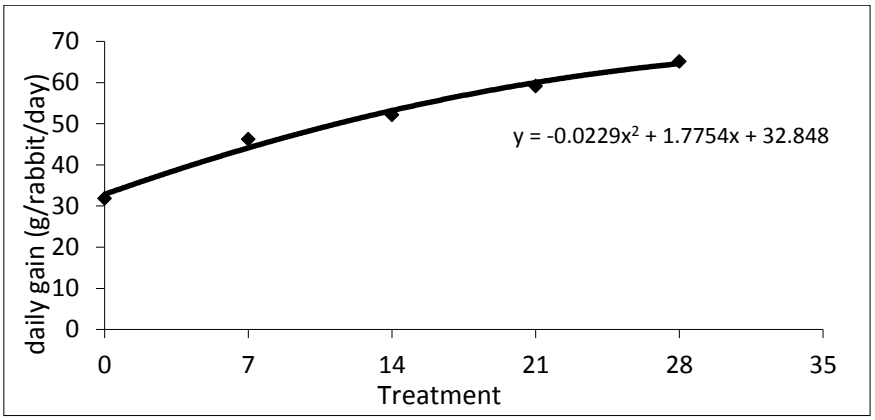


Fig 2. Daily gain of various treatments giving FOS (Fructooligosaccharide) from banana peel.

The Fig. 2, shown the quadratic is $Y=0.0229x^2+1.7754x+32.848$, at the optimum point of the 39th day of observation, the growth of daily gain is 67.26 g/rabbit/day. And the determinant correlation (R^2) between the influence of FOS to the daily gain is 99%. Feed conversion is the ratio between the amount of feed consumtion to produce one kilogram of slaughter weight (Raharjo , 2005). Rabbits have a unique behavior that is re-eating feces (*coprophagy* or *caecotrophy*), that rich of vitamin, Niacin, Riboflavin, Pantothenic Acid, Cyanocobalamin (B12), and VFA. According to Raharjo (2005) the composition of fat, cholesterol and salt are low in rabbit carcasses. High-quality feeding with good management can result a rabbit feed conversion of 2.80-4.00 (Aritonang *et al*., 1990). In this study, there was a lower converting mass of 2.04 and a further test using orthogonal polynomials is known that the treatment gives a quadratic effect at 1% level, as shown in Figure 3.

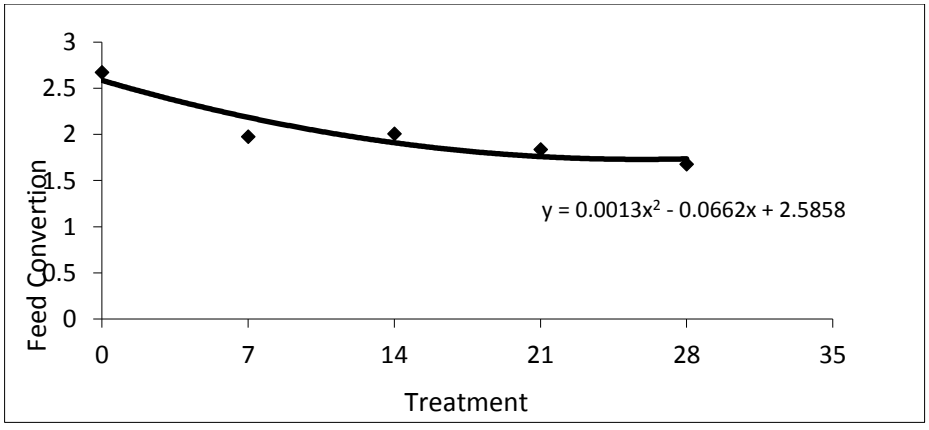


Fig. 3. Feed convertion of rabbit rations of various treatment giving FOS (Fructooligosaccharide) from banana peel

