University of Novi Sad - Univerzitet u Novom Sadu
Faculty of Agriculture - Poljoprivredni fakultet

# CONTEMPORARY AGRICULTURE
# *SAVREMENA POLJOPRIVREDA*

The Serbian Journal of Agricultural Sciences
*Srpski časopis za poljoprivredne nauke*

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# ANALYSIS OF THE INFLUENCE OF BEAN SEED INNOCULATION WITH RHIZOBIUM PHASEOLUS BACTERIA ON BEAN MORPHOLOGICAL AND CHEMICAL CHARACTERISTICS

MIRSAD VELADŽIĆ, REFIK ŠAHINOVIĆ, HALID MAKIĆ, AZRA BEĆIRAJ, SUZANA JAHIC, FATIMA MUHAMEDAGIĆ, MIRSAD IČANOVIĆ[1]

*SUMMARY: Experiment was carried out on the control surface of 1000 m² without bean (Phaseolus vulgaris) seed inoculation and on the experimental surface of 1000 m² with seed inoculated with bacteria Rhizobium leguminosarum bv. phaseoli. Common technology of bean cultivation was used, with absolutely identical interventions and circumstances, the only difference being the use of inoculated seed in the experimental cluster. This paper elaborates influences of inoculation on morphological properties (plant height, length of pod, size of bean) and chemical characteristics (proteins, raw fibres, ash, fats and humidity). Average values of the plant height in inoculated seed, size of the pod and average bean size are higher than in the control and this difference is statistically highly significant. Moreover, chemical analysis of bean (seed) showed 13,42% higher contents of proteins in inoculated seed, 5,68% higher ash contents, 10,34% higher fat contents, 1,26% higher celluloses contents and 8,68% higher humidity.*

***Key words:*** *bean, seed inoculation, Phaseolus vulgaris, Rhizobium leguminosarum bv. phaseoli.*

## INTRODUCTION

Bean (*Phaseolus vulgaris*) is annual herbaceous plant from the *Fabaceae* family, genus *Phasealus*. There are five phases of bean development: phase one involves swelling and germination of the seed and plant springing, the second phase is the phase of vegetational mass or leaves, the third phase is blooming, the fourth phase is pod develop-

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Original scientific paper / *Originalni naučni rad*
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ment and the fifth phase is full ripeness. Bean is a plant that requires a lot of warmth and humidity. It is cultivated in crop rotation and is good pre-crop for many agricultural and vegetable cultures. Bean on heavy, very acidic or extremely alkaline soils vegetates less effectively. Most suitable soils for bean are those with pH between 6,5 and 7,5 (Vasić et al., 2007). Among others, pH is a limiting factor in yield and quality of Leguminosae as well as the most of agricultural cultures (Tanaka et al., 1984). Nutritional value of bean is not contained only in its high contents of proteins (23 to 30%), but in its total contents of the most important amino acids (tyrosine, tryptophan, lysine, arginine, histidine, cysteine, methionine). Chemical characteristics are presented through the contents of proteins, celluloses, ash, fats and humidity. Morphological characteristics are the most important properties like: plant height, pod length, bean size and yield (Todorović et al., 2003). Bean belongs to legume family and has great agro technical significance because it leaves soil in very good physical and biological state, therefore it is an excellent pre-crop for many agricultural and vegetable cultures (Vidović and Todorović, 1988). It makes symbiosis with certain microorganisms of *Rhizobium* genus that have the ability of nitrogen fixation, that is the use of nitrogen from air. Bacteria from *Rhizobium leguminosarum bv. phaseoli.* are symbiotic gram-negative microorganisms of the soil that influence formation of root nodules. The efficiency of nitrogen fixation of nodular bacteria depends on bacteria specificity to live in symbiosis with only one type of plants (bean, pea, soy, clover), virulence – bacteria's ability to penetrate the root and reproduce, and activity – bacteria's ability to fix gaseous nitrogen from air in nodules. According to various sources, nitrogen quantity that remains in soil after bean sowing is some 3-170 kg/ha (average is 50 kg/ha). During a year, depending on ecological circumstances, Leguminosae in symbiosis with *Rhizobium* fix up to 400 kg N/ha (Veladžić et al., 2003). Bean's nodular bacteria exist even in our soils, but not in optimal numbers nor the most efficient strains. In the inoculum, there are selected strains that fixate gaseous nitrogen in the most intensive way and leave the greatest quantities of nitrogen in soil after bean plucking (Marinković, 2006; Milošević and Marinković, 2009). Rhizobia stimulate plant growth and development producing biologically active matters (vitamins, gibberellins and auxins) too (Alami et al., 2000; Egamberdiyeva, 2007).

Nodule bacteria beans in our soils are in small numbers, particularly relating to land with an acid reaction (Burdman et al., 1996). Entering effective and acidoresistant strains of these bacteria during sowing beans, increases nitrogen fixation and allows the cultivation of these plant species in less fertile soils. (Milutinovic et al. 1992). In acidic, poorly humic soils, nitrogen fertilizers do not inhibit symbiotic nitrogen fixators because the land does not have enough affordable or nutrients for microorganisms or plant. The amount of nitrogen fixed varies depending on the effectiveness of nodule bacteria, which enter the composition of microbial fertilizers, and also the genotype of the plant. (Milic et al. 2004).

## MATERIAL AND METHODS

The experiment was set up in 2009 in two parcels; each was made of 1000m$^2$, in Bihac municipality, Bosnia and Herzegovina. The first parcel used as a control and we used bean seed *Phaseolus vulgaris* that was not noculated. On the other parcel of the same size, seed inoculated with bacteria *Rhizobium leguminosarum bv. phaseoli* was used. On the experimental parcel, microbiological fertilizer with selected strains

of nodular bacteria was used and applied to bean seed just prior to seeding. Inoculum application was done in shade. It was applied to watered seed, in a ratio of 10g of inoculum per 1kg of seed (Institute of Field and Vegetable Crops, Novi Sad, nitragin). The seed was planted immediately after inoculation with bacterial preparation. Ploughing was done with ploughs reaching depth of 30cm. Fertilization was made with well rotted sheep manure in a quantity of 1500 kg per 1000m$^2$ on both parcels. Seeding was done on 02.05. 2009 in beds 8 to 10 cm apart with a distance of 50 cm between rows. For fertilization before sowing was used was 2 kg of NPK fertilizer (12:12:12), was used for supplemental feeding around 0.10 gr kristalon (pure nitrogen fertilizer) to 1.5 liters of water.

Seeds were planted at a depth of 4 to 5 cm. During germination and due to occurrence of soil crust, hoeing up was undertaken. The first inter-row hoeing up was undertaken when first "troper" leaves were formed, the second and the third cultivation was undertaken in span of 12 to 16 days. Sixty samples of bean were taken for this experiment. Thirty of them from inoculated parcel and thirty from control one. For every taken bean sample plant height, length of pod and length of bean were measured. Apart from that, all pods were ingathered aside from each parcel and on the basis of their weighing, yield per hectare was calculated. Moreover, the qualitative features of bean seeds were identified (proteins, lipids, humidity, ash and cellulose). Identification of these qualitative features of bean seed was performed in laboratories of Agricultural Institute of Una-Sana Canton. Analyses of proteins, lipids, humidity and ash were done after the manual Instrumental Methods in Biological Control (Veladžić and Čaklovica 2001).

## RESULTS AND DISCUSSION

Individual data were processed by the utilisation of mathematical-statistical methods. Analysis of statistical-variational parameters of plant height, pod length and bean size for control and inoculated parcel was done (Table 1).

Table 1. The statistical variations of the parameters stem, pods and bean cultivation methods for both beans

*Tabela 1. Statističko varijacioni parametri za stabljiku, mahunu i zrno graha za oba načina uzgoja graha*

| Parcels / *Parcele* | X | SD | CV | Sx |
|---|---|---|---|---|
| Plant height (cm) / *Visina stabljike (cm)* | | | | |
| Control / *Kontrola* | 46,43 | 2,27 | 0,18 | 0,41 |
| Inoculated / *Inokulacija* | 52,35 | 6,13 | 0,18 | 1,12 |
| Pod length (cm) / *Veličina mahune (cm)* | | | | |
| Control / *Kontrola* | 10,14 | 1,32 | 0,18 | 0,24 |
| Inoculated / *Inokulacija* | 12,19 | 1,88 | 0,18 | 0,34 |
| Size of beans (cm) / *Veličina zrna (cm)* | | | | |
| Control / *Kontrola* | 1,15 | 0,20 | 0,19 | 0,04 |
| Inoculated / *Inokulacija* | 1,35 | 0,21 | 0,2 | 0,04 |

Where: **X** – mean, **SD** – standard deviation**, Cv** – coefficient of variation**, Sx** – error estimation

Gde je: ***X***-sr.vrednost, ***SD***-standardna devijacija, ***Cv*** -koeficijent varijacije, ***Sx*** -ocena greške

**Plant height:** There is increase in plant height in inoculated bean seed for 12.75% in relation to control. Calculated values of indicator $t_{izr}$ is 4.97 and is evidently larger than tabular value whose significance level is 0.1%. On that basis, it can be concluded that differences between arithmetic means of two samples are *very highly significant* and the null-hypothesis is rejected, $t_{calc}$= 4.97***.

**Pod length:** Average pod length in inoculated bean is for 20.22% larger than pods from control parcel. Calculated value of indicator $t_{calc}$= 4.88 and is also larger than tabular value of 0.1% significance level. On that basis, it can be concluded that differences between arithmetic means of two samples are *very highly significant* and the null-hypothesis is rejected, $t_{calc}$= 4.88***.

**Size of beans:** Average size of beans on inoculated parcel is for 17.39% larger than on control parcel. Calculated value of indicator $t_{calc}$= 3.33 and is also larger than tabular value of 0.1% significance level. On that basis, it can be concluded that differences between arithmetic means of two samples are *highly significant* and the null-hypothesis is rejected, $t_{calc}$ = 3.33**.

**Yield calculation** on the observed parcels of 1000m$^2$ (inoculated seed and control-not inoculated) gave the following results: Yield of inoculated bean is 98 kg, i.e. 980 kg/ha. Yield of control bean is 80 kg, i.e. 800 kg/ha. Yield on parcel with inoculated bean seed in comparison to control parcel is noticeably increased for 180 kg, i.e. for 22.5%.

If it is kept in mind that the effect of bean inoculation caused the increase of yield in the amount of 180 kg, and if that amount is multiplied with market price of bean, which is 2€ currently, it means that the effect of financial increase is 360€. Apart from yield increase, it is very important that the soil is deposited with important supply of nitrogen (Marinković, 2006). The story does not end with only these effects. It is rather important to give the values of deposited nitrogen in soil after bean yield.

Table 2. Qualitative properties of beans (protein, fat, moisture, ash, and cellulose)
*Tabela 2. Kvalitativna svojstva zrna graha (belančevine, masti, vlaga, pepeo i celuloza)*

| Chemical characteristics<br>*Hemijska svojstva* | Inoculated<br>*Inokulisano* | Control<br>*Kontrola* | Increase (%)<br>*Povećanje (%)* |
|---|---|---|---|
| Proteins (%)<br>*Belančevine (%)* | 28.32 | 24.97 | 13.42 |
| Ash (%)<br>*Pepeo (%)* | 4.84 | 4.58 | 5.68 |
| Lipids (%)<br>*Masti (%)* | 1.28 | 1.16 | 10.34 |
| Cellulose (%)<br>*Celuloza (%)* | 5.64 | 5.57 | 1.26 |
| Humidity (%)<br>*Vlaga (%)* | 13.40 | 12.33 | 8.68 |

On the basis of identified results, it is noted that there is increase in chemical characteristics of inoculated bean, that is the content of proteins and lipids increased for over 10%, ash for 5.68%, cellulose for 1.26% and humidity for 8.68% (Table 2). These data about increased contents of proteins, lipids, ash, cellulose and water are in full accordance with many researchers' findings that inoculation of Leguminosae just prior to sowing produces positive effects on number of nodules, increases growth and yield, savings on nitrogen mineral fertilizers, increases quality of beans and biological

activity of the soil (Milošević and Marinković 2009; Yazdani et al., 2009; Gholami et al., 2009; Zaidi et al., 2006).

## CONCLUSIONS

Regarding quantitative characteristics, there are statistically highly significant differences between inoculated and not inoculated beans regarding the increase of plant height, pod length and size of beans.

Yield analysis of inoculated bean showed yield of 980 kg/ha, and in control production yield of 800 kg/ha.

Regarding qualitative characteristics, positive effects on increase of contents of proteins 13.42%, ash 5.68%, lipids 10.34%, cellulose 1.26% and humidity 8.68% are identified.

Bean seed bacterisation with *Rhizobium leguminosarum bv. phaseoli*. prior to seeding should be considered obligatory and effective measure in bean production technology. It is particularly important on soils where bean has not been cultivated for longer period or at all.

The importance of bean inoculation is visible from decreased use of expensive nitrogen fertilizers, contribution to the improvement of soil quality, decreased burden of nitrogen mineral fertilizers on eco system and increased yield and quality of beans.

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## ANALIZA UTJECAJA INOKULACIJE SEMENA GRAHA BAKTERIJAMA *RHIZOBIUM LEGUMINOSARUM BV. PHASEOLI.* NA MORFOLOŠKE I HEMIJSKE KARAKTERISTIKE GRAHA

MIRSAD VELADŽIĆ, REFIK ŠAHINOVIĆ, HALID MAKIĆ, AZRA BEĆIRAJ, SUZANA JAHIĆ, FATIMA MUHAMEDAGIĆ, MIRSAD IČANOVIĆ

### Izvod

Eksperiment je proveden na površini od 1000 $m^2$ bez inokulacije semena graha Phaseolus vulgaris (kontrolna) i na površini od 1000 $m^2$ gdje je korišteno inokulisano seme bakterijama (eksperimentalna) Rhizobium leguminosarum bv. phaseoli. U tehnologiji uzgoja graha korištena je uobičajna praksa, sa potpuno identičnim zahvatima i okolnostima, uz jedinu razliku što je u eksperimentalnoj skupini izvršena inokulacija semena. U radu su obrađeni utjecaji inokulacije semena na morfološke karakteristike (visina stabljike, dužina mahune, veličina zrna) i na hemijske (belančevine, sirova vlakna, pepeo, masti i vlagu). Srednje vrednosti visine stabljike kod inokulisanog semena, veličina mahune i prosečna veličina zrna su veće od kontrole i ta razlika

je visoko statistički značajna. Također je hemijskom analizom zrna (semena) utvrđen veći sadržaj belančevina kod inokulisanog semena za 13,42%, sadržaj pepela za 5,68%, sadržaj masti za 10,34%, sadržaj celuloze za 1,26% i sadržaj vlage za 8,68%.

**Ključne reči**: grah, inokulacija semena, *Phaseolus vulgaris, Rhizobium leguminosarum bv. phaseoli.*

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# AHP–GROUP DECISION-MAKING IN SELECTING TREE SPECIES FOR URBAN WET SITES*

MILENA LAKIĆEVIĆ, BOJAN SRĐEVIĆ[1]

*SUMMARY: This paper deals with the problem of tree species selection for urban wet sites. The Analytic Hierarchy Process (AHP) is used in its standard version and the aggregation of individual decision makers' preferences in mixing selected tree species is performed by geometric averaging method. For the hierarchy of the problem with four criteria and seven tree species, optimal tree species composition is obtained. The proposed approach aims at encouraging landscape planners to adopt contemporary decision-making methodologies in performing related engineering tasks. The numerical example is illustrative.*

***Key words:*** *urban wet sites, tree species selection, Analytic Hierarchy Process, group decision making*

## INTRODUCTION

The selection of tree species is essential for successful urban greening. In cities, trees are endangered by severe environment conditions and the most common problems are air pollution, drought, insufficient space for the development of root system and crown and a lack of sunshine. It is widely accepted that plants have to be very tolerant and adaptive to survive in urban areas, and therefore landscape planners have to carefully select plants species; otherwise, plants might become unhealthy or even die. Multi-criteria analysis and optimization in selecting and mixing tree species can be helpful in fulfilling real-life urban planning requirements and reaching best interests of a society.

This paper considers the problem of landscaping urban wet zones influenced by both surface and underground waters, which make tree survival (and selection) even harder. The problem solution is finding the optimal (in multi-criteria sense) mixture of seven tree species (*Chamaecyparis lawsoniana, Thuja occidentalis*, *Acer saccharinum*, *Platanus x acerifolia*, *Quercus robur*, *Salix babylonica* and *Taxodium distichum*), all recognized as compatible in greening urban wet sites (Gerhold and Porter, 2007). The problem is stated as a group decision-making problem and solved by the Analytic hier-

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archy method, a well known decision support tool. Four landscape architects evaluated the tree species against mutually agreed four selection criteria (adaptability to the environment site conditions, resistance to damaging agents – pests and diseases, ornamental value and costs of establishment and management). Individual decisions (mixtures of tree species) are aggregated geometrically.

## MATERIALS AND METHODS

Hierarchy of the decision-making problem is shown in Fig.1.

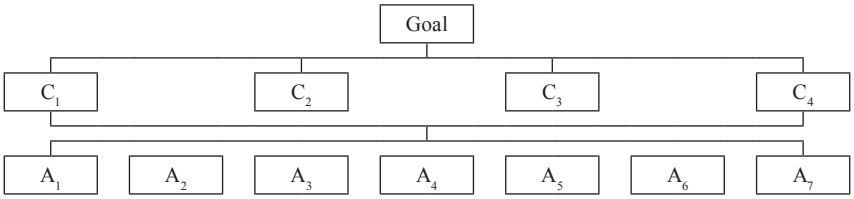


Fig.1. Hierarchy of the problem (Lakićević et al, 2011)

The overall goal is stated as determining the best mixture of seven tree species. A set of four evaluation criteria is stated as:

($C_1$) Adaptability to the environment site conditions

($C_2$) Resistance to damaging agents – pests and diseases

($C_3$) Ornamental value

($C_4$) Cost of establishment and management.

The tree species are considered as alternatives, namely:

($A_1$) *Chamaecyparis lawsoniana* (Murr.) Parl. is an evergreen tree growing to 40 m at medium rate, with a symmetrical, pyramidal and dense crown. Lawson's Cypress is native to North America. This is the most commonly cultivated plant from this genus in Serbia. It is a shade and wind tolerant and long-lived tree distributed over wet areas. It is well adapted to the environment conditions in cities (Vukićević, 1996).

($A_2$) *Thuja occidentalis* L. is an evergreen medium-sized tree, up to 20 m tall. Although originally cultivated in North America, nowadays it is a common plant in Middle and Southern Europe. It is most often associated with cool, moist, nutrient-rich sites, particularly organic soils near streams or other drainageways, or calcareous mineral soils. Northern white cedar grows best in full sun, but will tolerate some shade (Jovanović, 2000).

($A_3$) *Acer saccharinum* L. is a deciduous large-sized tree common in North America. It is also called silver, river, swamp, water and white maple. It is ideal for wet lowland sites, and will easily recover from periods of extended flooding. Due to its high ornamental values, this species often occurs in urban parks. Frequently it is planted as a shade tree on account of its rapid growth. It is a soft wood tree, hence susceptible to wind and storm damages (Vukićević, 1996).

($A_4$) *Platanus x acerifolia* (Ait.) Willd. is a deciduous tree growing to 35 m with a widely spreading, high domed crown. London plane is usually considered to be an interspecies hybrid between *Platanus orientalis* and *Platanus occidentalis*. This species prefers deep, sufficiently moist, well-drained soil. It is generally very adaptable in city en-

vironments and also a smoke and frost tolerant tree (Knežević and Šijačić-Nikolić, 2005).

($A_5$) *Quercus robur* L. is a deciduous tree up to 50 m tall. Pedunculate Oak is native to Europe and western Asia. This species is not that flexible when it comes to soil, requiring deep and fertile soils which are influenced by underground waters and occasionally flooded. The largest areas covered by Pedunculate Oak forests in Serbia are in the valleys of the Sava (Srem is the area with the best quality Pedunculate Oak forests), the Danube and the Morava Rivers (Batos et al, 2010).

($A_6$) *Salix babylonica* L. is a deciduous tree growing to 18 m at a fast rate with an open crown and pendulous branches. Weeping willow is of Asiatic origin and it was introduced to Europe centuries ago. It is a short-lived and rapidly growing tree that can exist even on wetlands. This is a light-demanding species, susceptible to windthrow (Vukićević, 1996).

($A_7$) *Taxodium distichum* Rich. is a deciduous large-sized conifer native to swamps of the United States. Bald Cypress is reputed to be a slow growing and very long-lived tree. When near water, its root form "knees" which stick up above the water level (Phillips and Grant, 1983). It is a light-demanding species, successfully adapted and cultivated in Serbia.

The decision makers were four landscape architects, identified herein as DM1-DM4, all academic professionals.

**Analytic hierarchy process in individual and group decision making**

The Analytic hierarchy process (AHP) (Saaty, 1980) is the most commonly used multi-criteria based tool for supporting decision-making processes in both individual and group frameworks. It requires a well-structured problem represented as a hierarchy with the goal at the top and, at following levels downward, criteria (and sub criteria, if they are specified) and the alternatives at the bottom level.

The AHP determines preferences among the set of decision elements at the given level of a hierarchy against each element at the upper level. The decision maker performs pair-wise comparisons by using the Saaty's importance scale given in Table 1 and creates a comparison matrix. An upper triangle of the matrix contains assigned values to semantic comparisons (Cf. Table 1) made by the decision maker. The main diagonal entries are 1s, and the lower triangle contains reciprocal values from the upper triangle (symmetrically).

Table 1. Saaty's Importance Scale

| Semantic definition | Assigned value |
|---|---|
| Equally important | 1 |
| Weak importance | 3 |
| Strong importance | 5 |
| Demonstrated importance | 7 |
| Absolute importance | 9 |
| Intermediate values | 2,4,6,8 |

The given comparison matrix, a priority vector which contains weights of compared elements, can be computed as a principal (right) eigenvector as suggested by Saaty (1980). Although there are other methods to identify priority vector from the given comparison matrix, the eigenvector method (EV) is the most commonly used (Srdjevic, 2005).

The AHP synthesis is performed once all priority vectors are computed within a hierarchy. Simple additive weighting procedure provides the final utilities (weights) of alternatives at the bottom level versus the overall goal at the top of a hierarchy.

Standard AHP evaluates consistency at all levels of the hierarchy and computes consistency ratio (CR) for the whole hierarchy, unlike other multi-criteria decision-making methods (Lakićević and Srđević, 2011). If CR is smaller than prescribed value 0.10, originally proposed by Saaty (1980), then the decision maker is considered consistent within tolerant limit. If not, the inconsistent decision maker should re-evaluate her/his judgments; in practice, inconsistent matrices are sometimes accepted if CR tolerant value of 0.10 is not significantly violated (Jandrić and Srđević, 2000).

In AHP-group decision-making it is necessary to aggregate information obtained by decision makers (Srđević, 2006). There are different ways to do this (e.g., Forman and Peniwati, 1998). A trustful method is to geometrically average the final individual priority vectors, i.e. the weights of alternatives versus goal, by applying formula (1):

$$w_i^G = \prod_{k=1}^{K} [w_i(k)]^{\alpha_k} \tag{1}$$

where $K$ is the number of decision-makers, $w_i(k)$ is the weight of alternative $i$ computed for the decision-maker $k$, $\alpha_k$ is the weight of the decision-maker, and $w_i^G$ is the the final (group) weight.

## RESULTS AND DISCUSSION

AHP method is applied for the hierarchy in Fig. 1 by all four decision makers (DM1-D4) and results are presented in Tables 2 and 3.