In Figure 3, the quadratic equation that $y = 0.0013x^2+0.0662x+2.586$, shows the optimum point of the 26th day of observations 1.74 feed conversion, and determinant correlation (R^2) between the former use of FOS to feed conversion by 94%. In this study, rabbits were given FOS can increase the feed intake, body weight gain, but has low feed conversion. FOS (Fructooligosaccharide) is a type of short polysaccharide chain, is fiber food that is undigested that helps maintain the health of the digestive tract but can be utilized by lactic acid bacteria in the colon, especially *Bifidobacterium sp* and *Bacteroides sp* and will inhibit the growth of pathogenic bacteria (Yun, 1996 ; Murniasih, 2010), are the source of energy for monogastric animals herbivores other than carbohydrates, the VFA (Volatile Fatty Acids) such as butyric acid, propionate and acetate has been absorbed in the digestive tract that will be a source of energy (Lebas, 1996).

Carcass Production

Slaughter weight (g), carcass weight (g), meat weight (g), bone weight (g), and fat weight (g) were significantly different at 1% (Table 2). Nevertheless, the values of the four treatments were increasing in slaughter weight (g), carcass weight (g), carcass percentage (%), and meat weight (g) but decrease in fat weight (g) treated with FOS and has not difference in bone weight (g) as shown in Table 2.

Table 2. The Effect of Treatment on Slaughter Weight, Carcass Weight, Carcass Percentage , Bone Weight, Meat Weight, and Fat Weight in the New Zealand White Crossbred Male Rabbit.

No	Variable	Treatments				
		P0	P1	P2	P3	P4
1	Slaughter Weight (g)	1439.20**	1858.60**	2087.60**	2307.00**	2435.00**
2	Carcass weight (g)	713.2**	946.80**	1086**	1205.60**	1414.40**
3	Carcass percentage (%)	49.6	50.9	52.0	52.3	57.6
4	Bone weight (g)	251.37tn	250.41 tn	251.47 tn	253.33 tn	254.56 tn
5	Meat weight (g)	418.60**	528.00**	762.60**	798.00**	935.00**
6	Fat weight (g)	80.84**	75.48**	73.35**	63.38**	63.24**

Note: tn = non significant, ** = significancy level 1%

The treatment of FOS (Fructooligosaccharide) isolated from banana peel (*Musa paradisiaca var. Sapientum (L.)* Kunt, increasing the slaughter weight, carcass weight, carcass percentage, bone weight, the meat weight, and lower carcass fat weight. Slaughter weight affected by the age, type, and ration. Young rabbits will produce low slaugher weight compared with the mature rabbit. Growth can occur due to an increase of the cells number and also the body cell size. The process occurs in line with the age and condition of the rabbit (Yurmiaty, 2006),. The means of slaughter weight (2029.08 g), are consistent with Hernandez *et al*., (2001) which uses four types of rabbit (California, Chinchilla, New Zealand age 80 days and NZW age 90 days) has the average slaughter weight (1900-2000 g) and carcass weight (1100 - 1180 g). Based on the result of the analysis, it is known that the treatment has effect on the slaughter weight. Further trials using orthogonal polynomials, the treatment is a quadratic on the level 1%, (Figure 4).

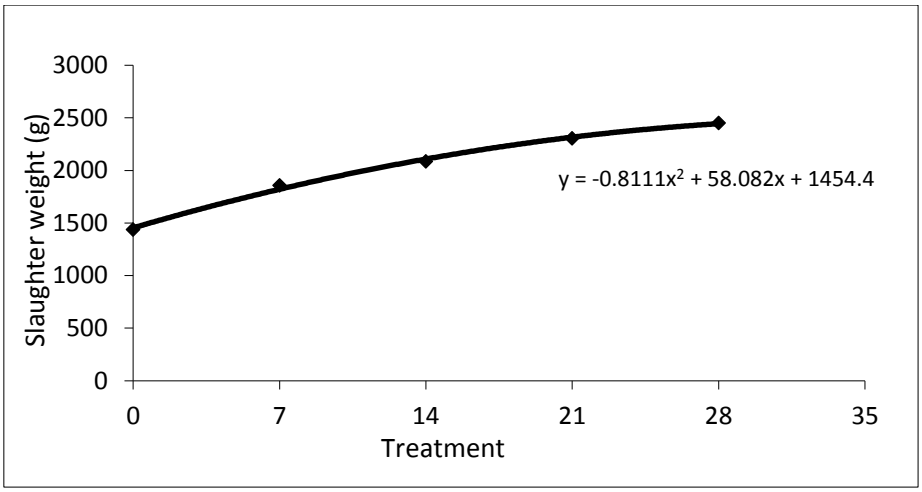


Fig. 4. Slaughter weight of rabbit rations of various treatments giving FOS (Fructooligosaccharide) from banana peel

In the Fig. 4, shows the quadratic is $y = 0.8111x^2+8.082x+1454.4$ at the optimum point of the 35th day, the slaughter weight (2494.20 g). And the determinant corelation of FOS to slaughter weight is 99% . Carcass weight and carcass percentage is affected by slaughter weight (Metzger *et al*., 2003). The carcass weigh, without the blood, head, skin, liver, tail, digestive tract and its contents, and chest cavity contents, except the kidneys, (Rao, *et al.* 1978). The highest carcass weight was obtained in the P4 treatment (1414.40 g), and the lowest in treatment P0 (713.2 g), while the average carcass weight (1078.4 g). The results is higher than Agustin *et al* (2017), that fed of corn oil in the feed (975.51 g). The slaughter weight, higher than the carcass weight. The carcass production is influenced the slaughter weight. Brahmantiyo *et al*. (2010) states that the carcass weight is higher than the slaughter carcass weight. Haryoko and Warsiti (2008), stated that the carcass component consists of meat, bone and fat. Based on the result of the analysis, it is known that the treatment has effect on the carcass weight. A further test using orthogonal polynomial is known that the treatment is linearly at 1% level, as shown in Figure 5, with equation of $y = 20.26x + 797.6$. The percentage was influenced by the length use of FOS on carcass weights; has a determinat correlation (R^2) of 95%. Rabbits with heavy body weight can produce a large percentage of carcasses, the increasing of live weight will increase the feed consumption. Rabbit consume more feed have a tendency to accumulate more protein as a growth response and increased the carcass weight. Carcass weight and carcass percentage is highly dependent on breed, environment, live weight, and nutrient in feed. The percentage of carcass has a positive relationship to the energy content in the ration.

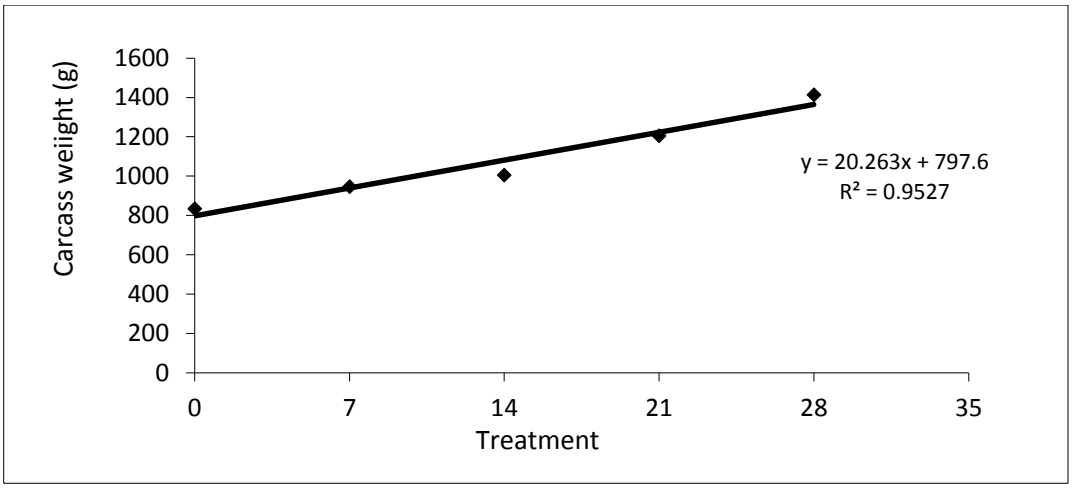


Figure 5. Carcass weight of rabbit rations of various treatment giving FOS (Fructooligosaccharide) from banana peel