Table 2. Weights of criteria with respect to goal

| Criteria | Weights of criteria | | | |
|---|---|---|---|---|
| | DM1 | DM2 | DM3 | DM4 |
| $C_1$ | 0.527 | 0.168 | 0.240 | 0.063 |
| $C_2$ | 0.291 | 0.464 | 0.160 | 0.080 |
| $C_3$ | 0.115 | 0.329 | 0.372 | 0.421 |
| $C_4$ | 0.067 | 0.039 | 0.228 | 0.436 |

Table 3. Weights of alternatives (tree species) with respect to criteria

| Alt. | DM1 | | | | | DM2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ | $C_4$ | | $C_1$ | $C_2$ | $C_3$ | $C_4$ |
| $A_1$ | 0.074 | 0.252 | 0.040 | 0.043 | | 0.087 | 0.285 | 0.030 | 0.037 |
| $A_2$ | 0.058 | 0.201 | 0.028 | 0.056 | | 0.068 | 0.161 | 0.024 | 0.044 |
| $A_3$ | 0.056 | 0.158 | 0.278 | 0.225 | | 0.103 | 0.085 | 0.283 | 0.233 |
| $A_4$ | 0.105 | 0.105 | 0.268 | 0.132 | | 0.073 | 0.121 | 0.072 | 0.127 |
| $A_5$ | 0.380 | 0.057 | 0.083 | 0.231 | | 0.367 | 0.055 | 0.136 | 0.251 |
| $A_6$ | 0.196 | 0.033 | 0.207 | 0.231 | | 0.212 | 0.050 | 0.274 | 0.251 |
| $A_7$ | 0.130 | 0.195 | 0.096 | 0.081 | | 0.090 | 0.243 | 0.181 | 0.056 |
| Alt. | DM3 | | | | | DM4 | | | |
| | $C_1$ | $C_2$ | $C_3$ | $C_4$ | | $C_1$ | $C_2$ | $C_3$ | $C_4$ |
| $A_1$ | 0.292 | 0.152 | 0.258 | 0.044 | | 0.321 | 0.037 | 0.307 | 0.048 |
| $A_2$ | 0.177 | 0.026 | 0.024 | 0.047 | | 0.180 | 0.245 | 0.126 | 0.043 |
| $A_3$ | 0.356 | 0.457 | 0.160 | 0.223 | | 0.321 | 0.183 | 0.091 | 0.225 |
| $A_4$ | 0.058 | 0.185 | 0.050 | 0.116 | | 0.059 | 0.031 | 0.029 | 0.116 |
| $A_5$ | 0.052 | 0.109 | 0.085 | 0.219 | | 0.053 | 0.058 | 0.095 | 0.237 |
| $A_6$ | 0.023 | 0.045 | 0.260 | 0.287 | | 0.023 | 0.161 | 0.299 | 0.246 |
| $A_7$ | 0.042 | 0.025 | 0.163 | 0.064 | | 0.042 | 0.285 | 0.052 | 0.086 |

The weights of alternatives versus goal, individually obtained by standard AHP synthesis, are presented in Table 4. These values represent preferences of the decision makers if they would individually define an optimal (in multi-criteria sense) mixture of tree species in a given wet urban area.

For equally 'weighted' decision makers ($\alpha_1=\alpha_2=\alpha_3=\alpha_4=0.25$), the geometrically averaged weights are presented in the last column of the Table 4. These values represent the final 'participation' of assessed tree species in the mixture as the joint group decision.

Table 4. The final AHP results – individual and group

| Alt. | Tree species | Weights | | | | |
|---|---|---|---|---|---|---|
| | | DM1 | DM2 | DM3 | DM4 | Group |
| $_A1$ | Chamaecyparis lawsoniana | 0.120 | 0.158 | 0.200 | 0.173 | 0.165 |
| $A_2$ | Thuja occidentalis | 0.096 | 0.096 | 0.067 | 0.103 | 0.092 |
| $A_3$ | Acer saccharinum | 0.123 | 0.159 | 0.269 | 0.171 | 0.179 |
| $A_4$ | Platanus x acerifolia | 0.125 | 0.097 | 0.089 | 0.069 | 0.096 |
| $A_5$ | Quercus robur | 0.242 | 0.142 | 0.112 | 0.151 | 0.160 |
| $A_6$ | Salix babylonica | 0.152 | 0.159 | 0.175 | 0.247 | 0.185 |
| $A_7$ | Taxodium distichum | 0.142 | 0.190 | 0.089 | 0.085 | 0.123 |

Worthy of note is that the overall consistencies (CR) of decision makers were within tolerant limit of 0.10, except the decision maker 3 (Table 5). This situation can be considered as a usual one.

Table 5. Consistency Ratio (CR)

| DM | CR |
|---|---|
| DM1 | 0.067 |
| DM2 | 0.066 |
| DM3 | 0.151 |
| DM4 | 0.049 |

## CONCLUSION

The survival of trees in any urban environment is a demanding task. The selection of the most appropriate trees to create mixtures for specific urban sites, especially wet ones, is a task to be performed by landscape planners in attempt to maintain healthy and graceful green areas. To define the best tree mixture, it is important to take into account certain criteria and sometimes sub-criteria. Multi-criteria analysis and the Analytic hierarchy process, as recognized methods in the subject area, may help to compute the exact mixture share (percentage) of tree species.

An illustrative scientific experiment described in this paper demonstrates how the group decision making process can be performed in landscape planning, namely in mixing seven selected tree species in greening urban wet area. The result of this particular AHP-group application indicates that the approach we used could also be efficient if more different adaptable tree species were evaluated in order to preserve endangered biodiversity in cities.

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## AHP – GRUPNO ODLUČIVANJE O IZBORU VRSTA DRVEĆA ZA VLAŽNA STANIŠTA U GRADOVIMA

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**Izvod**

Razmatra se problem izbora vrsta drveća za vlažna staništa u gradovima. Kao naučni metod korišćen je analitički hijerahijski proces (AHP) u standardnoj verziji, a objedinjavanje preferenci donosilaca odluka vršeno je geometrijskim osrednjavanjem težina razmatranih alternativa pri individualnim primenama AHP. Za problem odlučivanja struktuiran kao hijerarhija sa četiri kriterijuma vrednovanja za sedam vrsta drveća, dobijen je, u višekriterijumskom smislu optimalan sastav drveća. Cilj rada je da ukaže na pristup koji bi pejzažne planere mogao da podstakne na savremeno rešavanje pripadajućih inženjerskih zadataka. Numerički primer ima ilustrativni karakter.

**Ključne reči:** vlažna gradska staništa, izbor vrsta drveća, analitički hijerarhijski proces, grupno odlučivanje.

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# IMPACT OF COPPER TO THE CHELATION EFFECT OF BOVINE SERUM ALBUMIN AND SPERMATOZOA MOTILITY PARAMETER *IN VITRO**

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*SUMMARY: The target of this in vitro study was to analyse the influence of copper on the spermatozoa motility in the presence of bovine serum albumin (BSA) as culture medium and to provide additional information on the interaction between serum albumin and copper (II) chloride ($CuCl_2$). The spermatozoa motility parameters was determined after exposure of $CuCl_2$ (3.9; 7.8; 15.6; 31.2; 62.5; 125; 250; 500; 1000 µmol/L) using the Sperm Vision™ CASA (Computer Assisted Semen Analyzer) system during different time periods (Time 0 h, 1 h, 2 h and 24). The culture medium containing 20% BSA, triladyl and 5% glucose increased the overall percentage of spermatozoa motility after 1 h of cultivation. The percentage of motility spermatozoa significantly ($P<0.001$) decreased after 2 h of cultivation at the concentrations ≥ 250 µmol/L of $CuCl_2$ in comparison with the control group (without $CuCl_2$ administration). The experimental administration at the doses ≤ 31.2 µmol/L of $CuCl_2$ stimulated the overall of motile spermatozoa (Time 24 h). Identical spermatozoa motility was detected also for the percentage of progressive motile spermatozoa during all time periods. Parameter of distance average path (DAP) showed increase in all $CuCl_2$ addition groups in comparison with the control group at Time 1 h. Concentration 125 µmol/L of $CuCl_2$ in various time periods of cultivation act stimulating on spermatozoa motility, but later (Time 24 h) inhibitory. Evaluation of velocity average path, showed similar results as for DAP. Measurement of the amplitude of lateral displacement (ALH) at Time 0 h as well as at Time 1 h was higher in all the experimental groups compared to the control group, but the differences were not significant ($P>0.05$). The experimental administration at the doses ≤ 62.5 µmol/L of $CuCl_2$ stimulated ALH during 24 h of cultivation. The results suggest that adding energy*

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*and protein substrate to the culture medium increases the spermatozoa motility parameters also the presence of high doses (≥ 125 µmol/L) of copper ions during short-term periods (Time 0 h, 1 h). Concurrently BSA maintained motility of spermatozoa (≤ 31.20 µmol/L of $CuCl_2$) during the long-term (Time 24 h) of cultivation, which confirms the protective effect of albumin binding to the copper ions.*

***Key words:*** *copper, bovine serum albumin, spermatozoa, motility.*

## INTRODUCTION

Essential trace minerals (ETMs) are among important factors in maintaining and recovering health (Hostetler et al., 2003). The necessity of ETMs for support of life is largely unquestioned, however their requirement for reproduction has not been as extensively studied (Lu et al., 2009). Copper (Cu) is an important microelement for the animal and human organism, because it has a great positive role in physiological and regulatory processes (Dobrzanski et al., 1996; Tang, 2005). It is involved in numerous biological processes (Goyer, 1991; Massanyi et al., 2003) and it is a component of a number of metalloenzymes and metalloproteins, such as superoxide dismutase (Cu/Zn SOD) (Agarwal et al., 1990), catalase, peroxidases, cytochrome oxidase, lysine oxidase, dopamine-β-hydroxylase and ceruloplasmin, which are involved in energy and antioxidant metabolism (Haliwell and Gutteridge, 2000; Aydemir et al., 2006). Copper plays an important role in male and female reproduction system (Ebesh et al. 1999; Wong et al. 2001). The excessive Cu intake has a negative effect on the organs of reproduction (Jockenhovel al et., 1990; Katayose et al., 2004). The high concentrations of copper ions ($Cu^{2+}$) have a toxic effect on the epididymis (Xu et al., 1985), testes, scrotum of mammals (Skandhan, 1992; Eidi et al., 2010), which may ultimately lead to a reduced fertility (Pesch et al., 2006). Several experimental studies demonstrated the adverse effects of $Cu^{2+}$ on spermatozoa motility (White and Rainbow, 1985; Viarengo et al., 1996; Wong et al., 2001; Machal et al., 2002; Roychoudhury and Massanyi, 2008; Roychoudhury et al., 2010; Knazicka et al., 2010a; Sakhaee et al., 2011), This element reduces oxidative processes and glucose consumption (Skandhan, 1992), consequently it minimizes or disrupts spermatozoa motility (Chen et al., 1989).

Spermatozoa are extremely sensible to *ex vivo* conditions and on the loss of exogenous energy sources therefore different culture media are used on the viability prolongation of spermatozoa. Semen culture media usually contain glucose or fructose as the dominant energy substrate (Matsuoka et al., 2006) and bovine serum albumin (BSA) as a protein alternative to egg yolk (Peters et al., 1975). Serum albumin is a multifunctional protein, which forms covalent adducts with various metals ($Cu^{2+}$, $Ni^{2+}$, $Hg^{2+}$, $Ag^{2+}$, $Au^{+}$) (Stamler et al., 1992; Simion et al., 2009). It provides a range of benefits including protection from oxidative damage, stabilization of other media components (i.e fatty acids, pyridoxal) and inactivating various toxic lipophilic metabolites (i.e bilirubin) (Emerson, 1989). Recently several researchers investigated interactions between $Cu^{2+}$ with serum albumin, due to the importance of Cu for various biological and chemical processes (Anzai et al. 1996; Schwarz et al., 2000; Yan et al., 2003; Pinto et al., 2008). However, in this field there is still a lack of information about the influence of BSA as a culture

medium component on the general spermatozoa viability. Therefore, the purpose of this *in vitro* study was to provide additional information on the interaction between serum albumin and copper (II) chloride on the spermatozoa motility parameters.

## MATERIAL AND METHODS

Bovine semen samples were obtained from 4 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic quality criteria given for the corresponding breed. The semen was obtained on a regular collection schedule using an artificial vagina. After collecting the samples, they were stored in the laboratory at room temperature (22-25°C). Each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medital, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration.

The culture medium of bovine serum albumin (BSA; final concentration of 20%; Sigma-Aldrich, USA) was prepared by dissolving protein into buffer (Phosphate Buffer Saline - PBS; Sigma, St. Louis, USA), triladyl® (MiniTüb; Tiefenbach Germany), glucose (5% D-glukosa monohydrate p.a; Penta Chrudim, Czech Republic) and redistilled water. Semen samples were added to the culture medium and cultivated with various concentrations of Cu (group I – 3.9; H – 7.8; G – 15.6; F – 31.2; E - 62.5; D - 125; C - 250; B - 500; A - 1000 μmol/L), in the form of copper (II) chloride ($CuCl_2$; Sigma-Aldrich, St. Louis, USA). Spermatozoa with $CuCl_2$ were incubated in the laboratory at room temperature (22-25°C) for 24 h. We compared the control (Ctrl) group (medium without $CuCl_2$) with the experimental groups (exposed to different concentrations of $CuCl_2$).

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision™ program (MiniTüb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Japan) at cultivation Times 0 h, 1 h, 2 h and 24 h. Each sample was placed into the Makler Counting Chamber (deph 10 μm, Sefi-Medical Instruments, Izrael) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5 μm/s; MOT); percentage of progressive motile spermatozoa (motility > 20 μm/s; PROG); distance average path (DAP; μm); velocity average path (VAP; μm/s) and amplitude of lateral head displacement (ALH; μm) This study was performed in three replicates at each concentration (n = 8).

Statistical analysis of the results was carried out using the statistical program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance (ANOVA) and the Dunnett´s multiple comparison test were used for statistical evaluations. The level of significance was set at $^{A}$ ($P<0.001$); $^{B}$ ($P<0.01$); $^{C}$ ($P<0.05$).

## RESULTS

The initial (Time 0 h) spermatozoa motility showed slightly increased values at doses ≥ 31.20 μmol/L of $CuCl_2$ but no significant differences ($P>0.05$) were found between these groups and the control group (without $CuCl_2$ administration). The percentage of motile spermatozoa decreased slowly after 1 h of cultivation compared to Time 0. The average motility values significantly ($P<0.001$) decreased during 2 h of cultivation

at the concentrations ≥ 250 μmol/L of $CuCl_2$ (Table 1) in comparison with the control group. However, the other concentrations stimulated the percentage of spermatozoa motility. The lowest spermatozoa motility was significantly (P<0.001) detected in the groups with the highest doses (≥ 125 μmol/L) of $CuCl_2$ (Time 24 h). The low concentrations increased the average motility values, especially in the groups H and I compared to the control group (69.65% and 70.04% versus 64.15%).

Table. 1. Spermatozoa motility (MOT; %) exposed to copper ($CuCl_2$) in BSA during different time periods.

| Groups | Control | 1000 | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 | 3.9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | A | B | C | D | E | F | G | H | I |
| CuCl2 (μmol/L) | | | | | | | | | | |
| **Time 0 h** | | | | | | | | | | |
| x | 87.53 | 90.38 | 91.53 | 90.36 | 88.32 | 87.71 | 88.40 | 85.58 | 86.75 | 85.67 |
| minimum | 80.43 | 84.50 | 88.65 | 85.36 | 81.35 | 76.59 | 80.85 | 76.19 | 75.75 | 74.19 |
| maximum | 100.0 | 97.95 | 93.33 | 95.23 | 97.05 | 96.55 | 96.06 | 94.68 | 96.29 | 94.73 |
| S.D. | 5.50 | 4.12 | 1.47 | 2.98 | 5.34 | 5.84 | 3.97 | 5.91 | 6.08 | 7.33 |
| CV (%) | 6.29 | 4.56 | 1.61 | 3.29 | 6.04 | 6.66 | 4.49 | 6.90 | 7.01 | 8.56 |
| **Time 1 h** | | | | | | | | | | |
| x | 81.32 | 85.55 | 85.72 | 82.45 | 85.84 | 84.96 | 82.60 | 82.37 | 83.65C | 83.53C |
| minimum | 70.00 | 75.47 | 75.00 | 77.77 | 73.07 | 70.52 | 70.58 | 69.23 | 69.23 | 70.00 |
| maximum | 88.52 | 93.54 | 93.57 | 90.41 | 96.03 | 96.10 | 93.13 | 92.39 | 96.93 | 94.73 |
| S.D. | 6.05 | 5.30 | 5.42 | 4.12 | 5.72 | 7.68 | 6.49 | 7.34 | 9.50 | 7.85 |
| CV (%) | 7.44 | 6.19 | 6.32 | 4.99 | 6.66 | 9.04 | 7.85 | 8.91 | 11.36 | 9.40 |
| **Time 2 h** | | | | | | | | | | |
| x | 75.87 | 70.05A | 73.88A | 74.58A | 76.07 | 76.78 | 79.50 | 79.27 | 80.90 | 79.62C |
| minimum | 61.11 | 56.66 | 55.17 | 51.35 | 61.64 | 54.54 | 67.92 | 60.00 | 65.11 | 70.07 |
| maximum | 90.00 | 82.81 | 86.20 | 92.17 | 89.58 | 92.72 | 88.65 | 94.59 | 91.52 | 90.74 |
| S.D. | 9.24 | 7.61 | 11.62 | 13.26 | 7.45 | 10.53 | 7.00 | 9.95 | 6.05 | 6.55 |
| CV (%) | 12.17 | 10.86 | 15.73 | 17.78 | 9.79 | 13.72 | 8.80 | 12.55 | 7.47 | 8.23 |
| **Time 24 h** | | | | | | | | | | |
| x | 64.15 | 2.78A | 6.03A | 11.38A | 38.86A | 58.97 | 66.47 | 68.45 | 69.65 | 70.04 |
| minimum | 41.17 | 1.07 | 2.63 | 5.00 | 30.76 | 27.27 | 30.43 | 47.05 | 60.56 | 50.00 |
| maximum | 79.74 | 5.49 | 9.25 | 17.33 | 41.38 | 67.30 | 78.37 | 79.72 | 77.61 | 86.66 |
| S.D. | 12.04 | 1.51 | 2.08 | 4.80 | 4.06 | 10.44 | 13.15 | 9.45 | 6.19 | 11.06 |
| CV (%) | 18.76 | 54.28 | 34.51 | 42.21 | 10.45 | 17.71 | 19.79 | 13.81 | 8.88 | 15.79 |

x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}$P<0.001; $^{B}$P<0.01; $^{C}$P<0.05

Identical spermatozoa motility was detected also for the percentage of progressive motile spermatozoa (> 20 μm/s) during all time periods (Table 2). A significant (P<0.001) decrease of progressive motility at the concentrations ≥ 62.50 μmol/L of $CuCl_2$ was detected during the long-term cultivation (Time 24 h). However, the experimental administration at the doses ≤ 31.20 μmol/L of $CuCl_2$ stimulated (P<0.001) the overall of progressive motile spermatozoa.

Table. 2. Progressive spermatozoa motility (PROG; %) exposed to copper ($CuCl_2$) in BSA during different time periods.

| Groups | Control | 1000 | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 | 3.9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | A | B | C | D | E | F | G | H | I |
| $CuCl_2$ (µmol/L) | | | | | | | | | | |
| **Time 0 h** | | | | | | | | | | |
| x | 84.69 | 86.72 | 88.49 | 87.56 | 85.41 | 85.36 | 86.84 | 83.70 | 84.00 | 82.87 |
| minimum | 79.06 | 78.66 | 86.48 | 82.85 | 76.31 | 75.75 | 79.04 | 73.07 | 66.78 | 74.19 |
| maximum | 97.82 | 97.95 | 89.53 | 90.47 | 97.05 | 93.84 | 92.41 | 93.13 | 93.82 | 91.89 |
| S.D. | 5.47 | 5.82 | 1.06 | 2.35 | 6.70 | 5.71 | 3.62 | 5.65 | 7.75 | 6.05 |
| CV (%) | 6.46 | 6.71 | 1.19 | 2.68 | 7.84 | 6.69 | 4.17 | 6.75 | 9.22 | 7.30 |
| **Time 1 h** | | | | | | | | | | |
| x | 79.36 | 82.04 | 81.84 | 79.70 | 82.56 | 80.35 | 79.77 | 79.97 | 80.16 | 81.33 |
| minimum | 66.66 | 73.33 | 72.22 | 71.26 | 67.85 | 70.83 | 72.91 | 69.23 | 65.38 | 67.64 |
| maximum | 88.00 | 92.30 | 92.66 | 87.67 | 93.06 | 88.70 | 90.81 | 90.19 | 93.12 | 91.76 |
| S.D. | 7.04 | 5.77 | 5.31 | 4.58 | 7.16 | 5.78 | 5.35 | 5.50 | 8.70 | 7.88 |
| CV (%) | 8.87 | 7.03 | 6.48 | 5.75 | 8.67 | 7.20 | 6.71 | 6.88 | 10.85 | 9.68 |
| **Time 2 h** | | | | | | | | | | |
| x | 70.54 | 66.52 | 69.26 | 70.55 | 70.87 | 71.19 | 74.96 | 75.61 | $75.28^{C}$ | $75.24^{C}$ |
| minimum | 58.91 | 48.48 | 45.71 | 48.58 | 58.90 | 50.90 | 64.58 | 63.63 | 62.79 | 68.42 |
| maximum | 84.00 | 83.63 | 87.01 | 86.95 | 85.41 | 85.45 | 84.44 | 89.18 | 84.84 | 85.18 |
| S.D. | 7.65 | 9.24 | 14.01 | 12.37 | 7.74 | 10.32 | 5.45 | 7.79 | 5.36 | 5.24 |
| CV (%) | 10.85 | 13.89 | 20.23 | 17.54 | 10.92 | 14.50 | 7.27 | 10.31 | 7.12 | 6.96 |
| **Time 24 h** | | | | | | | | | | |
| x | 59.87 | $1.20^{A}$ | $2.63^{A}$ | $3.02^{A}$ | $22.48^{A}$ | $46.84^{A}$ | $61.39^{A}$ | $62.90^{A}$ | $62.15^{A}$ | $66.92^{A}$ |
| minimum | 40.00 | 0.29 | 0.33 | 1.07 | 18.36 | 37.50 | 36.36 | 47.05 | 37.50 | 43.75 |
| maximum | 75.38 | 2.19 | 5.88 | 6.15 | 28.57 | 56.16 | 66.66 | 71.87 | 73.33 | 80.95 |
| S.D. | 11.78 | 1.06 | 2.30 | 1.64 | 3.10 | 6.22 | 9.23 | 8.06 | 12.32 | 10.20 |
| CV (%) | 19.68 | 87.77 | 87.39 | 54.19 | 13.77 | 13.28 | 15.04 | 12.81 | 19.83 | 15.25 |

x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Parameter of distance average path (DAP) showed increase in all $CuCl_2$ addition groups in comparison with the control group during 1 h of cultivation (Table 3). The DAP analysis revealed significant differences ($P<0.001$) at the concentrations $\geq$ 250 µmol/L of $CuCl_2$ in comparison to the control group at Time 2 h. Other data are not significant in comparison with the control group. Interestingly, concentration 125 µmol/L of $CuCl_2$ in short-term periods of cultivation act stimulating on the spermatozoa motility, but later (Time 24 h) inhibiting of selected parameter.

Table. 3. Distance average path (DAP; μm) exposed to copper ($CuCl_2$) in BSA during different time periods.

| Groups | Control | 1000 | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 | 3.9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | A | B | C | D | E | F | G | H | I |
| CuCl2 (μmol/L) | | | | | | | | | | |
| **Time 0 h** | | | | | | | | | | |
| x | 41.29 | 43.17 | 43.47C | 43.07 | 41.85 | 41.67 | 41.55 | 38.13A | 37.69A | 39.56 |
| minimum | 39.15 | 39.17 | 40.07 | 40.28 | 38.90 | 38.53 | 38.93 | 34.64 | 35.62 | 37.17 |
| maximum | 43.83 | 45.52 | 46.55 | 48.65 | 46.98 | 45.02 | 45.67 | 45.02 | 39.15 | 41.01 |
| S.D. | 1.46 | 1.58 | 1.83 | 2.77 | 2.87 | 1.87 | 2.50 | 3.81 | 0.99 | 1.16 |
| CV (%) | 3.52 | 3.66 | 4.20 | 6.43 | 6.87 | 4.48 | 6.01 | 9.98 | 2.62 | 2.94 |
| **Time 1 h** | | | | | | | | | | |
| x | 34.83 | 38.13A | 38.2A | 35.09A | 38.42A | 37.32C | 35.24 | 35.47 | 35.70 | 35.44 |
| minimum | 30.30 | 35.53 | 33.52 | 31.55 | 36.08 | 33.19 | 30.18 | 30.39 | 30.54 | 30.43 |
| maximum | 38.55 | 41.86 | 41.86 | 39.87 | 41.52 | 41.46 | 39.47 | 40.79 | 38.74 | 39.95 |
| S.D. | 2.47 | 1.38 | 2.59 | 2.43 | 1.67 | 2.17 | 3.42 | 2.92 | 2.52 | 2.62 |
| CV (%) | 7.10 | 3.63 | 6.77 | 6.93 | 4.35 | 5.80 | 9.71 | 8.24 | 7.07 | 7.39 |
| **Time 2 h** | | | | | | | | | | |
| x | 30.30 | 28.78A | 29.06A | 29.16A | 30.59 | 31.19 | 32.76 | 32.19 | 33.28 | 33.10 |
| minimum | 25.25 | 25.20 | 22.87 | 27.68 | 25.62 | 26.09 | 28.28 | 28.81 | 31.06 | 29.21 |
| maximum | 33.21 | 31.96 | 32.78 | 31.59 | 36.86 | 37.78 | 38.85 | 37.48 | 37.56 | 39.56 |
| S.D. | 2.33 | 2.63 | 2.84 | 1.46 | 3.09 | 3.57 | 2.30 | 2.46 | 2.31 | 3.33 |
| CV (%) | 7.68 | 9.14 | 9.76 | 5.01 | 10.09 | 11.43 | 10.07 | 7.65 | 6.93 | 10.06 |
| **Time 24 h** | | | | | | | | | | |
| x | 20.11 | 3.88A | 6.05A | 11.57A | 13.69A | 20.14 | 22.77 | 24.70C | 25.46A | 29.45A |
| minimum | 16.92 | 2.45 | 3.00 | 9.43 | 11.78 | 17.93 | 16.64 | 14.17 | 15.62 | 23.53 |
| maximum | 23.47 | 5.60 | 8.40 | 12.89 | 14.94 | 22.66 | 27.68 | 28.37 | 31.23 | 34.10 |
| S.D. | 1.84 | 1.21 | 2.34 | 1.87 | 1.11 | 1.68 | 4.25 | 5.06 | 5.25 | 3.05 |
| CV (%) | 9.16 | 31.10 | 38.59 | 16.18 | 8.09 | 8.34 | 18.67 | 20.50 | 20.60 | 10.36 |

x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Evaluation of velocity average path (VAP), showed similar results as for DAP (Time 0 h, 1 h). After 2 h of cultivation we proved that the experimental administration at the highest dose (1000 μmol/L) of $CuCl_2$ significantly ($P<0.001$) decreased of selected parameter. Parameter of velocity average path detected that spermatozoa exposed to low copper concentrations (≤ 31.20 μmol/L of $CuCl_2$) after 24 h of cultivation ($P<0.001$) are more active as those in control group, but in relation to higher copper concentration (≥ 500 μmol/L of $CuCl_2$) significant ($P<0.001$) decrease was observed.

Table. 4. Velocity average path (VAP; μm/s) exposed to copper ($CuCl_2$) in BSA during different time periods.

| Groups | Control | 1000 | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 | 3.9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | A | B | C | D | E | F | G | H | I |
| $CuCl_2$ (μmol/L) | | | | | | | | | | |
| **Time 0 h** | | | | | | | | | | |
| x | 97.98 | 101.20 | 103.00 | 100.10 | 99.29 | 98.04 | 99.27 | 92.69 | 95.55 | 93.61 |
| minimum | 91.44 | 93.41 | 92.25 | 90.95 | 91.64 | 85.72 | 91.05 | 76.39 | 78.43 | 85.44 |
| maximum | 102.50 | 111.40 | 119.10 | 105.50 | 107.00 | 116.20 | 109.90 | 108.60 | 113.60 | 101.80 |
| S.D. | 3.70 | 4.51 | 8.77 | 4.22 | 5.77 | 8.99 | 6.28 | 10.79 | 10.70 | 5.82 |
| CV (%) | 3.78 | 4.46 | 8.51 | 4.22 | 5.81 | 9.17 | 6.32 | 11.64 | 11.20 | 6.21 |
| **Time 1 h** | | | | | | | | | | |
| x | 80.63 | 89.34$^{A}$ | 89.16$^{C}$ | 83.34 | 90.02$^{A}$ | 85.68 | 83.59 | 83.86 | 85.09 | 85.12 |
| minimum | 72.47 | 79.16 | 76.29 | 70.20 | 73.85 | 75.20 | 71.44 | 76.61 | 65.07 | 70.18 |
| maximum | 88.46 | 99.12 | 99.63 | 100.60 | 107.40 | 98.98 | 99.05 | 98.05 | 97.92 | 99.63 |
| S.D. | 5.70 | 5.93 | 7.35 | 10.22 | 10.16 | 7.04 | 9.43 | 6.37 | 10.35 | 9.04 |
| CV (%) | 7.07 | 6.64 | 8.25 | 12.26 | 11.28 | 8.22 | 11.28 | 7.59 | 12.17 | 10.62 |
| **Time 2 h** | | | | | | | | | | |
| x | 74.19 | 66.41$^{A}$ | 67.82$^{C}$ | 68.50 | 72.19 | 74.66 | 75.05 | 75.20 | 76.98 | 76.54 |
| minimum | 58.64 | 51.56 | 53.36 | 60.23 | 54.77 | 62.91 | 65.27 | 66.45 | 70.24 | 61.41 |
| maximum | 88.34 | 79.97 | 78.53 | 88.34 | 83.06 | 81.99 | 86.66 | 89.90 | 92.12 | 94.27 |
| S.D. | 8.01 | 8.80 | 8.20 | 7.98 | 7.39 | 5.32 | 6.99 | 7.22 | 6.14 | 7.69 |
| CV (%) | 10.80 | 13.37 | 12.09 | 11.65 | 10.24 | 7.12 | 9.31 | 9.60 | 7.98 | 10.05 |
| **Time 24 h** | | | | | | | | | | |
| x | 41.87 | 10.79$^{A}$ | 18.51$^{A}$ | 25.15 | 31.56 | 45.61 | 54.75$^{A}$ | 56.19$^{A}$ | 57.30$^{A}$ | 65.56$^{A}$ |
| minimum | 36.50 | 7.89 | 9.68 | 19.51 | 27.02 | 38.65 | 31.65 | 29.41 | 33.43 | 50.28 |
| maximum | 48.95 | 14.78 | 27.67 | 28.63 | 38.70 | 59.84 | 67.94 | 67.88 | 69.74 | 76.90 |
| S.D. | 3.57 | 2.83 | 7.14 | 4.93 | 3.86 | 6.21 | 12.24 | 13.35 | 12.71 | 7.59 |
| CV (%) | 8.52 | 26.22 | 38.57 | 19.60 | 12.21 | 13.63 | 22.36 | 23.76 | 22.18 | 11.58 |

x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Measurement of the amplitude of lateral displacement (ALH) at Time 0 h as well as at Time 1 h was higher in all the experimental groups compared to the control group but the differences were not significant ($P>0.05$). The experimental administration at the doses ≤ 250 μmol/L of $CuCl_2$ stimulated ALH during 2 h of cultivation. Similar results were observed after 24 h, with the exception of groups A ($P<0.05$), B, C and D with the high copper concentrations, which decreased of ALH (Table 5).

Table. 5. Amplitude of lateral head displacement (ALH; μm/s) exposed to copper ($CuCl_2$) in BSA during different time periods.

| Groups | Control | 1000 | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 | 3.9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | A | B | C | D | E | F | G | H | I |
| CuCl2 (μmol/L) | | | | | | | | | | |
| **Time 0 h** | | | | | | | | | | |
| x | 4.68 | 5.02 | 5.09 | 5.07 | 5.03 | 4.84 | 4.88 | 4.76 | 4.79 | 4.64 |
| minimum | 3.66 | 4.20 | 4.18 | 3.95 | 3.48 | 3.56 | 3.72 | 3.49 | 3.15 | 3.19 |
| maximum | 5.89 | 5.70 | 6.25 | 6.25 | 6.21 | 6.21 | 6.04 | 6.36 | 6.02 | 6.21 |
| S.D. | 0.76 | 0.43 | 0.74 | 0.79 | 0.78 | 0.93 | 0.81 | 0.90 | 0.77 | 0.85 |
| CV (%) | 16.20 | 8.55 | 14.61 | 15.67 | 15.55 | 19.33 | 16.55 | 18.96 | 16.02 | 18.20 |
| **Time 1 h** | | | | | | | | | | |
| x | 5.10 | 5.56 | 5.49 | 5.22 | 5.64 | 5.42 | 5.30 | 5.37 | 5.28 | 5.28 |
| minimum | 4.58 | 4.80 | 3.88 | 3.98 | 5.22 | 4.05 | 4.10 | 3.59 | 4.15 | 4.15 |
| maximum | 5.62 | 7.91 | 8.01 | 6.30 | 7.01 | 8.40 | 6.36 | 6.65 | 6.81 | 5.80 |
| S.D. | 0.39 | 0.74 | 1.02 | 0.65 | 0.51 | 1.02 | 0.66 | 0.78 | 0.75 | 0.38 |
| CV (%) | 7.67 | 13.39 | 18.55 | 12.49 | 9.01 | 18.79 | 12.93 | 14.57 | 14.12 | 7.20 |
| **Time 2 h** | | | | | | | | | | |
| x | 4.87 | 4.80 | 4.85 | 4.90 | 5.03 | 5.09 | 5.11 | 5.17 | 5.19 | 5.11 |
| minimum | 4.29 | 4.22 | 3.58 | 3.59 | 3.09 | 4.69 | 4.23 | 3.93 | 4.50 | 4.74 |
| maximum | 5.75 | 5.43 | 5.91 | 6.00 | 6.57 | 6.34 | 7.07 | 6.38 | 7.08 | 5.76 |
| S.D. | 0.58 | 0.42 | 0.74 | 0.72 | 0.95 | 0.54 | 0.80 | 0.69 | 0.89 | 0.30 |
| CV (%) | 11.95 | 8.85 | 15.30 | 14.64 | 18.82 | 10.64 | 15.57 | 13.42 | 17.18 | 5.95 |
| **Time 24 h** | | | | | | | | | | |
| x | 2.94 | 1.81C | 1.84 | 2.02 | 2.60 | 3.58 | 4.08C | 4.18A | 4.39A | 4.85A |
| minimum | 1.85 | 1.12 | 1.00 | 1.56 | 2.37 | 2.19 | 2.80 | 1.78 | 2.61 | 3.86 |
| maximum | 3.85 | 2.28 | 2.85 | 2.36 | 3.07 | 4.36 | 4.96 | 5.46 | 5.44 | 5.57 |
| S.D. | 0.81 | 0.47 | 0.67 | 0.30 | 0.22 | 0.65 | 0.77 | 1.05 | 0.96 | 0.53 |
| CV (%) | 27.45 | 25.74 | 36.56 | 14.76 | 8.38 | 18.06 | 18.79 | 25.08 | 21.91 | 10.88 |

x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

## DISCUSION

In the present study we evaluated the spermatozoa motility in the presence of $CuCl_2$ with culture medium addition in composition of 20% BSA, triladyl and 5% glucose. This culture medium increased the bovine spermatozoa motility during the short-term of cultivation (Time 0 h, 1 h) in spite of the presence of high doses (≥ 125 μmol/L) of $CuCl_2$. This observation could be explained binding of copper ions to albumin, which confirms previous experimental studies (Bradshaw et al., 1968; Masuoka and Saltman, 1994). The role of albumin is protective as a result of its ability to trap toxic substances in the culture media (Yamane et al., 1976; Fox and Flynn, 2003). The binding of metals to proteins is a defense to reduce toxicity by preventing availability of the metals (Davidson et al., 2007) and it is vital to our understanding of the relationship between its structure and function (Masuoka, et al., 1993; Masuoka and Saltman, 1994).

Several authors consider bovine serum albumin as a suitable protein supplement for the long-term spermatozoa cultivation, because it has protective functions (Yamane

et al., 1976) and in addition a good essential amino acid profile (Peters et al., 1975). Regarding our results we can confirm, that the overall percentage of motile spermatozoa at doses ≤ 31.20 μmol/L of $CuCl_2$ was maintained during the long-term (Time 24 h) of *in vitro* cultivation. It could be explained by a high concentration of energy and protein substrates in the medium. From our study, the highest sensitivity of spermatozoa to Cu was found when activating in BSA containing the highest doses (500 μM; 1000 μM) of $CuCl_2$.

Several investigators have found that the incorporation of BSA in semen diluents can protect and stimulate the spermatozoa of many species. Klem et al. (1986) confirmed that BSA increases of equine the spermatozoa motility. It is likely that higher values of bovine spermatozoa motility characteristics obtained in our study may be at attributed to the stimulating effect of BSA. Equally, Bakst and Cecil (1992) reported the possible effects of BSA on the turkey spermatozoa viability. Similar observations have been recorded with spermatozoa of different animal species (Harrison et al., 1978; 1982). An appropriate energy substrate (Knazicka et al., 2010b), protein supply, as well as optimal laboratory conditions are important factors for a successful *in vitro* spermatozoa motility and viability (Tvrda et al., 2010).

There are still questions about the optimal BSA concentration for spermatozoa cultivation, since high concentrations of any substance may be toxic (Tvrda et al., 2010). The aim of the investigation of Serniene et al. (2001) was to study the effect on semen quality caused by the addition of BSA to boar semen and to determine the optimal dose of the BSA. The analysis revealed that addition of BSA, spermatozoa storage time and their interaction had significant effect only on the agglutination rate. In their conclusion addition of 0.5 g BSA to the insemination dose significantly decreased the agglutination rate of spermatozoa and did not significantly affect the motility, vigor rate and a number of viable/non damaged spermatozoa per ejaculation. El-Kon (2011) conducted to test the post-thaw spermatozoa characteristics through addition of different concentrations BSA to buffalo semen. Observed data from this study demonstrated that spermatozoa motility (58.20±4.60% and 59.40±4.80%) and viability (69.30±4.10% and 69.20±4.20%) were significantly ($P<0.05$) higher in the 10% and 15% BSA groups than in the tris-egg yolk control group and other samples (0.5; 1.0 and 5.0% BSA) containing BSA. These findings are in agreement with the previous results by Matsuoka et al. (2006), which studied the effects of different BSA concentrations (0; 0.3; 1; 5; 10 and 15%) on the post-thaw viability of ram spermatozoa. Our own results argue in favour of 20% BSA which has a stimulating function on the spermatozoa motility.

Copper in ionic form rapidly becomes toxic to a variety of cells (Eidi et al., 2010), including human spermatozoa (Holland and White, 1998; Wong et al., 2001). Rebrelo et al. (1996) observed the effect of $Cu^{2+}$ on the motility, viability, acrosome reaction and fertilization capacity of human spermatozoa *in vitro*. Motility, viability and acrosome reaction in spermatozoa incubated for 5 h were significantly affected by $Cu^{2+}$ at a concentration of 100 μg/mL, but not at lower concentrations. Incubation for 24 h did not affect the motility and viability of spermatozoa incubated in the presence of $Cu^{2+}$ ranging from 10 ng/mL to 10 μg/mL, but the concentration of 100 μg/mL caused a significant decrease of both parameters. Dhami et al. (1994) stressed the impact of Cu on spermatozoa motility. Katayose et al. (2004) claimed that higher concentrations of Cu had significant adverse effects on the spermatozoa motility. Similar results were also observed in our previous study with copper sulphate ($CuSO_4$) on the bovine spermatozoa motility (Knazicka et

al., 2010a). A significant ($P<0.05$) decrease in spermatozoa concentration, motility and viability after experimentally induced $CuSO_4$ poisoning in male rats was seen the study of Sakhaee et al. (2011). The data obtained their study show that $CuSO_4$ at a dose of 200 mg/kg/day caused testicular atrophy and induced structural abnormalities in spermatozoa. The authors stated that the spermicidal effect of $CuSO_4$ may be responsible for these effects. Meeker et al. (2008) found evidence of an inverse association between high Cu levels and semen quality, which is consistent with a number of animal and human studies (Battersby et al., 1982; Skandhan, 1992; Huang et al., 2000; Massanyi et al., 2004; Yuyan et al., 2007).

## CONCLUSION

The data obtained from this *in vitro* study proved that adding energy and protein substrate to the culture medium increases the spermatozoa motility parameters also the presence of high doses (≥ 125 μmol/L) of copper ions during short-term periods (Time 0 h, 1 h). Therefore we may assume that 20% BSA stimulating the spermatozoa metabolism. Concurrently BSA maintained motility of spermatozoa (≤ 31.20 μmol/L of $CuCl_2$) during the long-term (Time 24 h) of cultivation, confirms the protective effect of albumin binding to the copper ions. Findings of the present study demonstrated the importance metal-protein interactions.

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# UTICAJ BAKRA NA HELACIONI EFEKTA GOVEĐIH SERUMSKIH ALBUMINA I PARAMETRE POKRETLJIVOSTI SEPERMATOZOIDA *IN VITRO*

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**Izvod**

Cilj ovog in vitro istraživanja je bio da se analizira uticaj bakra na pokretljivost spermatozoida u prisustvu bovinog serum albumina (BSA), kao medijuma za kultivaciju, kao i da se dobiju dodatne informacije u vezi sa interakcijom između serum albumina i bakar (II) hlorida ($CuCl_2$). Parametri pokretljivosti spermatozoida su utvrđeni posle izlaganja delovanju $CuCl_2$ (3.9; 7.8; 15.6; 31.2; 62.5; 125; 250; 500; 1000 μmol/L), upotrebom Sperm Vision™ CASA (Computer Assisted Semen Analyzer) system, tokom različitih vremenskih perioda (0 h, 1 h, 2 h and 24). Medijum za kultivaciju je sadržao 20% BSA, triladyl i 5% glukoze. Procent pokretnih spermatozoida je signifikantno ($P<0.001$) opao posle 2h kultivacije, kada je koncentracija $CuCl_2$ iznosila $\geq 250$ μmol/L, u poređenju sa ontrolnom grupom (bez dodatka $CuCl_2$). Dodatak $\leq 31.2$ μmol/L of $CuCl_2$ je stimulisao pokretljivost spermatozoida, posle 24h.
Identična pokretljivost spermatozoida je ustanovljena tokom svih ispitivanih perioda. Ovi rezultati pokazuju da dodavanje energije i proteina, kao i prisustvo visokih doza bakarnih jona*(≥ 125 μmol/L) tokom kratkog vremenskog perioda (oh i 1h)* u kultivacione medijume povećavaju pokretljivost spermatozoida. Dodavanje BSA održava pokretljivost spermatozoida tokom dužeg vremena kultivacije (24h), što potvrđuje zaštitni efekt albumina vezanih za jone bakra.

**Ključne reči:** bakar, goveđi serum albumin, spermatozoidi, pokretljivost.

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# THE EFFECT OF TREHALOSE, CAFFEINE AND GLUTATHIONE ON BOVINE SPERMATOZOA: 2. MORPHOLOGY AND OXIDATIVE STATUS *IN VITRO**

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*SUMMARY: The aim of this study was to examine the effects of glutathione, trehalose and caffeine on selected bovine spermatozoa morphology parameters, as well as lipid peroxidation following an in vitro cultivation at different temperatures and time periods. Morphological analysis showed similar occurrence of morphological abnormalities in all group with no significant differences (p>0.05). The TBARS assay revealed that the selected concentrations of trehalose, caffeine and glutathione did not have a significant effect (p>0.05) on lipid peroxidation as a process leading to oxidative stress development in spermatozoa.*

***Key words:*** *trehalose, caffeine, glutathione, spermatozoa, bulls, morphology, MDA.*

## INTRODUCTION

Artificial insemination (AI) has become one of the most important pillars in animal biotechnology. Especially in the cattle AI, bull semen quality is highly important to ensure a good biological material for breeding as well as a certain biodiversity protection (Ibrahim et al., 2000).

Progress in the use of AI has been related to search for semen extenders with a potential ability to stimulate the viability and to enhance the fertilizing ability of animal and human spermatozoa (Pivko et al., 2009; Spalekova et al., 2011). A special attention is dedicated to substances with antioxidant properties, as sperm cell membranes contain high concentrations of polyunsaturated fatty acids susceptible to lipid peroxidation. Inversely, the seminal plasma possesses a wide antioxidant system to prevent the

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oxidative cellular damage (Kefer et al., 2009). Nevertheless, antioxidants present in the seminal plasma, are usually attenuated by dilution of the semen during the preparation of insemination doses (Pivko et al., 2009).

It was confirmed that the addition of glutathione, as an antioxidant and trehalose, a nonpermeant cryoprotectant has elicited beneficial effects in many facets of AI and *in vitro* fertilization: increase of semen quality, spermatozoa motility and viability. Furthermore, it was documented that caffeine as a cyclic nucleotide phosphodiesterase inhibitor markedly increased and maintained the respiration and motility of ejaculated spermatozoa, which resulted in a higher fertilization rate of oocytes (Tatham et al, 2003; Spalekova et al, 2011).

As a follow-up of our previous study (Massanyi et al., 2011), we examined the effects of glutathione, trehalose and caffeine on selected bovine spermatozoa morphology parameters, as well as lipid peroxidation following an *in vitro* cultivation at different temperatures and time periods.

## MATERIAL AND METHODS

Bovine semen samples (n=48) were obtained from 6 randomly selected adult breeding bulls (Slovak Biological Services, Nitra, Slovakia) on a regular collection schedule using an artificial vagina. The semen was cooled down to 4˚C and transported to the laboratory, where the samples were divided into four main groups, according to the concentration of the used experimental supplement and the cultivation temperature together with the time intervals of analysis.

Basic spermatozoa diluent medium consisted of Triladyl® *(*250 mL; Minitüb, Tiefenbach Germany*),* distilled water (750 mL) and egg yolk (62.5 mL). The treatment was based on the addition of trehalose (Sigma-Aldrich, St. Louis, USA) caffeine (Sigma-Aldrich, St. Louis, USA) and glutathione (Sigma-Aldrich, St. Louis, USA) into the semen diluent medium at dosages of 0 (Control), 1 (Group 1) and 2 (Group 2) mg/mL. Fresh semen was added to each medium with a final dilution rate of 1:50. Group A was cultured at 37 °C and analyzed at time intervals of 0h, 1h, 2h, 3h and 4h after the experiment had started. Group B was cultured at 5°C and analyzed at 24h, 48h, 72h and 168h after the experiment had begun.

For the analysis of morphologically altered spermatozoa the semen samples were fixed in Hancock's solution and stained according to the Giemsa–Romanowski staining method. The frequency of abnormal spermatozoa was quantified microscopically at 500x magnification, and the following abnormal morphological changes were evaluated: knob-twisted flagellum, separated flagellum, flagellum torso, broken flagellum, retention of cytoplasmatic drop, acrosomal changes, large head, small head, flagellum ball, and other abnormal spermatozoa Morphologically changed spermatozoa were sorted to the classification table of morphological malformed forms of spermatozoa (Lukac et al., 2009).