Body size increase proportionally with the body weight of a livestock (Rao *et al.,* 1978). The carcass percentage is in line with the carcass weight, as the carcass percentage, is the ratio between carcass weight and weight live when slaughter, multiplied by 100% (Santoso, 2010). In this study, the highest percentage of carcass was found in the treatment R4 (57.6%), and the lowest in treatment R0 (49.6%). The carcass percentage, still higher than that of Ozimba and Lukefahr (1991) which obtained a 55% carcass percentage on NZW, California and California NZW cross rabbits. The carcass production of New Zealand White rabbit, local rabbit, NZW Crossbred and Chinchilla crosses, are 45.8, 42.6, 48.9 and 46.7% respectively (Diwyanto *et al*., 1985). Commercial carcass production, is strongly influenced by the slaughter weight (Rao *et al*., 1978), and the commercial parts of carcass weight, foreleg, rack, loin and hindleg (Blasco *et al*., 1992).

Conclusion

Feeding FOS (Fructooligosaccharide) from the isolation of *Ambon* banana peel (*Musa paradisiaca var. Sapientum (L.)* Kunt., has effected the growth performance (feed intake, slaughter weight, daily gain, and feed conversion) in quadratic and carcass production (carcass weight, meat weight, bone weight, and fat weight) linearly in New Zealand White Crossbred male rabbits, and further studies related to the use of FOS as prebiotics in rabbit feed.

Bibliography

2. Agustin Pratiwi, Supadmo, Andriyani Astuti, dan Panjono. 2017. *Growth Performance and Carcass Production of Rex Rabbits Feed With Corn Oil Supplementation.* Bulletin of Animal Science. Vol 41(2):119-125.
3. Aritonang, D. T., N. A. Tul Rofiah., T. Pasaribu, dan Y. C. Raharjo. 1990. *Laju pertumbuhan kelinci Rex, Satin dan persilangannya yang diberi Luctosym dalam sistem pemeliharaan intensif*. Balai Penelitian Ternak, Bogor.
4. Blakely, J.D,H., and Bade.1994. *Ilmu Peternakan.* Edisi keempat. Gadjah Mada University Press, Yogyakarta.
5. Blasco, A., J. Ouhayoun and G. Masoero. 1992. *Study of rabbit meat and carcass: Criteria and terminology*. J. Appl. Rabbit Res. 15: 775 – 786.