Decomposition of unstable peroxides derived from lipid peroxidation results in the formation of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA). The measurement of these is a well-established method for screening and monitoring lipid peroxidation. Cayman's Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit (Cayman chemical company, Ann Arbor, Michigan, USA) was used for the MDA evaluation. The MDA-TBA

product formed by the reaction of MDA and TBA at high temperatures (90-100°C) and acidic conditions was measured colorimetrically at 530-540 nm.

Statistical analysis of the obtained data was carried out using the SAS statistical program (SAS Institute Inc., Cary, USA). Basic statistical parameters (mean, standard deviation, coefficient of variation) were calculated at first. Subsequently, a paired t–test and Scheffe's test were used to compare the results between the control and experimental groups. The level of significance was set at *** ($p < 0.001$); ** ($p < 0.01$); * ($p < 0.05$).

## RESULTS AND DISCUSSION

### Group 1 (1 mg/mL)

The morphological analysis showed that more than 85% of the examined spermatozoa were intact (Table 1). The highest percentage of normal spermatozoa was found after trehalose (88.88%) and glutathione treatment (88.75%). The percentage of abnormal (morphologically changed) spermatozoa was almost equal in the control (14.62%) and caffeine (14.87%) group. The dominant abnormalities included flagellum torso and broken flagellum, as well as a small head in case of caffeine and glutathione treatment.

Table1. Spermatozoa morphology (in %) in groups with 1 mg/mL of supplement addition (mean±SD)

| Morphology | C1 | T1 | K1 | G1 |
|---|---|---|---|---|
| TN | 13.73±5.62 | 11.62±3.74 | 17.75±3.96 | 13.62±4.00 |
| AC | 0.82±0.75 | 1.25±1.16 | 1.12±1.13 | 1.62±0.92 |
| SF | 2.73±1.10 | 2.00±1.07 | 3.50±1.31 | 2.62±1.41 |
| KTF | 2.64±2.46 | 1.50±1.31 | 2.00±1.37 | 2.37±1.30 |
| FT | 2.18±1.47 | 2.37±1.06 | 2.62±1.19 | 2.12±1.81 |
| FB | 0.64±0.92 | 0.75±0.89 | 1.75±1.04 | 1.50±1.31 |
| BF | 1.45±1.29 | 0.88±0.83 | 1.62±0.52 | 0.88±1.36 |
| RCD | 0.55±0.93 | 0.38±0.52 | 0.38±0.52 | 0.50±0.53 |
| SH | 2.45±1.21 | 1.62±0.92 | 3.25±1.04 | 1.50±1.07 |
| LH | 0.27±0.65 | 0.50±0.76 | 1.12±1.36 | 0.50±0.76 |
| OPS | 0.00±0.00 | 0.38±0.52 | 0.38±0.74 | 0.00±0.00 |

TN -Total number of pathological spermatozoa, SF - Separated flagellum, KTF - Knob-twisted flagellum,

FB - Flagellum ball, BF - Broken flagellum, RCD - Retention of cytoplasmic drop, FT - Flagellum torso,

AC - Acrosomal changes, SH - Small head, LH - Large head, OPS - Other pathological spermatozoa

C – control group; T – trehalose; K – caffeine; G – glutathione

### Group 2 (2 mg/mL)

The examination demonstrated that over 80% of the observed spermatozoa showed normal morphology (Table 2). A higher number of normal spermatozoa was found in the group with trehalose addition (88.38%), the lowest number was detected after caffeine supplementation (82.25%). The dominant morphological abnormalities in all of the groups included a separated flagellum, knob-twisted flagellum, as well as flagellum torso in case of trehalose and a small head in the case of caffeine. Overall, no significant

differences were observed comparing the experimental groups with the control group.

Table 2. Spermatozoa morphology (in %) in groups with 2 mg/mL of supplement addition (mean±SD)

| Morphology | **C2** | **T2** | **K2** | **G2** |
|---|---|---|---|---|
| TN | 13.73±5.62 | 11.62±3.74 | 17.75±3.96 | 13.62±4.00 |
| AC | 0.82±0.75 | 1.25±1.16 | 1.12±1.13 | 1.62±0.92 |
| SF | 2.73±1.10 | 2.00±1.07 | 3.50±1.31 | 2.62±1.41 |
| KTF | 2.64±2.46 | 1.50±1.31 | 2.00±1.07 | 2.37±1.30 |
| FT | 2.18±1.47 | 2.37±1.06 | 2.62±1.19 | 2.12±1.81 |
| FB | 0.64±0.92 | 0.75±0.89 | 1.75±1.04 | 1.50±1.31 |
| BF | 1.45±1.29 | 0.88±0.83 | 1.62±0.52 | 0.88±1.36 |
| RCD | 0.55±0.93 | 0.38±0.52 | 0.38±0.52 | 0.50±0.53 |
| SH | 2.45±1.21 | 1.62±0.92 | 3.25±1.04 | 1.50±1.07 |
| LH | 0.27±0.65 | 0.50±0.76 | 1.12±1.36 | 0.50±0.76 |
| OPS | 0.00±0.00 | 0.38±0.52 | 0.38±0.74 | 0.00±0.00 |

TN -Total number of pathological spermatozoa, SF - Separated flagellum, KTF - Knob-twisted flagellum,

FB - Flagellum ball, BF - Broken flagellum, RCD - Retention of cytoplasmic drop,

FT - Flagellum torso, AC - Acrosomal changes, SH - Small head, LH - Large head,

OPS - Other pathological spermatozoa, C – control group; T – trehalose; K – caffeine; G – glutathione

Generally, no significant differences were observed ($p>0.05$), even though a tendency of a higher occurrence of morphological abnormalities with a higher dosage of the supplements (especially caffeine) was detected.

Discussing our results with other authors, our data showed that the highest percentage of morphologically intact spermatozoa in both groups was detected after trehalose supplementation. These results agree with the study of Yildiz et al. (2000). According to the authors, the proportion of spermatozoa with morphological abnormalities, especially damaged acrosomes was significantly reduced by sugars added to the extender, especially trehalose, galactose, lactose or sucrose. Furthermore, Woelders et al. (1997) showed that sugars have a protective influence against the morphological damage occurring in spermatozoa exposed to *ex vivo* conditions and that sucrose could be even more protective than trehalose for bull spermatozoa. Chen et al. (1993) stated that trehalose improved post-thaw survival of bovine spermatozoa stored at 25°C for 24h. Moreover Storey et al. (1998) reported that trehalose increased the proportion of intact mouse spermatozoa after cryopreservation.

Concerning caffeine, there are controversial data about its effects. Although caffeine may stimulate spermatozoa motility and activity, according to Pivko et al. (2009), it increased the occurrence of spermatozoa with swollen or damaged acrosome or spermatozoa with pseudoacrosomal reaction formed by exocytotic vesicles and accompanied with a loss of acrosomal content, leading to impaired membrane integrity and damages of spermatozoa head membranes. Also Harrison et al. (2003) reported that exposure of spermatozoa to high levels of caffeine may produce surface morphological damage which was more pronounced the longer spermatozoa spent incubated with caffeine. Disruption of the spermatozoa head and swelling of the mid-piece were the most

characteristic features. On the other hand, Barkay et al. (1984) state that no *in vitro* caffeine treatment of fertile donor semen does not damage the spermatozoa, as observed by the electron microscopy.

An efficiency of the addition of antioxidants such as glutathione into ram semen was demonstrated by Sarlos et al. (2002), as following the addition of these substances the frequency of spermatozoa abnormalities was decreased. Pivko et al. (2009) agree and state that glutathione, as an implementor added to insemination doses, did not influence the frequency of spermatozoa with intact head and acrosome but decreased the occurrence of swollen and damaged spermatozoa, ultimately leading to an improvement of spermatozoa membrane stability and maintenance of their functional state. Furthermore Lenzi et al. (1994) reported on a placebo-controlled crossover trial of 600 mg of glutathione, administered intramuscularly on alternate days, for a period of two months, to a group of 20 patients: ten with varicocele, and ten with 'germ-free genital tract inflammation'. The treatment resulted in a significant reduction in the proportion of forms with abnormal morphology.

**MDA Assay**

The MDA concentration as a marker of the lipid oxidative stress was the highest of the G2A group (68.40 μM) and the T1A group (60.36 μM). The lowest MDA concentration was recorded in the T2B group (45.85 μM) and the K1A group (47.40 μM). Significant results were not detected ($p>0.05$), neither in comparison with the control, nor within a concrete condition (Table 3). Based on this results we can assume that that the supplements did not have any effect on spermatozoa lipid peroxidation. On one hand, no enhanced peroxidation was observed, on the other hand no specific antioxidant properties of the substances were revealed.

Table 3. Malondialdehyde concentration (in μM) in the control/experimental groups (mean±SD)

| Group | Control | Trehalose | Caffeine | Glutathione |
|---|---|---|---|---|
| 1 mg/mL, 37°C | C1A | T1A | K1A | G1A |
| | 46.82±8.77 | 60.36±1.33 | 47.40±17.19 | 48.35±16.70 |
| 1 mg/mL, 5°C | C1B | T1B | K1B | G1B |
| | 53.94±13.41 | 58.76±11.97 | 49.55±18.10 | 57.04±11.62 |
| 2 mg/mL, 37°C | C2A | T2A | K2A | G2A |
| | 53.83±8.68 | 54.43±0.77 | 52.50±15.01 | 68.40±34.60 |
| 2 mg/mL, 5°C | C2B | T2B | K2B | G2B |
| | 50.26±13.31 | 45.85±3.57 | 54.10±7.23 | 50.12±8.65 |

Our resuts did not confirm the data from Aboagla and Terada (2003) who suggested that trehalose played a major role in increasing membrane fluidity, which led to greater endurance of spermatozoa against oxidative stress. Trehalose had a protective action related to the osmotic effect and to specific interactions with membrane phospholipids, thereby minimizing the degree of spermatozoa oxidative injury. Alternatively, the authors concluded that if trehalose directly protected lipids against peroxidation, there might be a diminished ROS propagation.

Arabi and Shahrokhie (2007) observed the effects of caffeine on lipid peroxidation of bovine spermatozoa without or with the presence of taurine or albumin. All the caffeine treatments showed a significant increase in the MDA level/LPO rate. Also, Arabi et al. (2003) investigated the effects of different concentrations of caffeine (5, 7

and 9 mM) on lipoperoxidation as a marker of spermatozoa integrity of normospermic men. They found out that caffeine is able to produce a mild state of oxidative stress and could be regarded as a potential infertility inducer. On the contrary our results show that tat the caffeine treatment led to a lower MDA concentration, which shows a certain protection of the spermatozoa lipids against oxidative stress development.

Brezezinska-Slebodzinska et al. (1995) studied the protective effects of reduced glutathione against lipid peroxidation in boar semen. The lipid peroxidation was measured by the TBARS assay doubled in the presence of the lipid peroxidation $Fe^{2+}$-sodium ascorbate-inducing system. The ascorbate-induced TBARS was inhibited by about 57% by glutathione. The protective role of glutathione with respect to boar semen against fatty acid peroxidation have been evidenced, even though we detected a higher oxidative state at 2 mg of glutathione supplementation.

## CONCLUSION

Our experiments analysis showed that morphological abnormalities of spermatozoa were elevated especially in case of higher trehalose, caffeine and glutathione concentrations. The TBARS assay revealed that the selected concentrations of the substances did not have a significant effect on lipid peroxidation as a process leading to oxidative stress development in spermatozoa. Based on these results we may conclude that all of the substances are not harmful for the spermatozoa viability and may be suitable for either a short-term (caffeine and glutathione) or a long-term (trehalose) spermatozoa cultivation. Still, only a few of our results were statistically significant, therefore further experiments have to be done with a higher variability in the supplements concentrations and cultivation conditions to see a more significant positive or negative effect on the spermatozoa morpoholgy characteristics and oxidative status.

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# UTICAJ TREHOLAZE, KAFEINA I GLUTATIONA NA SPERMATOZOIDE GOVEDA: 2. MORFOLOGIJA I OKSIDATIVNI STATUS *IN VITRO*

PETER MASSÁNYI, EVA TVRDÁ, MARTINA RAFAJOVÁ, NORBERT LUKÁČ

**Izvod**

Cilj ovog rada je bio da se ispita uticaj glutationa, treholaze i kafeina na morfološke parametre spermatozoida bikova, kao i lipidne peroksidacije, posle in vitro kultivacije tokom različitih perioda i na različitim temperaturama. Nije bilo značajnih razlika ($p>0.05$) u morfologiji spermatozoida, između eksperimentalnih grupa. TBARS isoitivanje nije pokazalo da postoji značajan uticaj ($p>0.05$) ispitivanih koncentracija trehaloze, kafeina i glutationa na lipidnu peroksidaciju, kao procesa koji dovodi do oksidativnog stresa kod spermatozoida.

**Ključne reči:** trehalosa, kafein, glutation, spermatozoidi, bikovi, morpolog, MDA.

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# SERUM ELECTROLYTES CHANGES IN HEALTHY DOGS SUBMITTED TO DOBUTAMINE STRESS TEST

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*SUMMARY: The aim of this study was to investigate hematologic and serum chemistry changes in healthy dogs subjected to conventional protocol of dobutamine stress test. The study was performed in ten healthy German Shepherd dogs. The dobutamine stress test was carried out as three minute stages protocol, from the starting dose of 7.5 μg/kg/min until the maximum dosage of 42.5 μg/kg/min. Blood samples were collected before and after the dobutamine stress test for complete blood count and chemistry analyses. Statistically significant changes of potassium concentration ($p<0.05$), sodium concentration ($p<0.05$) and chloride concentration ($p<0.001$) were registered after the dobutamine stress test. The registered changes could be explained by the dobutamine effects through β1 adrenergic receptors.*

***Key words:*** *dobutamine stress test, electrolytes, dog*

## INTRODUCTION

Dobutamine is widely utilized as a drug in human pharmacological stress testing, but dobutamine stress test (DST) is still in the investigational phase in canine medicine. Dobutamine is a potent β1 adrenergic agonist with minimal β2 and α adrenergic effects. Studies in normal conscious dogs (Vatner et al., 1974; Hinds and Hawthorne, 1975; Liang and Hood, 1979) have shown that dobutamine augments cardiac output and myocardial contractility, but has little effect on the heart rate (HR) and aortic blood pressure in the presence of an intact baroreceptor reflex (Liang et al., 1981). The primary effect of dobutamine infusion, as a diagnostic tool, constitutes an increase in heart rate and systolic blood pressure which results in the increase in myocardial oxygen demand, similar to that occurring during moderate physical exercise (Wackers, 1993). A high HR seems to be essential for the ability of the stress test to detect cardiac dysfunction. In order to reach high target HR, many DST protocols use high doses of the drug,

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potentially increasing the risk of adverse effects and complications (Lima et a., 2008). However, because of its brief duration of action dobutamine can be used with relative safety (Jewitt et al., 1974; Wackers, 1993).

By choosing the protocol appropriate for a clinical use, we investigated complete blood count (CBC) and serum chemistry changes in healthy German Shepherd dogs subjected to DST. These parameters represent basic circulatory and metabolic parameters and some of them, like electrolytes, are important for cardiac electrical activity. Former researches of DST in dogs have been conducted in certain breeds (Beagle, Doberman) and no information exist about DST in German Shepherd dogs.

## MATERIALS AND METHODS

### Animals

Ten German Shepherd dogs (8 males and 2 females) were studied; they were one to nine years old. All dogs were without clinical symptoms of cardiovascular or respiratory diseases. The dogs were evaluated by clinical examination including the history of each dog, physical examination, complete blood count and serum chemistry, electrocardiography, 2D and M-mode echocardiography. All examinations were performed with a manual restraint of the animals, without uses of sedation or anesthesia. The experimental protocol was approved by the the Ethical Committee of the Faculty of Veterinary Medicine University of Belgrade (approval number 01-3 / 2008).

### Dobutamine Stress Test

The dobutamine stress test was performed as conventional DST by dobutamine administered via a cephalic vein (Dobutamine, Panpharma S.A., France, 250 mg/ 20ml). The dobutamine initial dose of 7.5 μg/kg/min was increased at 3-minute intervals, by 5 μg/kg/min, until a maximum dosage of 42.5 μg/kg/min was achieved, or when one of the following signs was reached: tachycardia over 200/min, severe arrhythmia or uncontrollable excitement of the animal. Before dobutamine administration baseline blood samples were taken for a complete blood count (CBC): red blood cell (RBC), hemoglobin (Hgb), hematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular Hgb (MCH), mean corpuscular Hgb concentration (MCHC), and serum chemistry analyses: albumin, protein, blood urea nitrogen (BUN), creatinine, creatin kinase (CK), lactate dehydrogenase (LDH), potassium, sodium, chloride, bicarbonate. Right after termination of dobutamine application (<1 minute), blood samples were taken again and same parameters as before DST were evaluated. Blood samples were assayed on automated analyzers Cell Dyn 3700 (Abbott Diagnostics) and Olympus AU400 (Olympus). During DST cardiac echo and continuous lead II electrocardiogram were monitored in order to recognize the peak of DST and terminate dobutamine infusion.

### Statistical Analyses

The results were analyzed by statistical package Statistica 8. The data are reported as mean ± SD. All values before and after DST were compared by Student's t-test for dependant samples. The differences were taken to be significant at $p < 0.05$ and $p < 0.001$.

## RESULTS

This DST study was carried out in 10 German Shepherd dogs of different age, showing no cardiovascular and respiratory clinical signs. In a clinical examination of the cardiovascular system, physiological heart sound was observed with no heart murmurs and with regular or regularly irregular rhythm. The arterial and venous pulse were normal. On the basis of the clinical examination (CBC, serum chemistry analysis, electrocardiography, echocardiography) all the dogs were considered free of cardiac diseases and other systemic diseases that could influence the cardiovascular system structure and function.

All dogs tolerated the DST well. The following adverse effects were registered during DST: panting, mild excitement, nausea, weakness of short duration. None of these side effects interrupted the experimental protocol.

The CBC changes after DST consisted of red blood cell increase, hematocrit increase, as well as hemoglobin concentration increase. However, differences in the CBC values before and after DST were not significant. CBC values both before and after DST were within the reference values range.

Table 1. Hematological parameters of dogs before and after DST
*Tabela 1. Vrednosti hematoloških parametara kod pasa pre i posle DST*

| DST | Red blood cell/ *Eritrociti* ($x10^{12}$/l) | Hemoglobin/ *Hemoglobin* (g/l) | Hematocrit / *Hematokrit* (l/l) | MCV/ *MCV* (fl) | MCH/ *MCH* (pg) | MCHC/ *MCHC* (g/l) |
|---|---|---|---|---|---|---|
| Before/ *Pre* ($\bar{x}$ ±SD) | 6.31 ± 0.89 | 147.20 ± 19.61 | 0.43 ± 0.05 | 68.53 ± 2.10 | 23.41 ± 0.61 | 341.50 ± 6.26 |
| After/ *Posle* ($\bar{x}$ ±SD) | 6.60 ± 0.87 | 150.89 ± 23.57 | 0.45 ± 0.05 | 68.09 ± 2.29 | 22.81 ± 1.23 | 335.22 ± 22.16 |

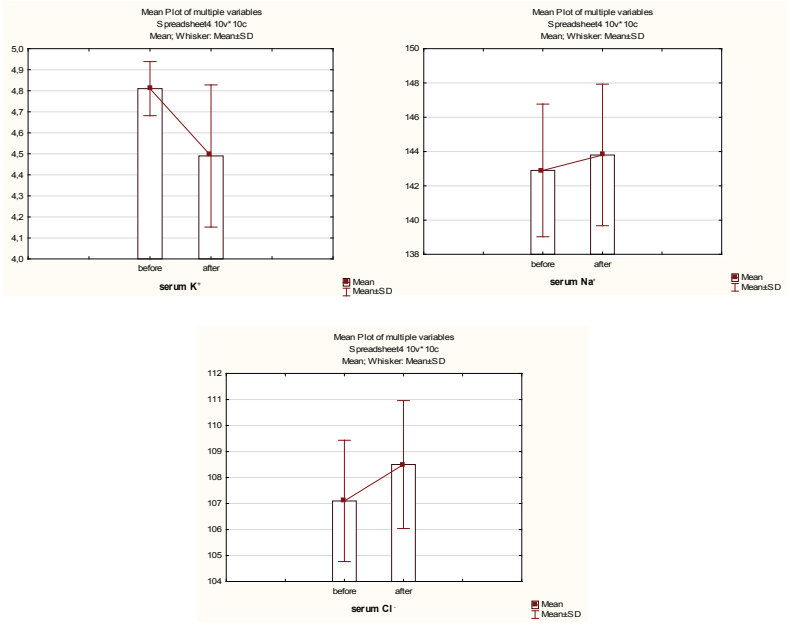
The concentration of serum protein, the serum enzyme values (CK and LDH), as well as creatinine and BUN concentrations were in the extent of normal values for dogs. The serum chemistry analyses of the dogs before and after DST demonstrated significant differences in potassium, sodium and chloride concentrations. Serum potassium concentration significantly decreased in DST (4.81 ± 0.14 mmol/l before DST versus 4.59 ± 0.29 mmol/l after DST; $p = 0.046$). A significant increase in serum sodium concentration was observed after DST in dogs (142.90 ± 3.87 mmol/l before DST versus 143.80 ± 4.13 mmol/l after DST; $p = 0.0187$). As far as serum chloride concentration is concerned, it was significantly increased after DST (107.10 ± 2.33 mmol/l before DST versus 108.50 ± 2.46 mmol/l after DST; $p < 0.001$).

Table 2. Values of serum biochemical constituents in dogs before and after DST

*Tabela 2. Vrednosti biohemijskih analiza seruma kod pasa pre i posle DST*

| Parameter / *Parametar* | Before DST/ *Pre DST* | After DST/ *Posle DST* | Parameter / *Parametar* | Before DST/ *Pre DST* | After DST/ *Posle DST* |
|---|---|---|---|---|---|
| Albumin/ *Albumini* (g/l) | 24.50±2.72 | 24.50±3.10 | Potassium / *Kalijum* (mmol/l) | 4.81±0.13 | 4.59±0.29* |
| Protein/ *Proteini* (g/l) | 60.70 ±3.30 | 59.20±4.94 | Sodium / *Natrijum* (mmol/l) | 142.90±3.87 | 143.80±4.13* |
| BUN / *Urea* (mmol/l) | 8.78±2.41 | 8.82±2.53 | Calcium / *Kalcijum* (mmol/l) | 2.33±0.10 | 2.31±0.13 |
| Creatinine/ *Kreatinin* (µmol/l) | 87.40±12.14 | 85.80±14.08 | Chloride / *Hloridi* (mmol/l) | 107.10±2.33 | 108.50±2.46** |
| CK/ *CK* (IU/l) | 59.90±9.93 | 60.80±17.72 | Bicarbonate/ *Bikarbonati* (mmol/l) | 20.20±4.59 | 20.10±4.01 |
| LDH/ *LDH* (IU/l) | 57.20±17.97 | 55.40±23.98 | * (p<0.05) | ** (p<0.001) | |

A B C




Graph. 1. Serum electrolytes concentrations before and after DST in the dogs (A – serum potassium concentrations; B – serum sodium concentrations; C – serum chloride concentrations)

*Graf. 1. Koncentracije elektrolita seruma kod pasa pre i posle DST (A – koncentracije kalijuma; B – koncentracije natrijuma; C – koncentracije hlorida)*

## DISCUSSION

It is very important to consider shortness of DST protocol in veterinary medicine because of uncooperativity of dogs. Until now, different protocols of DST were reported in canine cardiology. There were protocols of low dose dobutamine infusion of 5 μg/kg/min (Minors and O'Grady, 1998; Koplitz et al., 2004) which lasted 15 minutes, as well as protocols of incremental dobutamine infusion (McEntee et al., 1996, 1998, 2000, 2001; Sousa et al., 2005) which lasted 40 to 75 minutes.

In this study, criteria for choosing DST protocol were appliance in clinical practice. Thus, the minimal dose (7,5 μg/kg/min) was in the therapeutic range for dobutamine dosage; and afterwards it was gradually increased every 3-minute by 5 μg/kg/min. Maximum duration of DST was 24 minutes. Toleration of this DST protocol by the dogs was good.

The CBC changes (RBC, Hgb, PCV, MCV, MCH, MCHC) associated with DST were similar with ones in treadmill running physical exercise (Strasser et al., 1997). However, these CBC changes were not significant.

DST induces significant serum electrolyte changes in dogs. Sodium concentration after DST was significantly higher ($p=0.0187$) than before DST. Increased sodium concentration is in agreement with sodium concentration changes ($134.59 \pm 9.46$ mmol/l vs. $137.12 \pm 10.17$ mmol/l) in exercise stress testing (Strasser et al., 1997). Chloride concentration was significantly increased ($p < 0.001$) after DST. Opposite, exercise stress testing with treadmill running in study of Strasser et al. (1997) induced decreasing of chloride concentration ($122.28 \pm 6.19$ mmol/l vs. $122.00 \pm 6.35$ mmol/l). Potassium concentration after DST in dogs was significantly lower ($p=0.046$) than before DST. Increased potassium concentration ($4.04 \pm 0.27$ mmol/l vs. $4.21 \pm 0.30$ mmol/l) was registered in dogs after treadmill stress testing in study of Strasser et al.(1997). Decreased potassium concentration after DST in dogs is in agreement with DST study in horses (Frye et al., 2003). Dobutamine infusion induces decrease plasma potassium concentration, with a further decrease 10 minutes later. The maximum decrease in potassium occurs in the patients who received the highest dose of dobutamine (Coma-Canella, 1991). This phenomenon has been partially attributed to potassium influx induced by β adrenergic stimulation of the $Na^+ – K^+ - 2Cl^-$ cotransporter. Because of the activity of sodium-potassium–adenosine triphosphatase ion pumps, the opposite occurs with sodium (delCastillo et al., 1999). Chloride levels will follow sodium levels except in the case of acid-base imbalances. On the other hand, activation of adrenergic receptor stimulates adenyl cyclase with resultant increases in cAMP and protein kinase activity. Furthermore, outward potassium current (Ik) is diminished which leads to prolonged repolarization and action potential prolongation (Thomas et al, 2004). As a result, prolong cardiac repolarization may cause early afterdepolarization, thereby initiating development of cardiac arrhythmias.

In this study no arrhythmias were recorded during DST. Although serum electrolytes concentrations have been significantly changed, these changes were not clinically significant. Since this study was performed in healthy dogs, further studies are needed to investigate the possibilities of induction of arrhythmias based on changed electrolytes concentrations, especially potassium, in dogs with cardiac diseases.

## CONCLUSION

The conventional DST protocol in dogs leads to serum electrolytes changes. The changes of major electrolytes could be explained by dobutamine effects as β1- adrenergic agonist. Adverse effects associated with hypokalemia in DST need additional study so that safety of DST in dogs could be documented.

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## PROMENA KONCENTRACIJE ELEKTROLITA SERUMA KOD PASA PODVRGNUTIH DOBUTAMIN STRES TESTU

LJUBICA SPASOJEVIĆ KOSIĆ

### Izvod

Cilj ovog rada je da ispita hematološke i biohemijske promene seruma zdravih pasa podvgnutih konvencionalnom protokolu dobutamin stres testa. Ispitivanje je izvedeno na 10 zdravih nemačkih ovčara. Dobutamin stres test je izveden tako što je na svaka 3 minuta doza dobutamina povećavana počevši od 7,5 μg/kg/min do 42,5 μg/kg/min. Uzorci krvi su prikupljani pre i posle dobutamin stres testa, kako bi se uradila analiza kompletne krvne slike i biohemijskih parametara seruma. Statistički značajne promene koncentracije kalijuma ($p<0,05$), natrijuma ($p<0,05$) i hlorida ($p<0,001$) su registrovane nakon dobutamin stres testa. Registrovane promene mogu da se objasne delovanjem dobutamina preko β1 adrenergičnih receptora.

**Ključne reči:** dobutamin stres test, elektroliti, pas.

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# THE FEED CONVERSION, DAILY GAIN, AVERAGE BACKFAT THICKNESS AND MEAT PERCENTAGE IN PERFORMANCE TEST OF LANDRACE BOARS

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*SUMMARY: This paper analyzes the characteristics of the performance test and Landrace boars to: Dutch, Swedish, German and Danish, originating from large farms in Serbia in order to analyze the variability of different characteristics of the aggregate genotype: feed conversion, daily gain and thickness of the back side of bacon and lean meat. Systemic genetic factors (race and fathers) and environmental factors (farm) have ahighly significant effect (P<0.01) on the properties of the aggregate genotype (feed conversion and meat percentage) except for the traits daily gain and backfat thickness of the side, where impact of race was not significant (P>0.05). The tested properties of the aggregate genotype showed a high degree of heritability (heritability) with a high standard error. Heritability for feed conversion was 0.84 ± 0.21 for daily gain 0.73 ± 0.18,backfat thickness of 0.74 ± 0.19, the thickness of the side of bacon 0.76 ± 0.19 and percent of meat 0,66 ± 0.18. The positive genetic correlations between traits in the aggregate genotype (from 0.266 between feed conversion and backfat thickness side to 0.984 between backfat thickness and the thickness of the side), and negative genetic correlations between the percentage of meat and feed conversion, daily gain and thethickness of the back side of bacon thickness (-0.155, -0.344, -0.904 and -0.858), were found.*

***Key words:*** *Landrace, boars, performance test, genetic parameters, daily gain, percentage of meat.*

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## INTRODUCTION

Effects can be used in models for genetic evaluation of tested animals are numerous, given that different factors influence the varying properties of growth, feed efficiency and carcass quality of pigs (Mijatovic et al., 2006). The improvement of beef traits, which include weight gain, feed conversion and meat percentage, represents a significant fattening factor for the increased pig production (Imboonta et. al., 2007). These traits have medium and high heritability and can be measured directly or indirectly on the animals. Because of the high degree of heritability, genetic improvement can be achieved using the results of performance test. ,

Candidates for the next generation of parents are selected on the basis of the data's many features (Vidovic and Košarčić, 1998). In some European countries (the Netherlands), selection in the nucleus herds for fattening and carcass quality traits using information obtained in performance and sib testing.

Central test stations offer many advantages compared to on-farm testing. One of them is that you need to enable greater standardization (control) test environment and improving the testing procedure by minimizing the potential effects of uncontrolled factors (Mijatovic et al., 2005). However, testing in a central test stations is limited by capacity, in order to provide a larger number of animals tested in many countries to apply the test farm (Sckolling at al., 1981; Hudson and Kennedy, 1985; Merx, 1987; Vidovic et al.,1993; Trivunović, 1996), while in commercial gilts and sows (f1 generation), applies only phenotypic selection (Vidovic et al., 2011). In Serbia since the beginning of the introduction of testing to date, several methodologies were applied to the test (Markovic et al., 1963, Markovic et al., 1976, Drobnjakovic et al., 1988, Vidovic et al., 1993).

## MATERIALS AND METHODS

Investigations were made on four breeds Landrace boars from Vojvodina in the period since 2007. through 2011, during the performance test. Included are farms (total18) which are registered at the Department of Animal Husbandry, Faculty of Agriculture in Novi Sad, as the Main Breeding Organization for AP Vojvodina (Serbia). The study included a total of 699 tested Landrace boars of four breeds: Dutch (51); Sweden (337), Germany (306) and Danish Landrace (5). During the performance test, the aggregate genotype boars consisted of the following features: feed conversion (FC, kg) daily gain (DP, kg), average back fat thickness, (LS, mm), average thickness of the side of bacon, (BS, mm) and the percentage of meat, (PM, %).

### Ocena genetskih faktora i sistemskih faktora spoljne sredine

Za ispitivanje uticaja farme, rase i očeva na osobine iz agregatnog genotipa, korišćen je metod najmanjih kvadrata (Statistika 10), a statistički metod je sledeći:

### Rating genetic factor and systematic environmental factors

Testing effects of farm, breed and fathers on the properties of the aggregate genotype, performed by the method of least squares (statistics 10), a statistical method is as follows:

$$Y_{ijklm} = \mu + F^{i} + R^{j} + O^{k} + S\, b\, (X - \overline{X}) + e^{ijklm}$$

Where is: $Y^{ijklm}$ - expression of traits;

μ – overall mean value;

$F^i$- fixed-effect i - farm;

$R^j$ - fixed-effect j - race;

$O^k$ - fixed-effect k - father;

b - coefficient of linear regression of the impact of final the mass;

$e^{ijklm}$ - random error.

The model assumes that random error is independent and normally distributed effects for all N(0, σ ²).

**Genetic parameters**

Ratings of genetic parameters of traits in the aggregate genotype were calculated using the method of least squares of the components of variance and covariance of the half-sib by fathers (Harvey, 1990)., A statistical model was as follows:

$$Y_{ijklm} = \mu + F_i + R_j + O_k + S\, b\, (X - \overline{X}) + e_{ijklm}$$

The formulas used to calculate genetic parameters are as follows (Harvey, 1990):

Heritability $h^2 = \frac{4\sigma_s^2}{\sigma_s^2 + \sigma_e^2}$

Where is:

$\sigma_S^2$ - variance between fathers (family);

$\sigma_e^2$ - variance between fathers (family) or a random error

$r_g = \frac{Cov_{S(xy)}}{\sigma^2_{S(x)} \times \sigma^2_{S(y)}}$

Genetic correlation

Where is: $Cov_{S(xy)}$ - family covariance for the traits x i y;

$\sigma^2_{S(x)}$ - variance between fathers (family) for property x;

$\sigma^2_{S(y)}$ - variance between fathers (family) for property y.

$r_p = \frac{Cov_{P(xy)}}{\sigma^2_{P(x)} \times \sigma^2_{P(y)}}$

**Phenotypic correlation**

Where is: $Cov_{P(xy)}$ - phenotypic covariance for the traits x i y;

$\sigma^2_{P(x)}$ - phenotypic variance of traits x;

$\sigma^2_{P(y)}$ - phenotypic variance of traits y.

$$r_e = \frac{Cov_p - Cov_{s(xy)}}{\sqrt{\left[\sigma^2_{p(x)} - \sigma^2_{s(x)}\right] \times \left[\sigma^2_{p(y)} - \sigma^2_{s(y)}\right]}}$$

Environmental correlations

Strength of correlation was determined based on Romer-scale Ophterove (Latinović,1990).

## RESULTS AND DISCUSSION

### The rating system of genetic and environmental factors

The influence of race and fathers as a system of genetic factors and the farm as asystem of environmental factors on the traits of the aggregate genotype of boars is shown in Tables 1 - 4

Table 1. Influence of farm, breed and fathers on feed conversion in the test
*Tabela 1. Uticaj farme, rase i očeva na konverziju hrane u testu*

| Source of variation / *Izvor varijabilnosti* | SS - race *SS – rase* | df-race *df-rase* | MS - race *MS – rase* | F |
|---|---|---|---|---|
| Farma / *Farm* | 10,290 | 17 | 0,605 | 5,886** |
| Rasa / *Race* | 1,111 | 3 | 0,370 | 3,252* |
| Očevi / *Fathers* | 34,860 | 153 | 0,228 | 2,73** |

* Marked effects are significant at p<0.050, *Vrednosti su statistički značajne p<0,050,

** Marked effects are significant at p<0.010, **Vrednosti su statistički značajne p<0,010

**SS-sums of squares df-degrees of freedom, MS-square environment, **SS-sume kvadrata; df-stepeni slobode; MS-sredine kvadrata

By comparing the experimental F values shown in Table 1, the tabular values, it is evident that the farm and fathers significantly (P <0.01) effect on feed conversion in boars tested, while race is influenced significantly (P <0.05).

Table 2. Influence of farm, breed and fathers on daily gain in the test
*Tabela 2. Uticaj farme, rase i očeva na dnevni prirast u testu*

| Source of variation / *Izvor varijabilnosti* | SS - race *SS – rase* | df-race *df-rase* | MS - race *MS – rase* | F |
|---|---|---|---|---|
| Farma / *Farm* | 1574020 | 17 | 92589 | 5,519** |
| Rasa / *Race* | 93084 | 3 | 31028 | 1,671 *N* |
| Očevi / *Fathers* | 5010221 | 153 | 32747 | 2,23** |

** Marked effects are significant at p<0.010, **Vrednosti su statistički značajne p<0,010

**SS-sums of squares df-degrees of freedom, MS-square environment, **SS-sume kvadrata; df-stepeni slobode; MS-sredine kvadrata

In table 2 we can see that the farm and fathers significantly (P <0.01) affected daily gain in performance testing of boars, and the influence of race on the daily weight gain was not significant (P> 0.05).

Table 3. Influence of farm, breed and fathers on backfat thickness of boars in the test
*Tabela 3. Uticaj farme, rase i očeva na debljinu leđne slanine nerastova u testu*

| Source of variation / *Izvor varijabilnosti* | SS - race *SS – rase* | df-race *df-rase* | MS - race *MS – rase* | F |
|---|---|---|---|---|
| Farma / *Farm* | 2404,85 | 17 | 141,46 | 6,864** |
| Rasa / *Race* | 173,85 | 3 | 57,95 | 2,476 *N* |
| Očevi / *Fathers* | 6519,00 | 153 | 42,61 | 2,341** |

** Marked effects are significant at p<0.010, **Vrednosti su statistički značajne p<0,010

**SS-sums of squares df-degrees of freedom, MS-square environment, **SS-sume kvadrata; df-stepeni slobode; MS-sredine kvadrata

As in daily gain, systemic factors, farm and fathers were highly significant (P <0.01) affected the thickness of back fat, however, the impact of race was not significant (P>0.05) effect on this traist (table 3).

Table 4. Influence of farm, breed and fathers on thickness of the side of bacon of boars in the test
*Tabela 4. Uticaj farme, rase i očeva na debljinu bočne slanine nerastova u testu*

| Source of variation / *Izvor varijabilnosti* | SS - race *SS – rase* | df-race *df-rase* | MS - race *MS – rase* | F |
|---|---|---|---|---|
| Farma / *Farm* | 2479,28 | 17 | 145,84 | 7,292** |
| Rasa / *Race* | 215,89 | 3 | 71,96 | 3,1491* |
| Očevi / *Fathers* | 6259,75 | 153 | 40,91 | 2,266** |

* Marked effects are significant at p<0.050, *Vrednosti su statistički značajne p<0,050,

** Marked effects are significant at p<0.010, **Vrednosti su statistički značajne p<0,010

**SS-sums of squares df-degrees of freedom, MS-square environment, **SS-sume kvadrata; df-stepeni slobode; MS-sredine kvadrata

Sistemski faktori farme i očeva su visoko signifikantno (P< 0,01), a rase signifikantno (P< 0,05) uticali na osobinu debljine bočne slanine kod performans testiranih nerastova.

Systemic factors (farm and fathers) were significantly (P <0.01), and race significantly (P<0.05) affected the thickness of bacon side of the performance tested boars (table 4).

Table 5. Influence of farm, breed and fathers on the percentage meat of boar in the test
*Tabela 5. Uticaj farme, rase i očeva na procenat mesa kod nerastova u testu*

| Source of variation / *Izvor varijabilnosti* | SS - race *SS – rase* | df-race *df-rase* | MS - race *MS – rase* | F |
|---|---|---|---|---|
| Farma | 2254,5 | 17 | 132,6 | 7,13** |
| Rasa | 285,9 | 3 | 95,3 | 4,53** |
| Očevi | 5761 | 153 | 38 | 2,24** |

** Marked effects are significant at p<0.010, **Vrednosti su statistički značajne p<0,010

**SS-sums of squares df-degrees of freedom, MS-square environment **SS-sume kvadrata; df-stepeni slobode; MS-sredine kvadrata

All of the systemic factors (farm, breed and fathers) were significantly (P <0.01) affected the percentage of meat performance tested boars (table 5).

As can be seen from the results, the results of testing boars affect systemic genetic influences such as the influence of race and fathers and systematic environmental factors, such as a farm. All of these factors during testing, the impact on the expression characteristics of the aggregate genotype, except for daily gain and backfat thickness of the side where the race was not significant (P> 0.05) affected.

Similar results have come many authors (Merx, 1986; Petrovic et al., 1991; Radivojevic et al., 1992; Tuan, 1992; Sreckovic et al., 1993; Das and Mishra, 1993; Merx and Oijen, 1994; Park et al., 1994; Short et al., 1994; Trivunovic, 1996; Brkic et al., 2000; Mijatovic et al., 2005; Mijatovic et al., 2006; Deer et al., 2007). It is therefore necessary to introduce the methods of estimation of breeding values that will eliminate these influences in order to obtain pure additive value of the animal. One of these methods is the BLUP (best linear objective indicator) and AM (individual model).

**Heritability, genetic and phenotypic correlations**

Heritability and standard error of the heritability of traits and genetic and phenotypic correlations between observed traits in the aggregate genotype are shown in Table 6.

Table 6. Heritability and standard error of heritability (on diagonal), genetic correlations and standard errors of genetic correlations (below diagonal) and phenotypic correlations (above diagonal).

*Tabela 6. Heritabilnost i standardna greška heritabilnosti (na dijagonali), genetske korelacije i standardne greške genetskih korelacija (ispod dijagonale) i fenotipske korelacije (iznad dijagonale).*

| Traits / *Osobine* | KH | DP | LS | BS | PM |
|---|---|---|---|---|---|
| KH | **0,84** ± 0,21 | 0,495 | 0,096 | 0,136 | -0,085 |
| DP | 0,513 ± 0,15 | **0,73 ± 0,18** | 0,316 | 0,269 | 0,053 |
| LS | 0,328 ± 0,19 | 0,700 ± 0,13 | **0,74 ± 0,19** | 0,686 | -0,529 |
| BS | 0,266 ± 0,19 | 0,545 ± 0,16 | 0,984 ± 0,03 | **0,76 ± 0,19** | -0,731 |
| PM | -0,155± 0,21 | -0,344± 0,20 | -0,904± 0,18 | -0,858± 0,21 | **0,66 ± 0,18** |

We can see that all the properties of the aggregate genotype boars show a high degree of heritability with high standard error. Heritability for feed conversion is 0.84 ± 0.21 for daily gain 0.73 ± 0.18, backfat thickness, 0.74 ± 0.19, the thickness of the side of bacon 0.76 ± 0.19 and percent of meat 0.66 ± 0.18. The obtained values for the heritability of properties from the aggregate genotype, are located within the literature cited by many (Biyelis at al., 2000; Chen at al., 1999; Huff-Lonergan at al., 2002; Schwab at al., 2010).

Heritability of traits indicates the degree of genetic variability in the total phenotypic variability. If heritability is high, meaning that differences between individuals are genetically determined, and based on the phenotype of animals we can determine the genotype. This further means that animals are the best phenotype, and also represent genetically superior animals. So, as well as phenotypic variability, high heritability, indicating that it is possible to apply the successful selection of properties from the aggregate genotype.

Genetic correlations between feed conversion and daily gain, feed conversion, and backfat thickness, feed conversion and backfat thickness of the side were positive and high (rg = 0.513 ± 0.15, rg = 0.328 ± 0.19, rg = 0.266 ± 0.19). Between feed conversion and lean meat, genetic correlations were negative and weak (rg = -0.155 ± 0.21) Genetic correlations between daily gain and backfat thickness and backfat thickness of the side are positive and strong (r g = 0.700 ± 0.13, rg = 0.545 ± 0.16), while the genetic correlation between daily gain and percentage of meat were negative (rg = -0.344 ± 0.20). Genetic correlation between backfat thickness and backfat thickness of the side showed a positive and complete (rg = 0.984 ± 0.03), while between backfat thickness and lean meat were also full, but negative (rg = -0.904 ± 0.18). Between the backfat thickness of the side and percentage of meat, genetic correlations were negative and very strong (rg = -0.858±0.21)

Positive and high phenotypic correlations were found between feed conversion and daily gain (r p = 0.495), and positive and the poor between feed conversion and backfat thickness of the side (rp = 0.136). Phenotypic correlations between feed conversion and backfat thickness, feed conversion and percenatge of meat were not established. Phenotypic correlations between daily gain and backfat thickness and backfat thickness of the side were high and positive (rp = 0.316, rp =0.269). There were no phenotypic correlation between daily gain and percentage of meat. Positive and strong phenotypic correlations were found between backfat thickness and the backfat thickness of the side of bacon (rp = 0.68), while between backfat thickness and persenateg of meat were negative and high (rp = - 0.52). Negative and strong phenotypic correlations were observed between the backfat thickness of the side and percenatage of meat (rp = - 0.73).

The established genetic and phenotypic correlations between traits of the aggregate genotype in this study are the same sign and phenotypic correlations were lower than genetic, but the correlation between the percentage of meat and other traits which are negative. The lower phenotypic correlation of genetic, can be explained by the influence of environmental factors.

Correlations obtained in this study are in agreement with results of other authors (Gajic et al., 1975; Pohar et al., 1977, Young et al., 1978; Pathiraja et al., 1991; Trivunović, 1996).

On the basis of phenotypic and genetic correlations necessary to define the breeding program to achieve the desired breeding objective.

## CONCLUSION

By analyzing the results of performance test of boars and features included in the aggregate genotype (feed conversion, daily gain, backfat thickness, backfat thickness of the sid, meat percentage), genetic parameters and based on the cited literature, can be drawn the following conclusions:

- Systemic genetic factors (race and fathers) and environmental factors (farm) are highly significant (P<0.01) effect on the traits of the aggregate genotype, except for daily gain and backfat thickness of the side, where the race was not significant (P>0,05) affected. It is therefore necessary to introduce the methods of estimation of breeding values (BLUP – best linear objective indicator) and AM (individual model) that can as much as possible to eliminate these impacts.
- The tested properties of the aggregate genotype showed a high degree of heritability (heritability). Heritability for feed conversion was 0.84 ± 0.21 for daily gain 0.73 ± 0.18, backfat thickness of 0.74 ± 0.19, the thickness of the side of bacon 0.76 ± 0.19 and percent of meat 0.66 ± 0.18. This allows the work to improve the properties of the aggregate genotype based on the selection.
- The established genetic and phenotypic correlations between traits in the aggregate genotype in this study are the same sign and phenotypic correlations were lower than genetic, but the correlation between the percentage of meat and other traits which are negative. The lower phenotypic correlation of genetic, despite the fact that the phenotype includes genotype, can be explained by the influence of environmental factors. The existence of positive and negative genetic and phenotypic correlations, indicating that the simultaneous assessment of the breeding values and selection of traits from the aggregate genotype, it is necessary to have information about correlations and include them in the method of assessment of breeding values.
- For the application of BLUP methods need to be in the elite boars for AI centers, This would be the model for the evaluation of breeding values include the effect of the farm, which would contribute to greater accuracy of assessment.
- To allow simultaneous selection on multiple traits, with respect to heritability, genetic, phenotypic and environmental correlations, as well as economic value of traits, recommended the use of multiple trait BLUP (AM) model.