6. Brahmantiyo B, Raharjo YC, Martojo H, Mansjoer SS. 2010. *Performa produksi kelinci rex, satin dan persilangannya*. *JITV*. 15(2): 131-137.
7. Cheeke and Patton. 1981. *The Rabbit: An Emergency livestock species*. Feedstuff. p:25-26.
8. Diwyanto, K. Sunarlim and P. Sitorus. 1985. *Pengaruh persilangan terhadap nilai karkas dan preferensi daging kelinci panggang*. Ilmu dan Peternakan 1(10): 427 – 430.
9. Ensminger, M. E. 1991. *Animal Science* 9th Edition. The Interstate Printer and Publisher Inc. Danville Illinois. USA.
10. Evans, E. 1981. *Effect of dietary energy and fibre levels on performance of fryer rabbits.* J. Appl Rabbit Res. 4 (2) : 41-43.
11. Haryoko. I. and Titik Warsiti. 2008. *Pengaruh Jenis Kelamin dan Bobot Terhadap Karateristik Fisik Karkas Peranakan New Zealand White*. J. Produksi Peternakan 2(10): 85 – 89.
12. Hernandez, J. A., M. S. Rubio Lozano R. D. And Carregai. 2001. *Effect of Breed and Sex On Rabbit carcass Yield and Meat Quality*. Meat Science Labolatory, Facultad de Medicina Veterinaria y Zootecnia Universidad Nacional Autonoma de Mexico, Mexico
13. Kaffi S.S.,Zulfahmi and Hertini Rani, 2014. *HAKI Metode Proses Isolasi FOS dari Kulit Pisang* no POO201406459 tertanggal 23 Oktober 2014. Departemen Hukum dan HAM Republik Indonesia. Jakarta.
14. Lang, I. 1981. *The nutrition of the commercial rabbit. Part I. Physiology Digestibility and Nutrient Requirement*. Nutrition Abstract and Reviews series B. Commonwealth Bureau of Nutrition. 51 (4) : 197-204.
15. Lebas, F., P. Coudert, R. Rouvier and H.de Rohambeau. 1996. *The Rabbit Husbandry, Health and Production*. FAO Animal Production and Health Series No.21. Rome. Italy.
16. Lukefahr, S.D., W.D. Hohenboken, P.R. Cheeke and N.M. Patton. 1983. *Characterisation of straight bred and crossbred rabbits for milk production and assosiative traits*. Journal of Animal Science. 5: 71-100.
17. Metzger, S., K. Kustos, Z. Szendro, A. Szabo, C. Eiben, and I. Nagy. 2003. *The effect of housing system on carcass traits and meat quality of rabbit*. World Rabbit Sci 11: 1-11.
18. Murniasih, T. 2010. *Fruktooligosakarida (FOS) and Galaktosakarida (GOS) as Functional Food.*
19. J Mol catal B-Enzym 59:76-81.
20. 18.Musita, N. 2008. *Kajian dan Karakteristik Pati resisten dari Beberapa Jenis Pisang*. Tesis. Universitas Lampung. Bandar Lampung.
21. 19.National Research Council (NRC).1977. *Guide for the Care and Use of laboratory animals.* 8th Ed. Washington: National Academy Press.
22. Ozimba, C.E. and S.D. Lukefahr. 1991. *Evaluation of purebreed and crossbreed rabbits for carcass merit*. J. Anim. Sci. 69: 2371 – 2378
23. Raharjo. Y. C. 2005. *Prospek, Peluang dan Tantangan Agribisnis Ternak Kelinci.* Proseding Lokakarya Nasional Potensi dan Peluang Pengembangan Usaha Kelinci. Pusat Penelitian dan Pengembangan Peternakan. Bogor.
24. Rao,. D. R, Sunki G.R, Johnson W.H, Chen C.P. 1978. *Effect of weaning and slaughter age on rabbit meat productions II.Carcass, Quality and Composition*. J Animal Sci 5: 578-582.
25. Santoso, U and Sutarno. 2010. *Bobot Potong Dan Karkas Kelici New Zealand White Jantan Setelah Pemberian Ransum Kacang Koro (Mucuna pruriens var. utilis).* Bioteknologi. 7(1):19-26.
26. Sarwono, B. 2002. *Kelinci Potong dan Hias*. Agromedia Pustaka. Jakarta.
27. Spreadbury, D. 1978. *A study of the protein and amino acid requirement of the growing New Zealand White rabbit with emphasison lysine and sulphur containing amino acids*. 1. Nutr. 39 : 610-613.
28. Templeton, G. S. 1968. *Domestic Rabbit Production*. The Interstate Printers and Publisher, Inc. Danville, Illinois.
29. Yun, Jong Won. 1996. *Fructooligosaccharides-Occurance, preparations, and applications*, Elsevier Science Inc.
30. Yurmiaty. H. 2006. *Hubumgan Berat Potong dengan Berat, Tebal Pelt Kelinci.* Jurnal Ilmu Ternak. 1 (6) : 48 -52.