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## KONVERZIJE HRANE, DNEVNI PRIRAST, PROSEČNA DEBLJINA SLANINE I PROCENAT MESA U PERFORMANS TESTU NERASTOVA RASE LANDRAS

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**Izvod**

U radu su analizirane osobine iz performans testa nerastova rase landras i to: holandski, švedski, nemački i danski, poreklom sa nekoliko farmi na teritoriji AP Vojvodine, u cilju analize varijabilnosti različitih osobina iz agregatnog genotipa: konverzije hrane, dnevnog prirasta debljine leđne i bočne slanine i procenta mesa. Sistemski genetski faktori (rase i očevi) i faktori spoljne sredine (farma) imaju visoko signifikantan uticaj ($P< 0,01$) na osobine iz agregatnog genotipa (konverzija hrane i procenat mesa), izuzev na osobine dnevnog prirasta i debljine bočne slanine na koje rasa nije signifikantno uticala ($P>0,05$). Ispitivane osobine iz agregatnog genotipa pokazuju visok stepen heritabilnosti (naslednosti) sa visokom standardnom greškom. Heritabilnost za konverziju hrane iznosila je 0,84±0,21, za dnevni prirast 0,73±0,18, debljinu leđne slanine 0,74±0,19, debljinu bočne slanine 0,76±0,19 i procenat mesa 0,66±0,18. Ustanovljene genetske korelacije između osobina iz agregatnog genotipa su pozitivne i kreću se od 0,266 između konverzije hrane i debljine bočne slanine do 0,984 između debljine leđne slanine i debljine bočne slanine. Ustanovljene su negativne genetske korelacije između procenta mesa i konverzije hrane, dnevnog prirasta, debljine leđne i debljine bočne slanine (-0,155; -0,344; -0,904 i -0,858).

**Ključne reči:** Landras, nerastovi, performance test, genetski parametri, dnevni prirast, procenat mesa.

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# VETERINARY AND ZOOTECHNICAL SITUATION IN ARTIFICIAL INSEMINATION AT SWINE FARM UNITS IN VOJVODINA (SERBIA)*

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*SUMMARY: The primary conditions for successful pig artificial insemination (AI), are adequate health protection treatment of breeding animals, high hygiene of animals, buildings, equipment and people, and the precise application of modern AI technology measures. The aim of this paper is to establish the veterinary and zootechnological situation at the investigated farm units. Estimated results demonstrate that hygiene, health protection and AI technology, performed at investigated farm units, are not fully in accordance with modern principles of health protection and modern AI technology. The improvement of health protection and AI technology would significantly contribute to improving the health status and reproductive efficiency of breeding animals in the reproductive herds at farm units in Vojvodina.*

***Key words:*** *AI, health, zootechnology, farm unit, pig.*

## INTRODUCTION

The technology of artificial insemination (AI) is used in domestic animals for over 60 years and is the most important biotechnological method, which has made a huge impact on the development of domestic animals (Foote, 2002). The significance and scope of application of artificial insemination, is well illustrated by the fact that, today, the world's annual production of over 90 million doses of boars semen (Thibier and Wagner, 2002). AI technology is constantly evolving, with the aim to: (a) achieve the maximum degree of fertility of inseminated females, (b) maximize the number of insemination doses per ejaculate, (c) to ensure maximum hygiene application of AI, (d)

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to prevent the spread of infectious diseases, and (e) to achieve maximum economic efficiency of the AI technology (Ponsart et al., 2004; Stančić et al., 2008; Stančić et al., 2010; Stančić et al., 2011). The success of artificial insemination is usually measured by sows farrowing rate (Tomes and Nielsen, 1982; Thompson, 2002). This value is significantly influenced by many factors, such as the quality of ejaculate used for AI (Spronk et al., 1997), and the moment of insemination techniques (Wabersky and Weitz, 1996; Stančić et al., 2010), health status of sows and boars (Hoyt, 1998, Stančić et al., 2011), as well as hygiene of insemination procedure (Flowers, 1992; Stančić, 2004; Stančić, 2006).

The aim of this paper is to obtaine results about veterinary and zootechnical situation on the intesive pig production farms in AP Vojvodin. The results could be used to correct the current situation and defining policies and procedures that would significantly improve the health status of the breeding herd and the efficiency of artificial insemination.

## MATERIAL AND METHODS

The survey was conducted during the year 2011, at 8 large pig farms in AP Vojvodina, where artificial insemination (AI) is provide. Based on answers to questions, set up in the survey, the following data, about sanitary and hygienic measures providing on farms, were collected: (1) treatments of primary health care of breeding animals, (2) records of illnesses, that significantly reduce the reproductive efficiency of herds, (3) sanitary-hygienic measures and procedures that are performed in boxes with animals, (4) sanitary and hygienic measures and procedures that are performed throughout the facility, (5) sanitary-hygienic measures and procedures performed in the room (laboratory) for manipulating sperm, (6) sanitary-hygienic measures in the process of taking sperm, (7) measures in the sperm dilution procedure, (8) measures in the formation and storage of AI doses, (9) measures in the insemination procedure of sows and gilts, and (10) measures in the process of handling sows after insemination. Also reviewed are the procedures zootechnological in the VO on the farms.

The survey results are analyzed and presented so as to gain a detailed insight into the true situation regarding the veterinary medical and zootechnical measures, undertaken in the technology of artificial insemination on our farms.

## RESULTS AND DISCUSSION

Veterinary (sanitary-hygienic) measures and procedures that are performed on the studied farms, are shown in table 1, and zootechnological aspects of AI procedures on farms, are shown in table 2.

Tabele 1. Veterinary (sanitary-hygienic) measures and procedures

| Measures and procedures | | No. of farms that performing |
|---|---|---|
| **1. Primary health care** | | |
| Vaccination | *Escherichia coli* | 0,0% (0/8)B 50% (4/8)S 75% (6/8)G |
| | *Mycoplasme hyopneumoniae* | 0,0% (0/8) |
| | *Morbus Aujecky (MA)* | 50% (4/8) |
| | *Erysepelotrix rhusiopathiae* | 75% (6/8) |
| Anti ecto an endoparasitic boars tretment | | 100% (8/8), 2 time per year |
| Bors status for stress sensitivity is known (PPS) | | 0,0% (0/8) |
| Postoji tačna evidencija zdravstveni status vašeg zapata, u vezi sa: | *PPV* | 25,0% (2/8)B 37,5% (3/8)S 37,5% (3/8)G |
| | *PRRS* | 75,0% (6/8) 50,0% (4/8) 50,0% (4/8) |
| | *PCV-2* | 37,5% (3/8) 37,5% (3/8) 50,0% (4/8) |
| | *MA* | 50,0% (4/8) 37,5% (3/8) 37,5% (3/8) |
| | *Brucelozom* | 62,5% (5/8) 50,0% (4/8) 50,0% (4/8) |
| | *Leptospirozom* | 62,5% (5/8) 50,0% (4/8) 50,0% (4/8) |
| **2. Disease that influence herds reproductive efficiency** | | |
| Male reprod. system (in ≥ 20% animals) | | 0,0% (0/8) |
| Endometritis (in ≥ 10% animals) | | 25,0% (2/8)S 25,0% (2/8)G |
| Pyometritis (in ≥ 5% animals) | | 37,5% (3/8) 0,0 (0/8) |
| Vulvovaginitis (in ≥ 20% animals) | | 12,5% (1/8) 0,0 (0/8) |
| Mastitis (in ≥ 10% animals) | | 12,5% (1/8) 12,5% (1/8) |
| Hypogalactia (in ≥ 10% animals) | | 25,0% (2/8) 12,5% (1/8) |
| Agalactia (in ≥ 5% animals) | | 12,5% (1/8) 12,5% (1/8) |
| MMA – syndroma (in ≥ 3% animals) | | 12,5% (1/8) 0,0 (0/8) |
| **3. Sanitary-hygienic measures and procedures that are performed in boxes with animals** | | |
| Cleaning once per day | | 75,0%B 75,0%S 100,0%G |
| Cleaning and washing by water once per day | | 25,0% 12,5% 12,5% |
| Desinfection, once per week | | 25,0% 25,0% 25,0% |
| **4. Sanitary-hygienic measures and procedures that are performed throughout the facility** | | |
| Cleaning and washing by water, once per month | | 87,5%B 87,5%S 87,5%G |
| Desinfection, once per month | | 87,5% 87,5% 87,5% |
| **5. Sanitary-hygienic measures and procedures that are performed in the room (lab) to manipulation of sperm** | | |
| Cleaning and washing by water once per day | | 85,7% |
| Desinfection one per day | | 25,0% |
| Separated entrance in AI laboratory (deso-bariera) | | 62,5% |
| Workers in the laboratory not in direct contact with animals and otherworkers which is in contact with animals | | 25% |
| Workers in the AI laboratory implement regulatory measures of personal hygiene and a prescribed work clothes and shoes | | 50% |
| **6. Sanitary-hygienic measures in the process of taking sperm from boars** | | |
| Mechanical (manual) to remove impurities, washing the foreskin with plain water, disinfection and drying of the foreskin with a clean cloth | | 25% |
| Sperm is collected manually, so it catches the penis clean and disinfected with the hand, of sterile polyethylene gloves. | | 50% (4/8) |
| To collect the semen, original factory equipment are used (disposable sermen collector, filter, gauze, plastic bags) | | 12,5% (1/8) |

| | |
|---|---|
| **7. Sanitary-hygienic measures in the semen dilution procedure** | |
| Original factory extenders are used for semen dilution | 100% |
| Factory original redsetilated water are used for extender dilution | 25% |
| The sanitation of own distillate aparate and quality control of distilled water are perform | 25% |
| **8. Sanitary-hygienic measures in the formation and storage of AI doses** | |
| AI dose are preserv in original disposable plastic bottles | 100% |
| **9. Sanitary-hygienic measures in the insemination of sows and gilts** | |
| Immediately before AI, only mechanically (by hand or with a dry cloth) removal of contaminants from the vulva | 62,5% |
| Sows are AI in his own individual boxes | 62,5% |
| Gilts AI are performe in the group boxes | 75% |
| Original sterilized disposable AI ctheters are use | 62,5% |
| Gilts AI are performe with special disposable ctheters for gilts | 37,5% |
| Adequate therapy are perfome in sows with vulval discharge 14 to 18 days after AI | 75% (6/8) |
| **10. Sanitary-hygienic measures in the females after AI** | |
| After AI, females are in the individual boxes for 30 days | 87,5% |

[B] Boars; [S] Sows; [G] Gilts.

Table 2. Zootechnological procedures in the AI process

| Procedures | | No. of farms that performing |
|---|---|---|
| Ejaculate frequency obtaine pr boar | Every day | 50% |
| | If necessary, at irregular intervals | 50% |
| For each ejaculate volume, progressive motility and total sperm count are recorded | | 25% |
| Method of sperm counting: | Hemocitometry | 25% |
| | Digital photometry | 25% |
| Number of AI doses from each ejaculate are counted according to total sperm number and percentage of progressive motility | | 25% |
| Average number of progressive motile sperm per AI dose (x109) | | 5 |
| Average doses number per ejaculate | | 10 (9-12) |
| AI doses are preserv at + 17oC from formation to using | | 100% |
| Period from formation to using AI doses | < 12 hours | 25% |
| | 1 day | 37,5% |
| | 2 days | 25% |
| | 3 days | 12,5% |
| Ultrasound pregnancy controll within 30 days after AI | | 25% |
| Rebreeding (return tu estrus) after AI, are performing once per day with full boar contact | | 87,5% |
| Separate facilities for boar housing | | 50% |
| Boars facilities with open area for walking | | 0,0% |

Vaccination against the main infectious diseases is not performed on all farms. The relatively small number of farms has a record of the presence of infectious diseases (PPV, PRRS, PCV-2, MA, Brucellosis and Leptospirosis), that causing significant disruption of reproduction. The emergence reproductive system diseases of sows and gilts, to an significant extent, is recorded on a relatively small number of investigated

farms (between 0 and 37.5%, depending on the disease, Tab. 1). Sanitary and hygienic measures carried out in facilities for the animals, the animals themselves, equipment, people, and in the process of artificial insemination, are not at a satisfactory level. This, in particular, relates to the hygiene of boar just before semen taking, hygiene of sperm collection and storage, insemination hygiene, hygiene of workers, who are in direct contact with animals, to the laboratory for semen manipulation, which are not completely isolated from other objects with the animals, that may result in transmission of infectious diseases in breeding animals (Table 1). On the farms do not apply all the principles of modern AI technology. For example, only 25% of the farms formed insemination doses based on the established progressive motile spermatozoa number in the ejaculate. Digital photometry for sperm counting, is used at only 25% of the total number of investigated farms. One ejaculate Relatively small number of AI doses (mean 10) is made per ejaculate, with an unnecessary large number of sperm in a dose (4 to $5x10^9$) (Table 2).

Infectious diseases, especially of viral and bacterial etiology, leading to a significant reduction in reproductive efficiency of the breeding herd (Bondurant, 1991; Hoyt, 1998; Stančić et al., 2010; Stančić et al., 2011). Uterine infections, viral and bacterial agents, is the main reproductive desease in the precticl conditions (Meredith, 1994). This has resulted in significant disruption of reproduction, which is most often clinically manifested as irregular returns to estrus (as a consequence of embryonic and fetal mortality), the birth of the dead, avital and mumifid piglets, in significant decrease of piglets born per litter, increased number of pseudopregnant sows and gilts, as well as in increase number of temporary or permanent subfertile or sterile females (Bondurant, 1991; Floss and Tubbs, 1993; Baysinger and Cooper, 1997; Hoyt, 1998; Stančić et al., 2010; Stančić et al., 2011). Therefore it is important, in our large pig farms, to significantly improve veterinary measures of general health care of breeding animals, to improve sanitary-hygienic measures in the process of artificial insemination, and to strictly comply with the implementation of all principles of modern technology of artificial insemination of swine. This would be: (a) significantly increased the degree of control the spread and eradication of infectious diseases in herds, and (b) increased success of artificial insemination, measured by the achieved level of fertility (farrowing rate and litter size) in the inseminated sows and gilts. In the zootechnological aspects, better sanitary and hygienic measures of facilities, animals, equipment, people, and the process of insemination, mast be implemented. Strict application of the principles and measures of AI technology, is the primary factor in the success of AI (Wabersky and Weitz, 1996; Stančić and Šahinović, 1998; Stančić, 2005). Therefore, according to obtained result in the present study, to improve the zootechnological aspect of AI, it is necessary to: (1) use the disposable equipment (catheters, spermcollectors, plastic bottles for the insemination dose, gloves, etc.), (2) to perform detailed quality control of each ejaculate, using contemporary method (digital photometry) and (3) control of early pregnancy diagnosis using ultrasound method. Improved health care, sanitation and hygiene measures and the implementation of all principles of modern technology in the swine AI, would significantly increase the level of reproductive efficiency of our swine herd, both in its zootechnological, veterinary, and economic terms.

## CONCLUSION

Based on the results obtained in the present study, at the pig farm units in AP Vojvodina, it can be concluded:

1) Sanitary-hygienic measures of breeding animals is not performed in the scope and manner, that can significantly increased efficiency of control and eradicate the major diseases that impact reproduction in our swine herds.
2) Sanitar and zootechnical measures, performed in the process of artificial insemination, is not in accordance with modern AI principles and practices.
3) Improving of sanitary-hygienic, health protection and AI technology measures, as well as higher education of workers, would significantly improve the health and reproductive efficiency of breeding herds on farms in AP Vojvodina.

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## VETERINARSKA I ZOOTEHNOLOŠKA SITUACIJA U VEŠTAČKOM OSEMENJAVANJU SVINJA NA VOJVOĐANSKIM FARMAMA

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**Izvod**

Primarni uslovi za uspešno veštačko osemenjavanje (VO) svinja su: adekvatna zadravstvena zaštita priplodnih životinja, visoka higijena životinja, objekata, opreme i ljudi, kao i precizna primena svih postupaka u tehnologiji VO. Cilj ovog rada je da se, putem ankete sprovedene na 8 intenzivnih vojvođanskih farmi svinja, ustanovi veterinarska i zootehnološka situacija na ispitivanim farmama. Dobijeni rezultati pokazuju da mere higijene, zdratvene zaštite i tehnologije VO, koje se izvode na ispitivanim farmama, nisu potpuno u skladu sa savremenim principima u tehnologiji veštačkog osemenjavanja. Njihovim unapređenjem, značajno bi se doprinelo poboljšanju zdravstvenog statusa i reproduktivne efikasnosti priplodnih životinja u zapatima vojvođanskih farmi.

**Ključne reči:** VO, zdravlje, zootehnologija, farma, svinja.

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# PORCINE REPRODUCTIVE RESPIRATORY SINDROME (PRRS)*

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*SUMMARY: Respiratory disease in pigs are major cause of morbiditiy,mortality, and major cause of economc losses. As results of this situation, it is necessary to cary out timely diagnoses, adequate therapy and on farms established profilacitc mesures. Our intention was to show in this paper , production indicators, such as consequence presence of PRRS in pig farms industrial type. The biggest losses in pigs were the first three month of out break in the period from 3-12 month mortality gradually descreased. The costs of prevention and treatment of secundary infections during the 12 month after outbreak of the disease were on average about 40 % higher in the period before in relation on period before appear disease.*

***Key words**: pigs, farm industrial type, PRRS.*

## INTRODUCTION

Respiratory disease of pigs have been expanded throught out the world and in past years has a been their tide, as it becomes increasingly diffuclt health problem in almost all technological stages of production. Appear in various scenarious, the disturbing frequency and intensity as a result of the interaction of multiple patogens. Our intention was that specific case of industrial swine farms show any health problems can cause reporductive respiratory syndrome, the presence of pigs and (PRRS) and the measures are applied.

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## RESEARCH IN PIGS REPRODUCTIVE RESPIRATORY SYNDROME (PRRS)

Reproductive cause respiratory syndrome is isolated from nasalsecretions, feces and urine. Infection is spread by direct contact with infected animals, a well as droplets. Experiments have demonstrated the transfer agent of boar semen(Petrujkić et.al., 2011). In addition, the virus can be transmitted transplacentary ( in the last trimester pregancy). The infection is characterized by the occurrence of abortion and irregular return to estrus (Blackbum, 1995; Petrujkić et.al., 2011).

The disease is diffusion worldwide. It is characterised by the emergance of abortion, premature farrowing sows, birth nonvital animals or and a high percentage of deaths in the first days after farrowing. Cause of the virus was classified as a *Arteriviridae* family, genus *Arterivirus*, order *Nidovirales* (Cavanaugh, 1997). Clinical picture of reproductive respiratory syndrome varies considerably depeding on virulence strain, dose of virus, the immune status of individuals, categories of production animals and housing conditions. It has been shown that PRRS virus infection as multysistemic disease. Initially manifested viral load, and after distribution of the virus in many organs where the virus multiplies and causes pathological changes (pneumonia, vasculitis, myocarditis, lymmphadenopathy). Vasculitis varied in intensity and change and affect all dimensions of blood vessels. Based on findings of vasculitis in aborted fetuses, nursing and weaning piglets in the lungs and nervous system, it was felt that the blood vessels of the primary goal of PRRS virus.

Clinical symptoms depend on the stage of gestation and age animals. Abortion may occur sporadically or as a mass phenomenon. Usually, fetuses can be mummified at late abortion and weak piglets occure at premature farrowing. Piglets can be infected immediatly after farrowing, showing severe dyspnoea, conjunctivitis, eyelied edema, increased body temperature, tremors and slow blood coagulation (Jackson and Cockcroft, 2007). The clinical picture in suckling piglets are usually manifests several days after onset of symptoms in sows. In most cases the resulting respiratory symptoms, cough, inapatenca, tremors, cyanosis of ears, glowing conjunctiva, swelling of the eyelids, and behavioral disoders. Our observations indicate the occurrence of dark circles and eye sockets and ruffled hair. Diarrohea may be present in case there is no response to therapy , there is possibility of dehydration (Radojičić, 2009; Bojkovski et. al., 2008a,b, 2009a, and 2010a,b). First notice the changing appettie (one day of eating, not eating one day). Finally,the animals lose their appetite back. Body temperatures at 41°C . The animal enters into lethargy and dyspnea. Cough not always present symptoms. The apperance of the redness occurs on the skin, ears, back, hind limbs, vulva and perianal region. They can be caused premature births or abortions, as well as the increaesd number, weak piglets, mumified fruit and stillborn piglets. In the coming period may develop irregular estrus, hypo and agalctia, great loss newborn piglets (Radojičić, 2002, 2003 and 2009; Šamanc, 2009; Petrujkić et.al., 2011). Rarely leads to death from PRRS infected sows, but in acute PRRS in sows and gilts perecentage of deaths could be 8 % (Blackbum, 1995). Fig. 1 show pathology changes on lungs cused virus PRRS. It is noticeable reticulate appearnce of lung tissue as a result of the island interlobular conective-tissue compartment.

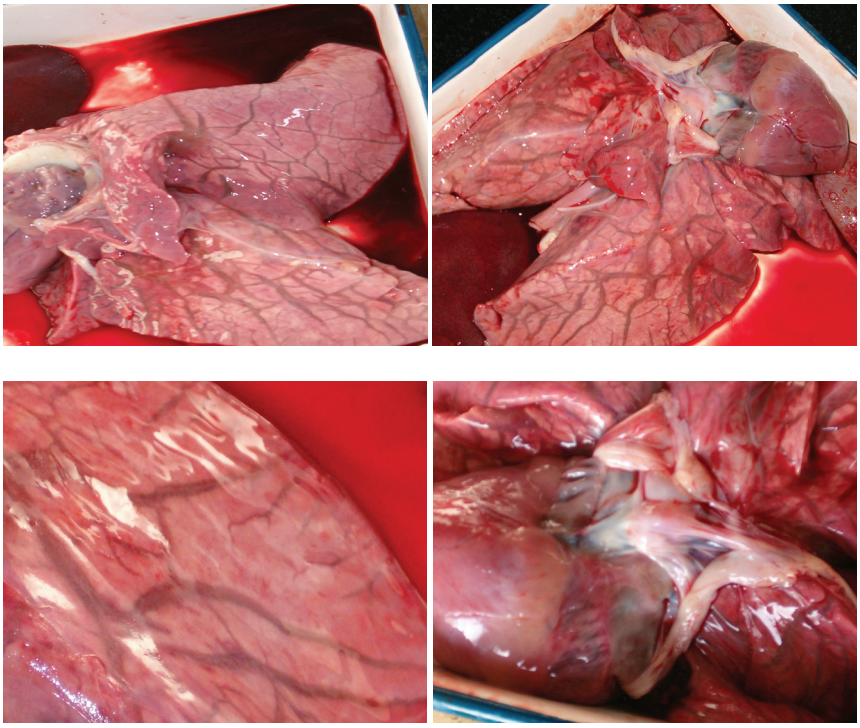

Fig. 1. Pathology changes on lungs cused virus PRRS (Dobrosavljević, orig .photo)
*Slika 1. Patološke promene na plućima izazvane virusom PRRS*

## PRODUCTION INDICATORS AS RESULT OF PRRS PRESENCE AT PIG FARM

Intensive pig production involeves a large concetration of pigs in a relatively small area, making it necessary to apply certain measures to perseve the herd to prevent the spread of disease in the herd and maintaining production. In suckling piglets, great importance is given to variatons of pathogenic microorganisms, not only the expression of resistence to drugs, but also the appreance of genetic recombination, which could affect the clinical course of disease and all of which makes diagnostic installing and imlementating treatment and prophylaxis (Bojkovski et.al.,1997, 2005 and 2011b).

### Case report on one farm in Serbia

At pig farm „A“ industrial type in December 2006.years there has been a suuden and massive outbreaks of symptoms typical of PRRS. Live stock consisted of 240 sows with an annual production of 4,000 hugs. Before the advent of on farm PRRS piglets is the percentage of death below 10 %, 6 % of weaned piglets and fattening pigs in the final 1,4 %. The average body weight at weaning pigs at 30 days was 7,1 kg. Respiratory form of PRRS is commonly encountered in suckling piglets and young pigs, with the emergence of anorexia, dispnoea, cough, and ocasional deaths.There is some pigs severe abdominal cyanosis and ears.

In december 2006 th in the course of disease in laboratory send 10 samples of weaning piglets with respiratory symptoms. Diagnosis was performed serologically and (Enzyme-linked immunosorbent assay – ELISA). Of the total number of serum samples send for testing anti PRRS antibody ELISA positive samples was 80 %. In this way confirmed the suspicion of reproductive respiratory syndrome and swine. In the first stage of the disease in sows in farrowing obsereved the following symptoms loss of appetite, premature farrrowing, stillborn, and mummified piglets, agalactia, mastitis, and individual cases of cough. At weaning piglets dominated the respiratorny symptoms, discharge from eyes and cyanosis of ears, cough and temperature above $40,8^0$ C.

The first symptoms were observed in sows farrowed in a form of premature farrowing. In December 2006 the 40 sows farrowed, 3 (7,5%) were farrowed 112 th day and 2(5%) 109 of pregnancy. Premature births were occurred in the next 3 month from the onset of disease.

Production indicators are given for the period prior to and during the 12 months from time of the outbreak. In the present form was no difference in conception in sows and it was 83%. The number of piglets born alive in the year before the outbreak was 5656 in the period after the outbeak of 55000, so the number of piglets born alive was reduced by 156 pigs. After the outbreak of stillbirths pigs increased to 5,05% (293 of total 5793) and the number of mummified piglets on 2,41% (140 of 5793). Pigs in relation to the period before the onset of the disease was 2,88% the stillbirths (168 of 5824) and 0,20% (12 of 5824 pigs) in mummified piglets.The percentage of stillborn and mummified piglets was constantly increased from the time an outbreak of infection during the period and not after 12 months returned to previous levels.The number of stillborn piglets per litter increased from 0,33 % to 0,57% in the period.In the first three month of the onset of clinical symptoms obsereved the occurrence of mastitis and agalctia in sows. Pigs less weight and weak pigs broke in hypoglycemia and have little chance ti survive. Before the outbreak of disese mortalitiy suckling piglets amount is less than 10% in the 12 months after onset was aproximatly 14 % (811 of 5793 piglets). The higest percentage of death was the first 3 month after the outbreak of 18,19 % (181 of 995 piglets). Percentage mortalitiy of weaned piglets from 6 % before the onset of the disease increased to 13,7% (564 of 4117 piglets) in the period after the onset of the disease. For such pigs were clearly marked respiratory symptoms and mortalitiy was consistently increased throughout the period of observation. Mortality in pigs before the onset of PRRS was 1,4% in the period after onset of illness was 4,26%. The biggest losses in pigs were the first three months of the outbreak in the period from 3-12 months mortality gradually decreased. The costs of prevention and treatment of secondary infections during the 12 month after the outbreak of the disease were on average about 40 % higher than the period before the outbreak of the disease.

## PROPUSRD MEASURES AND CONCLUSIONS

It is belived that PRRS is very complex disease and its controls in itself is complex. Since the primary mode of transmission of pathogens in direct contact with an infected animal, the main problem is how to protect healthy herd of entering pathogens. The measures used in the protection of the introduction an all other disease in the herd, and used here. They include only the purchase of pigs from herds free from PRRS, strict complience with quarantine institutions which in the case of PRSS a must persist for at

least 60 days, the production „all in all out“, cleaning and disinfection of fascilities at each introduction of new animlas, destruction of rodents, birds, cleaning and disinfection of transport. Among other measures recommended „multi site“ system of growing pigs, a partial early weaning, rearing depopulation medication early weaned piglets (Stanković, 2009).

Control strategy must be established for each farm separatly.There is no universal control program, each program must be based on the epidemiology of viruses, pathogenic agent that is active on the farm, their capabilities and managment. The application of medications helps to prevent the emergance of secundary infections. Due to the large differences in antigen structure , even within the same farm, vaccinated against PRRS is not given a satisfactory result. The effectiveness of vaccination ranges from 30 to 70 % and can not prevent infection. Efficiency vaccination against reflected mainly in reduction incidence of disease and mitigation of clinical symptoms. According to the findings (Gagrčin and Došen, 2004) medication or vaccination against PRRS, faild to reduce the losses incurred as result of the emergance of PRRS). In our studies (Rogožarski et.al., 2007; Savić et.al., 2009 and 2010.) medication and vaccination against mycoplasma with improving housing conditions have been able to reduce the losses that occur in the presence of PRRS virus on farms possible and successful business.

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# REPRODUKTIVNO RESPIRATORNI SINDROM SVINJA (PRRS)

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**Izvod**

Bolesti organa za disanje kod svinja su jedan od glavnih uzroka morbiditeta, mortaliteta i jedan od glavnih uzroka ekonomskih gubitaka. Kao rezultat takvog stanja, nephodno je blagovremeno sprovesti dijagnostiku, adekvatnu terapiju i na farmama uvdititi profilaktičke mere. Namera nam je bila da u ovom radu prikažemo proizvodne pokazatelje, kao polsedicu prisustva PRRS na farmi svinja indusrijskog tipa. Najveći gubici kod tovnih svinja bili su prva 3 meseca od izbijanja zaraze a u periodu od 3-12 meseci moratlitet se postepeno smanjivao. Troškovi prevencije i lečenja sekundarnih infekcija tokom 12 meseci posle izbijanja bolesti bili su u proseku oko 40 procenata veći u odonsu na period pre izbijanja bolesti.

**Ključne reči:** svinje, farma svinja industrijskog tipa, PRRS.

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# PATOMORPHOLOGICAL DIAGNOSIS OF HEMORRHAGIC PROLIFERATIVE ENTEROPATHY IN SWINE*

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JASNA KURELJUŠIĆ, MILENKO ŽUTIĆ, VOJIN IVETIĆ [1]

*SUMMARY: In this paper seven samples of the intestines originated from the naturally infected pigs, macroscopic, histochemically and immunohistochemically were examined. The caudal part of jejunum and ileum were affected. The affected intestine was thickened and dilated. The lumen of the ileum and colon contained one or more blood clots without feed contents. The rectum contained black tarry feces of mixed blood and digesta. The mucosal surface of the affected portion of intestine was markedly thickened without macroscopic erosions. Histological examination revealed: extensive degeneration, congestion, and hemorrhage within the proliferative epitelium, as well as a marked accumulation of the bloody cellular debris above affected mucosa. Clusters of argyrophilic, slightly curved rod-shaped microorganisms in the apical cytoplasm of enterocytes by Warthin-Starry silver stain were demonstrated. Immunohistochemical staining confirmed the presence of L. intracellularis in the apical cytoplasm of hyperplastic enterocytes and in lamina propria. In conclusion, diagnosis of hemorrhagic proliferative enteropathy is based on detection of the histologic lesions and detection of L. intracellularis by Warthin Starry silver stain, as well as by immunohistochemistry, where immunohistochemistry and Warthin-Starry silver method can be a complementary methods to confirm the diagnosis of L. intracellularis infection in pigs.*

***Key words:*** *hemorrhagic proliferative enteropathy, swine, Lawsonia intracellularis.*

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## INTRODUCTION

Porcine proliferative enteropathy (PPE) is the infectious, intestinal hyperplastic disease, characterized by thickening of the intestinal mucosa due to enterocyte proliferation. The disease affects weaned pigs, primarily growing and fattening. Porcine proliferative enteropathy is worldwide in distribution and occurs commonly in all pigs-rising regions and in all pig farm managment (McOrist et al., 2003). The incidence of lesions in pigs at normal slaughter age is generally low, at 0.7-2.0%, and therefore unreliable for farm monitoring (Jensen et al., 1999).

The etiological agent is the obligatory intracellular bacterium *Lawsonia intracellularis*, which preferentially grows within the cytoplasm of intestinal epithelial cells. *L. intracellularis* forms curved to straight vibrioid-shaped rods and measure 1.25-1.75 μm in length by 0.25-0.43 μm in width (Dale et al., 1998). From epizootiological point of view it is important that *L. intracellularis* can remain viable in feces at 5 to 15°C for 2 weeks (Collins et al., 2000). It is known from literature that infectious dose is relatively low and fecal excretion may be high in some infected "spreader" pigs (McOrist et al., 1993; Guedes et al., 2003). There are two forms of the PPE: clinical and subclinical. Clinical forms can be acute and chronic. Acute clinical form is hemorrhagic proliferative enteropathy. Chronic forms are: porcine intestinal adenomatosis, necrotic enteritis and regional ileitis. In each of these states, the characteristic lesion is present: thickened mucosa of the small intestine (mainly the ileal one), the caecum and/or the proximal colon. The main histological lesion of the PPE is the adenomatous hyperplasia of the intestinal crypt cells due to proliferation of the immature epithelial cells and almost lack of goblet cells. In most cases, no significant inflammatory reaction occurs and the organisms remain in the epithelium at this stage (Yates et al., 1979; Ivetić et al., 2006; McOrist and Gebhart, 2006; Ivetić et al., 2009). In severe cases of PPE, *L. intracellularis* can also be observed in the mesenteric lymph node and tonsils (McOrist and Gebhart, 2006). Because of the difficulty of culturing *L. intracellularis*, it has been necessary to develop alternative methods for its detection. Confirmation of clinical diagnosis may be obtained by demonstration of *L. intracellularis* in feces and intestinal tissue by PCR or by indirect serologic assays for detection of antibodies (Nathues, 2007). At necropsy, the use of modified Ziehl-Neelsen stain or the Giminez stain on mucosal smears to demostrate the intracellular organisms is a simple presumptive technique, requiring minimal time and equipment (Love et al., 1977). Histopahological examination of affected tissues will reveal the distinctive morphology of the proliferative lesions. Specific identification of *L. intracellularis* in these lesions can be achieved by immunohistochemical staining of fixed embedded tissues (Ladinig et al., 2009). In the absence of specific immunological reagents, modifications of the Warthin-Starry silver impregnation technique are satisfactory for routine use (Young, 1969). The affected crypts need to be examined carefully at high magnifications due to the small size of *L. intracellularis*.

In this work patomorpholological diagnosis included macroscopic examination and using histochemical and immunohistochemical methods for diagnose of this infection.

## MATERIAL AND METHODS

Intestine samples of seven growing pigs from two herds, on which necropsies were performed at Department of Pathology of the Institute of Veterinary Medicine of Serbia, were examined. Samples of the distal ileum originated from the infected pigs, macroscopic, histochemically and immunohistochemically were examined.

Samples of distal ileum were fixed in 10% buffered formalin, and after standard processing cast in paraffin blocks. Paraffin sections 3-5 μm thick were stained with hematoxylin and eosin (HE) and with Warthin-Starry silver stain for light microscopic examination. Three-step indirect immunohistochemical technique was performed at Veterinary Diagnostic Laboratory at Iowa State University, Ames, USA. After antigen retrieval and inactivation of endogenous peroxidase, the sections were incubated with primary non commercially monoclonal antibody against *Lawsonia intracellularis* diluted in PBS. All rinsing procedures and serum dilutions were done in PBS (pH 7.2). The detection kit was LSAB2 System-HRP, Rabbit/mouse (DAKO, K0675). Reactions were visualized by using DAB+ (Dako, K3468) and counterstaining with hematoxylin. Intestine sections of infected pigs were used as positive controls. Intestine section not treated with the primary antibody were used as negative controls.

## RESULTS AND DISCUSSION

Macroscopic examination revealed that the small intestine contained bile-stained mucus to the level of mid-jejunum where hemorrhage began and became gradually more copious with large blood clots. Caudal part of jejunum and ileum were affected. The affected intestine was thickened and dilated. The lumen of the ileum and colon contained one or more blood clots without feed contents (Figure 1). The rectum contained black tarry feces of mixed blood and digesta. The mucosal surface of the affected portion of intestine was markedly thickened without macroscopic erosions. Literature data shown the same macroscopic finding (McOrist and Gebhart, 2006). In this case there were no lesions in large intestine.

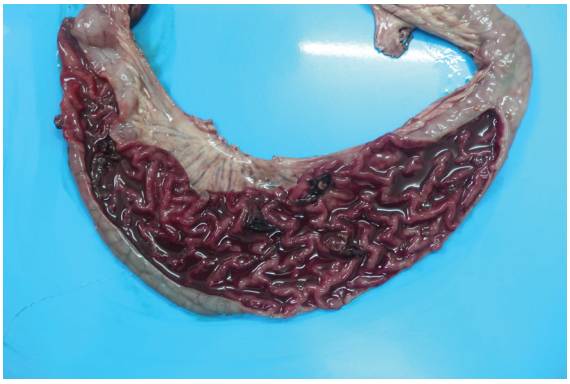

Figure 1. Swine ileum, thickened mucosa, hemorrhages and blood clots in the lumen
*Slika 1. Ileum svinje, sluznica je zadebljala, krvavljenje i krvni ugrušci u lumenu*

Histological examination revealed: extensive degeneration, congestion, and hemorrhage within the proliferative epitelium, as well as, a marked accumulation of the

bloody cellular debris above affected mucosa. In the cranial part of jejunum and in ileum there was flattening of villous architecture. The mucosa was markedly thickened by irregular hyperplastic crypts in which there was piling up of young epithelial cells, a high mitotic index, and a lack of goblet cells (Figure 2). Many crypts contained copious cellular debris and neutrophils and there was infiltration of the lamina propria by a mixed population of cells, including macrophages, lymphocytes, eosinophils, plasma cells and neutrophils. As well known, with severe disease, bacteria are present in macrophages in lamina propria. This may cause release of tumor necrosis factor-α, resulting in vascular permeability and hemorrhage (McGavin and Zachary, 2007). It is also known from literature that described lesions are characteristic for *Lawsonia intracellularis* infection in swine (Ivetić et al., 2006; McOrist and Gebhart, 2006; Ivetić et al., 2009).

Clusters of argyrophilic, slightly curved rod-shaped microorganisms in the apical cytoplasm of enterocytes by Warthin-Starry silver stain were demonstrated (Figure 3). Modifications of the Warthin-Starry silver impregnation technique are satisfactory for routine use (Young, 1969). However, this method is not specific for *L. intracellularis* and cannot always detect the organism in necrotic debris or in autolyzed tissue. More specific identification of *L. intracellularis* can be achieved by immunohistochemistry staining of fixed tissues. Immunohistochemical staining confirmed the presence of *L. intracellularis* in the apical cytoplasm of hyperplastic enterocytes and in lamina propria (Figure 4). This observation suggests that bacteria were mainly phagocytized and lysed within the cytoplasm of macrophages. It is known from literature that this technique is more sensitive than the silver stain because it reveals organisms within macrophages of the lamina propria during recovery from PPE (Ladinig et al., 2009). In addition, extracellular *L. intracellularis* can be identified either in exudate or necrotic debris in superficial mucosa. In a study comparing diagnostic methods, immunohistochemistry staining detected nearly twice as many pigs PPE lesions as did silver staining of formalinized tissues. The most sensitive tests for diagnosing PPE are mucosal PCR and tissue immunohistochemistry, but both require necropsy to perform. The antemortem methods are less accurate, with serology being most sensitive (Ladinig et al., 2009).

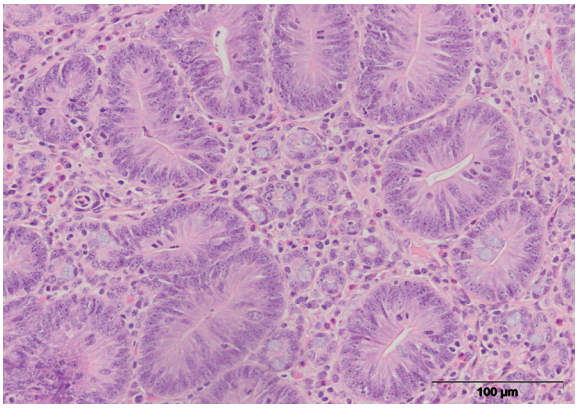


Figure 2. Swine ileum, hyperplastic crypts in which there is piling up of young epithelial cells, a high mitotic index, and a lack of goblet cells, HE stain

*Slika 2. Ileum svinje, hiperplastične kripte sa umnoženim mladim epitelnim ćelijama, visokim mitotskim indeksom i nedostatkom peharastih ćelija, HE bojenje*

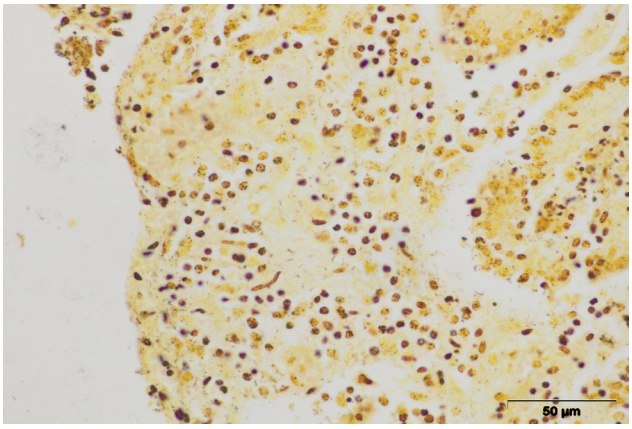


Figure 3. Swine ileum, argyrophilic, slightly curved rod-shaped microorganisms within epithelial cells, Warthin-Starry silver stain

*Slika 3. Ileum svinje, argirofilni, blago savijeni štapićasti mikroorganizmi u epitelnim ćelijama, Warthin-Starry bojenje*

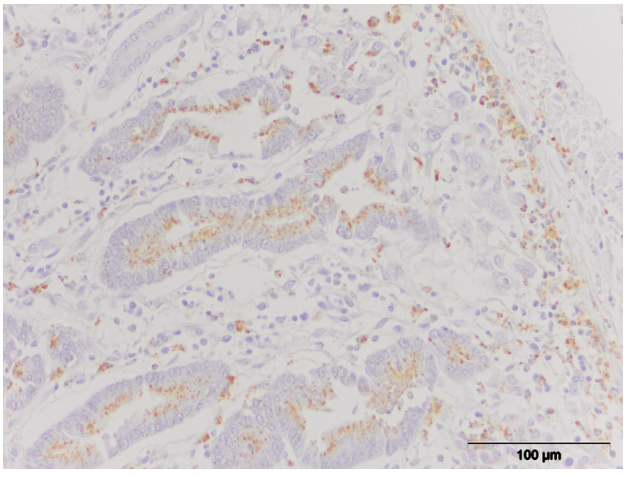


Figure 4. Swine ileum, clusters of immunopositive intracellular microorganisms (brown) are seen in the apical cytoplasm of hyperplastic epithelial cells and in lamina propria, Immunohistochemistry, LSAB2

*Slika 4. Ileum svinje, grupe imunopozitivnih intracelularnih mikroorganizama (smeđe) se uočavaju u apikalnoj citoplazmi hiperplastičnih epitelnih ćelija i u lamini propriji, Imunohistohemija, LSAB2*

## CONCLUSION

In conclusion, diagnosis of hemorrhagic proliferative enteropathy is based on detection of the histologic lesions and detection of *L. intracellularis* by Warthin Starry silver stain, as well as by immunohistochemistry, where immunohistochemistry and Warthin-Starry silver method can be a complementary methods to confirm the diagno-

sis of *L. intracellularis* infection in pigs. The "gold standard" for the histopathological diagnosis of *L. Intracellularis* infection is immunohistochemical examination, however, due to restricted availability of polyclonal antiserum and monoclonal antibodies, not all diagnostic laboratories can perform this test.

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# PATOMORFOLOŠKA DIJAGNOSTIKA HEMORAGIČNE PROLIFERATIVNE ENTEROPATIJE SVINJA

BRANISLAV KURELJUŠIĆ, BOŽIDAR SAVIĆ,
NEMANJA JEZDIMIROVIĆ,OLIVER RADANOVIĆ, JASNA KURELJUŠIĆ,
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**Izvod**

U ovom radu je makroskopski, histohemijski i imunohistohemijski ispitano 7 uzoraka creva prirodno inficiranih svinja. Patomorfološke promene u vidu zadebljanja crevnog zida i proširenog lumena su ustanovljene na kaudalnom delu jejunuma i ileumu. U lumenu ileuma i kolona su uočeni ugrušci krvi bez prisustva hrane. U rektumu je ustanovljen katranasti feces koji potiče od svarene krvi i crevnog sadržaja. Površina sluznice zahvaćenog dela creva je pokazivala značajno hiperplastično zadebljanje. Erozije sluznice nisu ustanovljene. Histološkim ispitivanjem u proliferisanom epitelu uočena je degeneracija epitelnih ćelija, kao i kongestija i obimna krvavljenja. Pored toga, zapaženo je i nakupljanje hemoragičnog ćelijskog debrisa iznad površine sluznice. Warthin-Starry bojenjem ustanovljene su grupe argirofilnih, blago savijenih štapićastih mikroorganizama u apikalnoj citoplazmi enterocita. Imunohistohemijskim ispitivanjem upotrebom monoklonskog antitela protiv *L. intracellularis* potvrđeno je prisustvo mikroorganizama u apikalnoj citoplazmi hiperplastičnih enterocita i u lamini propriji. Dijagnoza hemoragične proliferativne enteropatije je postavljena na osnovu karakterističnih histoloških lezija i korišćenjem Warthin-Starry bojenja, kao i upotrebom imunohistohemijske metode, korišćenjem monoklonskog antitela protiv *L. intracellularis*. Imunohistohemijska metoda i Warthin-Starry metod bojenja mogu biti komplementarne metode za potvrdu infekcije izazvane sa *L. intracellularis* kod svinja.

**Ključne reči:** hemoragična proliferativna enteropatija, svinja, *Lawsonia intracellularis*.

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# ELIMINATION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) WITH SERUMIZATION, NATURAL EXPOSURE AND VACCINATION ON SIX PIG FARMS IN SLOVENIA

MARINA ŠTUKELJ, ZDRAVKO VALENČAK[1]

*SUMMARY: Porcine reproductive and respiratory syndrome (PRRS) is currently the most important swine disease worldwide. A variety of strategies have been described for PRRS eradication. The study involved six one-site small pig farms where the reproductive problems were observed. The owners were acquainted with strict biosecurity and herd closure for at least 200 days. Serum samples were tested with IDEXX X3 PRRS ELISA for antibodies detection and with one step RT-PCR (Qiagen, Germany). Production results after serumization improved 3 months after on both farms. Herds were also without virus. On farm 1 new boar without quarantine and new gilts were introduced in to the breeding herd. Eleven months after serumization production results decreased and same strain of PRRS virus was present in herd. Breeding herd on farm 2 is 13 months without PRRS virus. Only in group of growers of 10 weeks PRRS virus persists but the subtype of it is new. After natural exposure on both farms number of seropositive pigs was decreased and fatteners were seronegative. Both farms were also without PRRS virus. After vaccination on farm 5 production results improved and also number of high seropositive animals decreased. Fourteen months after vaccination number of seropositive pigs increased and production results decreased. Results from second vaccinated farm were similar. Natural exposure with implementation the strict biosecurity protocols and improvement of management are the key factors for successful eradication of PRRS. At the moment serumization and vaccination are the methods with limited success.*

***Key words:*** *PRRS, elimination, serumization, natural exposure, vaccination.*

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## INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is economically the most important pig disease that occurs worldwide. Reproductive disorders 12%, increased mortality 43% and decreased feed efficiency 45% of total loss are consequence of PRRS (Zimmerman, 2008). Data based on ccalculations from the literature shows that losses due to PRRS outbreak on the farm with 50 breeding sows totals 20,363 EUR of which 55.30 EUR per farrowing/sow, which totals for farm with 50 sows 6.913 EUR, 4.60 EUR per weaner which totals 5.750 EUR and 7 EUR per prefattener and fattener which totals 7.700 EUR. Calculation is not absolute and it varies based on the phase of PRRS (acute or endemic) (Neumann et al., 2005). In United Status of America they believe that production of pig with PRRS is not economically effective and so they started with programs of PRRS eradication. Chile for instance succeeded to completely eradicate PRRS in 9 years by this method (Zimmerman, 2008). There are still few countries free of PRRS: Australia, New Zealand, Finland, Norway, Switzerland and Sweden. Due to enormous losses there are many attempts worldwide of control, elimination or eradication of PRRS (Jenny and Dee, 2006).

PRRS is a reason of frustrations both for pig producers as well as for veterinarians from the very first outbreak on. There are many successes in understanding of epidemiology, improvements in diagnosis and effective programs of disease control. But there are still many unsolved questions. With new knowledge about PRRS we are able to prevent, control and eradicate PRRS but in spite of all known measures of controlling, diagnostic and available vaccines achieving the goal is still difficult. Reasons are infection with few different subtypes of virus, homologous protection, permanently infected pigs, bad management and not known routes of infection (Štukelj, 2012). After PRRS outbreak there are various ways of intervention which depend on the size of a farm and prevalence of disease. We can either do nothing or we can start with control or elimination and eradication (Torremorell and Christanson, 2002; Taylor, 2006; Zimmerman et al., 2006).

*Control of the disease.* One possible measure is control of the disease. The goal here is reduction of losses due to virus circulation in breeding herd and stopping of vertical and horizontal shedding to achieve stabilization of breeding herd and development of specific immunity against farm's strain of virus with improvement of production results. This can be achieved by using only controlled negative semen, with acclimatization of gilts before introducing them to the farm or with vaccination (Zimmerman et al., 2006; Batista et al., 2004; Pesente et al., 2006; Vashisht et al., 2008; Scortti et al., 2006; Martelli et al., 2007; Kimman et al., 2009; Cesar et al., 2010).

Because PRRS virus is immunosuppressive there is increased manifestation of endemic diseases on the farm (streptococcal meningitis, porcine multisystem wasting syndrome, enzootic pneumonia, Glässer disease, parasitosis). Higher incidence of mentioned diseases leads to use of more antibiotics. Control of PRRS includes also control of endemic and secondary diseases.

*Elimination and eradication.* Elimination of the disease means that there are no clinically visible signs of the disease but there can still be present virus or specific antibodies. Eradication of the disease means absence of clinical signs, pato-anatomical signs and also absence of both the specific agent (virus) and specific antibodies. Elimination, eradication or disease free status can be achieved only through implemented strict biosecurity measures.

The first step in PRRS elimination is to find how the virus was introduced (Torremorell and Christianson, 2002). The next step is double closure of the farm which means both no introduction of new pigs on the farm and no introduction of farm's own gilts to the breeding herd for at least 200 days (Zimmerman et al., 2006; Cho and Dee, 2006). Strict biosecurity measures should be followed (Chappell et al., 2010; Pitkin et al., 2011).

Method of elimination/eradication of PRRS:

- depopulation/repopulation
- test and removal
- immunization: natural exposure, vaccination and serumization.

*Depopulation/repopulation.* Total depopulation is very radical and rather expensive method but very successful in fighting PRRS. With this method all pigs are removed from the farm, farm is cleaned and disinfected and only after that new negative pigs are introduced (Torremorell and Christianson, 2002; Corzo et al., 2010; Zimmerman et al., 2006; Cho and Dee, 2006).

*Test and removal of positive pigs.* This method can be used in case of low prevalence or when only few pigs are positive. Pigs are tested for antibodies and virus. The testing is finished when last positive pig is removed from the farm (Zimmerman et al., 2006; Dee, 2004; Cho and Dee, 2006).

*Immunization.*The goal of immunization is stabilization of breeding herd which means that all breeding pigs have present antibodies against PRRS virus but all are without PRRS virus. Immune sows protect their piglets with colostrum (Zimmerman et al., 2006).

*Natural exposure.* In small herds we can wait until all breeding pigs become immune to the virus through natural exposure. The time of immunization can differ. Negative effects of the disease are decreasing after the certain time (Corzo et al., 2010; Torremorell and Christianson, 2002).

First measure is herd closure for six months (Dee, 1998). Natural exposure is based on the fact that PRRS virus cannot exist in population in which all pigs have present specific antibodies. All breeding pigs have to be exposed to PRRS virus infection. The end result is that all breeding pigs are immune and no pig is excreting PRRS virus (Dee, 2009; Zimmerman et al., 2006).

*Vaccination.* On the market there are only two PRRS vaccines: against European (genotip I) and against American (genotip II) strains. In the period of 2009 – 2011 in Slovenia we have proved several strains of genotip I. In year 2011 also genotip II was proved. All registered vaccines for genotip I have one strain of PRRS virus (strain Lelystad). With live vaccine immunity is better but because new strain of PRRS virus is introduced in to the herd an outbreak of PRRS can be induced. Beside that in PRRS the protective immunity is homologous (only against herd strain) or against the strain which is genetically close to vaccine strain. Immunity against inactivated PRRS vaccines is weak but new PRRS outbreak is impossible (Kimman et al., 2009; Corzo et al., 2010; Stadejek et al., 2011).

Immunity protection after vaccination is complete only when there is no PRRS strain on the farm but the genetically close to vaccine strain (Martelli et al., 2007; Lager et al., 1997; Kimman et al., 2009). Complete protection can be achieved with herd vaccine.

*Serumization.* The easiest way to achieve homologous protection is serumization. Serumization is speeding the natural immunization. Blood of pigs with virus is used for

inoculation of all breeding pigs in one day (Štukelj et al., 2011).

*Partial depopulation.* This method is used as additional measure in PRRS eradication to stop the shedding of virus on the farm. Partial depopulation means that we remove different categories of pigs from the farm. With PRRS weaners are the critical category (Zimmerman et al., 2006; Torremorell and Christianson, 2002).

## MATERIAL AND METHODS

*Farms.* Farm 1 has 2 boars and 72 breeding sows, farm 2 has 4 boars and 130 breeding sows. Serumization was performed once on farm 1 and twice on farm 2 in three months period. Farm 3, farm 4 and farm 6 have each 15 breeding sows. Farm 5 has 20 breeding sows. The method of natural exposure was used on farm 3 and 4, and vaccination with live attenuated PRRS vaccine strain Lelystad was performed on farm 5 and 6. On all six farms double closure for six months was accepted. In this time import of new pigs to the farm and also replacement of gilts from their own farm to the breeding herd was prohibited. All farms are free of classical swine fever and Aujeszky disease.

*Sampling.* On all farms PRRS was proved with first sampling. At second sampling blood was taken from all breeding pig for determination of prevalence and to choose the appropriate method of elimination. Samplings were repeated every 3 months to control the process of elimination.

Table 1. Number of tested sera for detection of antibodies against PRRSV and number of tested sera for virus detection.

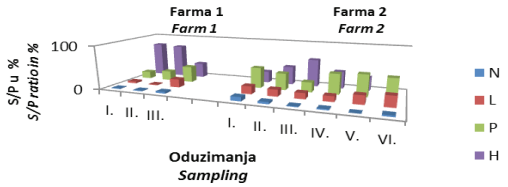
*Tabela 1. Broj uzoraka za antitela protiv virusa PRRS i broj pregledanih uzoraka na prisutnost virusa PRRS*

| Farm *Farma* | No. of samples tested with ELISA *Broj uzorka ispitan u ELISA ELISA* | No. of samples tested with RT-PCR *Broj pregledanih uzoraka s RT-PCR* |
|---|---|---|
| 1 | 292 | 40 |
| 2 | 519 | 271 |
| 3 | 64 | 20 |
| 4 | 31 | 10 |
| 5 | 94 | - |
| 6 | 80 | 20 |

*Methods.* Detection of antibodies by ELISA. Sera were tested with IDEXX PRRS ELISA (HerdChek, IDEXX Laboratories Westbrook, Maine, USA). S/P ratios were calculated according to test manual and were divided in N negative, L low positive – S/P less than 1, P positive S/P between 1 and 2, 4 – high S/P more than 2. Detection of nucleic acid of PRRS virus by RT-PCR in ORF 7 region of viral genome. Samples were tested by reverse transcription and polymerase chain reaction (RT-PCR) which enables detection of EU and USA strains of PRRS virus in well conserved region ORF 7 (Donadeu et al., 1999).

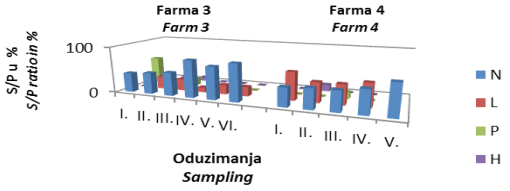
## RESULTS

On farm 1 three months after serumization improvement of production results and trend of stabilization of breeding herd were visible. Six months after serumization number of high positive samples decreased. Before third sampling new boar and fatteners without quarantine were added to the herd. Two months after third sampling production results were significantly lower. On farm 2 three months after serumization six breeding sows were still negative and 21 were with low S/P values, so we decided for second serumization. At third sampling, 3 months after second serumization, improvement of production results and trend of stabilization of breeding herd were visible. At fourth sampling, six months after serumization, breeding herd stabilization was evident (S/P values were lower, some pigs were negative) and production results were improved. 18 months after second serumization number of negative increased and number of high positive decreased. On all farms PRRS virus was from genotip I.



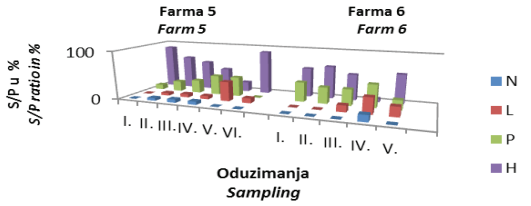

Graph 1. Results of serology of breeding herd from farm 1 and 2 after serumization
*Graf 1. Rezultati seroloških testiranja krmača na farmi 1 i 2 posle serumizacije*

Results of molecular testing on farm 1 and 2 proved absence of PRRS virus 3 month after serumization. On farm 1 eleven months after serumization same type of PRRS virus was proved. On farm 2 13 months after the serumization PRRS virus was not detected in breeding herds. Virus was persistently detected only in weaners at age 10 weeks. Ten months after second serumization new subtype of PRRS was proven by sequencing.




Graph 2. Results of serology of breeding herd from farm 3 and 4 after natural exposure
*Graf 2. Rezultati seroloških testiranja krmača na farmi 3 i 4 posle prirodnog prekuženja*

On farm 3 and 4 in samplings after natural exposure increasing of negative and also consequently decreasing of positive and low positive samples was evident. On both farms fatteners were also tested for antibodies and were respectively negative. On farm 3 and 4 virus was not detected.




Graf 3. Rezultati seroloških testiranja krmača na farmi 5 i 6 posle vakcinacije
*Graph 3. Results of serology of breeding herd from farm 3 and 4 after vaccination*

On farm 5 vaccination of breeding herd was performed twice in period of 3 months. Blood was taken before first vaccination (sampling I), before second vaccination (sampling II), two months after second vaccination (sampling III), five months after second vaccination (sampling IV), 10 months (sampling V) and 14 months after second vaccination (sampling VI). In table 4 it is visible that number of high positive was decreasing and number of positive was increasing and production results were improving until sampling VI. 14 months after vaccination number of high positive sample increased and production results drastically decreased.

On farm 6 pigs were vaccinated for substantial time before first sampling. Samplings were performed the following way: sampling II 1 month, sampling III 2 months, sampling IV six months and sampling V 11 months after our vaccination. Up until sampling V number of high positive samples was decreasing and number of positive samples was increasing and production results were improved. 11 months after vaccination number of high positive samples increased and production results decreased.

On farm 5 molecular testing was not performed. On farm 6 all 20 tested weaners of age 6 to 15 weeks were positive by RT-PCR before second sampling.

## DISCUSSION

Closure of farm for at least 200 days is necessary for a successful elimination of PRRS by any method (Yeske, 2008). Very important is also to abide strict biosecurity measures (Dee, 1998).

The best protection against PRRS virus is homologous, which can be achieved either by serumization or natural exposure. On farm 1 and 2 three months after serumization we succeed with herd stabilization (all pigs with unified titres and without virus). On farm 1 they didn't abide closure rules, so new outbreak occurred. On farm 2 closure is accepted but all in all out system is not practiced and so virus is persisting in category of weaners.

On farm 3 and 4 positive effect was visible 3 months after closure (Graph 3). With each testing number of negative samples was increasing. From literature antibodies are persisting for 300 days (Zimmerman et al., 2006) but from our study it looks that antibodies can persist for a year and eight months.

Results from sequencing show that subtypes of PRRS in Slovenia are not closely related to Lelystad virus. (Toplak et al., 2010) Relevance of homologous protection was visible also in our situation when six month after vaccination a new outbreak occurs. In many pigs there was no seroconversion also after numbers of repeated vaccination. Af-

ter vaccination there was only a short improvement of production results and alleviation of clinical signs. Corzo et al. (2010) reported on alleviation of clinical signs also in case of heterologous type of virus. Vaccination is suitable for control and elimination of farm strain (Gillespie and Carrol, 2003). In our study we proved improvement of production results but virus was still present on the farm after vaccination.

## CONCLUSION

Natural exposure with implementation of biosecurity rules and improvement of management are key factors in elimination and eradication of PRRS. All above mentioned measures are necessary for a successful pig production regardless of the herd health status. Serumization and vaccination are at this moment only methods with limited success.

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# ELIMINACIJA SVINJSKOG REPRODUKTIVNOG I RESPIRATORNOG SINDROMA (PRRS) SERUMIZACIJOM, PRIRODNIM PREKUŽENJEM I VAKCINACIJOM NA ŠEST FARMI U SLOVENIJI

MARINA ŠTUKELJ, ZDRAVKO VALENČAK

**Izvod**

Svinjski reproduktivni i respiratorni sindrom (PRRS) je najskuplja komercijalna svinjska bolest koja se javlja u svijetu. Kod izbijanja bolesti imamo na razpolaganje različite načine preduzimanja u odnosu na veličinu farme i prevalencu bolesti, možemo, da ne obavljamo nikakve radnje, ili poduzimamo različite akcije poput kontrole bolesti, eliminaciju bolesti i eradikaciju bolesti. Odabrali smo 6 farmi sa problemima u reprodukciji. Na svim farmama bilo je dvojako zatvaranje uzgoja za najmanje 6 meseci. Krvni uzorci su pregledani testom IDEXX PRRS ELISA. Dokaz nukleinskih kiselina PRRS virusa s RT-PCR metodom. Na farmi 1 i 2 je tri meseca nakon serumizacije došlo do poboljšanja proizvodnih rezultata i do trenda prema stabiliziranju uzgojnog stada, 6 meseci nakon serumizacije smanjio se broj visoko pozitivnih krmača i također su se poboljšali proizvodni rezultati. Rezultati molekularnog testiranja na farmi 1 i 2 potvrdili su nakon 3 meseca odsustvo PRRS virusa u priplodnom stadu. Jedanaest meseci nakon vakcinacije na farmi 1 još jednom smo dokazali prisutnost istog podtipa virusa. Na farmi 2 u uzgojnom stadu već godinu i mesec nismo dokazali PRRS virus a virus se održava u kategoriji zalučene prasadi u dobi od 10 tedana (novi podtip virusa). Na farmi 3 i 4 proveli smo prirodno prekuženje. Dobiveni rezultati pokazuju da nakon svakog oduzimanja povećan je broj negativnih krmača. Na obje farme testirani su i tovljenici na prisutnost protutela i svi su bili bez protutela protiv PRRS virusa. Na farmi 3 i 4 nismo dokazali prisutnost virusa. Na farmi 5 i 6 izvedena je bila vakcinacija krmača i stalno se smanjivao broj visoko pozitivnih krmača i povećao broj pozitivnih a također su poboljšani proizvodni rezultati. Jedanaest meseci nakon vakcinacije ponovo je došlo do porasta visoko pozitivnih krmača i pogoršanja proizvodnih rezultata. Prirodno prekuženje pridržavanjem bio-sigurnosih zahteva i poboljšanje managementa kod unutarnje bio-sigurnosti su ključni čimbenici eliminacije i eradikacije PRRS. Serumizacija i vakcinacija su trenutačno metode s ograničenim uspehom.

**Ključne reči:** PRRS eliminacija, serumizacija, prirodno prekuženje, vakcinacija.

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# MORPHOLOGICAL ANALYSIS OF BOAR SPERMATOZOA BY AGE AND BREED*

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*SUMMARY: The total of 56 boars from 12 farm units (3 to 7 boars per farm) were used for cytological and morphological examination of semen. Large White (LW, n = 18); Swedish Landrace (SL, n = 11), Duroc (OA, n = 12); German Landrace (NL, n = 6), crossbreeds (OST, n = 9) boars were used for examination. Sperm was stained with eosin/nigrosine in one step. According to the findings of spermatozoa with protoplasmic droplets (PPD), boars were divided into groups with ≤ 10% of the PPD and the group with > 10% of the PPD. The impact of the PPD rate to number of live born piglets per litter and correlation of PPD rate and findings of live sperm with intact akcrosoma (LIA), or normal apical ridge (NAR) were investigated. Farrowin rate and abnormal sperm with tail deformities was significantly (p <0.05) lower in the boars younger than two years, compared to the boars older than two years (farrowing rate: 74.32% vs. 62.82%). Statistically significant correlations were found between the findings of protoplasmatic droplets (PPD) on the tail of spermatozoa in native semen and number of live born piglets per litter (r = 0.44, p = 0.001). The medium correlation within these parameters were found in the Large White (r = - 0.57, p <0.05, n = 18), and Duroc boars (r = 0.68, p <0.05, n = 12). Other boar breds did not have significant correlation. The finding of cytoplasmic droplets on boar sperm tail is very stable and relatively easy to establish. It should be used as a practical mathod for control the quality of sperm as a selection parameter.*

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***Key words:*** *morphology, spermatoyoa, age, breed, boar.*

## INTRODUCTION

Artificial insemination is an inseparable biotechnology in modern swine production. Quality control of native and diluted semen includes macroscopic and microscopic evaluation. Morphological analysis of sperm structure is rarely practiced. Only a few papers have been published on cytoplasmic droplets and their effect on the fertility of different species. It seems more profitable to discard abnormal ejaculate than to use the ejaculate with many transient morphological abnormalities (eg, cytoplasmic droplets). Thus, the boars with abnormal ejaculate should be excluded from the breeding until production of normal sperm begins, or until tests indicate that the abnormalities are normal species variation with no negative effect on fertility. In addition, it is difficult to set a final diagnosis of subfertility, as most animals tend not to be constantly subfertile, and there are many factors, other than male, which contribute to poorer pregnancy and small litter size (Althouse, 1998).

On the farms, collecting of boars sperm for artificial insemination, the following measures are recommended: data about health condition, implementation of clinical and laboratory practice standards, and estimation of potential breeding value of boars are necessary in centers reproduction (Jovičin et al., 2008). Creating good practice with optimal hygienic conditions and installation of air conditioners will provide better utilization of genetic resources of imported boars (Jovičin et al., 2003).

According to Rouge (2004), the results of sperm morphology are expressed as the percentage of normal sperm present in ejaculate. Anomalies may be classified as abnormalities of the head, the connecting part or tail. The primary anomalies are more difficult and it is thought that they were created when the sperm was in testicular seminal tubules. Secondary abnomalities occur during the sperm passage through the epididymal channel or within semen handling process in vitro. Miljkovic (1969) emphasizes the importance of lipoprotein sperm membrane consisting of colloidal epididymal secretion channels. It makes sperm resistful and fertile, and it is the carrier of electrical load. Immature spermatozoa forms have proximal or distal protoplazmatic droplet (PPD) which is located below the head or the tail. It is the residue of protoplasm and lipoproteins. Immature forms of spermatozoa are poorly resistant and infertile. Hancock (1957, cit. Miljković, 1969) believes that the sperm of fertile boars normally contain 5.4% sperm with proximal droplet and 3.4%. with distal droplet. Constant number of spermatozoa with a droplets was found in some boars ejaculate, with no effect on the fertility (Paredis, 1961). The semen of fertile boars rarely contains more than 10% spermatozoa with protoplasmic droplets (Rothe, 1963). The boars that produce unacceptable ejaculate should be tested once a week. If improvement in the quality of the ejaculate is not reached within 3 months, the animal should than be excluded from the breeding program.

The aim of this paper was to demonstrate the importance of morphological examination of boar semen diluted in assessing the quality of sperm used for insemination of gilts and sows.

## MATERIALS AND METHODS

This study included data for a total of 56 boars, from 12 farms in the South Backa and Srem district (Serbia). The selection was made by using a stratified representative sample of active boar population, randomly selected within the group of boars from own breeding, purchased on our farms and from the imported boars. The data from 3-7 boars per farm were processed. The first division was made according to the breed, and the animals were divided in four groups: Large White (LW, n = 18); Swedish Landrace (SL, n = 11), Duroc (OA, n = 12); German Landrace (GL, n = 6); the fifth group consisted of crossbreeds (OST, n = 9). The second division was made according to age: boras younger than 2 years (n = 24) and older than 2 years (n = 32).

The score of sexual desire (*libido sexualis*) was determined in a scale 1 to 5, based on measuring the time for mounting and collecting the sperm. The samples of native sperm and diluted semen were taken in order to evaluate the progressive motility of spermatozoa and carry out cytological and morphological analyses. The analyses were performed aseptically in sterile plastic tubes. Supravital sperm staining and making of smears for morphological analysis was done by eosin-nigrozine staining technique in one step.

For the analyses 10 μL of well-mixed native sperm or 20 μL of diluted semen was taken and mixed with one droplet (20 μL) of eosin-nigrozina solution (Björndahl et al., 2003), a modified prescription by Mortimer (1994) using adjustable automatic micro-pipette (0 0.5- 10 μL Eppendorf Research adjustable-volume pipette, Eppendorf, Germany). The suspension was incubated at room temperature (20°C) for 30 seconds on the pre-heated microscopic glass plate. A doplet of the mixture (10 μL) was transferred to microscope marked slides, with prepared smears, spread the drops with a glass rod and then air dried. The smears were examined using the microscope Olympus BX 40 with a phase-contrast microscopy, 1000 × magnification, under immersion, with a 100 × lens.

The third division was made according to the morphological feature. The findings of spermatozoa with protoplasmic droplets (PPD) was performed on a group of boars (n = 36) and the values were ≤ 10% PPD, and a group of boras (n = 24) with the values> 10% PPD.

Livestock Selection Service has collected and processed data of the sows and breeding gilts inseminated with the sperm of these boars. The impact of the PPD on live born piglets per litter was analyzed and correlation between the PPD findings and findings of live sperm with intact acrosom (SIA), i.e. normal apical ridge (NAR).

Statistical analysis were done using the programs Prism and Sigma (Pad Prism. v 5.0, Graph Pad Software Inc., San Diego, CA, USA, and SigmaStat 3.5, v. 3.5.0.54, Systat Software, Inc., San Jose, CA, USA). T-test, the variance analysis of variance and correlation analysis according to race and for all boars were carried out.

## RESULTS

The examined boars (n = 56) were 161 to 1535 days of age (0.38 to 4.21 years, with average body condition score (BCS) = 3.09 ± 0.50 (2.0 to 4.0). The score of sexual desire (*libido sexualis*) was 3.39 ± 1.23. Time required to mount the sow lasted in average 140.79±93.11 seconds, or 2.35±1.55 minutes (0.42 to 8,00). Semen collecting lasted 382.61±154.21 seconds, or 6.38±2.57 minutes (2.92 to 19.52).

The volume of native ejaculate, after filtering through a double layer gauze, was 258.84 ± 103.41 mL (60-680). The number of spermatozoa in native semen was on average 251.66 ± 125.21 × $10^6$/mL (84-510). After diluting, in ratio 1:2 to 1:13, 362 insemination doses were formed. Progressive motility of spermatozoa in native semen (active movements, AK) was 76.34% ± 18.79% (10-95%). Progressive motility of diluted semen was 71.54% ± 18.49% (1-90%).

**The division according to age:**

Including boars ≤ 2 (N = 32) and > 2 years of age (n = 24).

1. The ability to inseminate was significantly lower in the boars younger than two years, compared to boars > 2 years (74.32% - 62.82% = 11.50%, t-test, p <0.05;*).
2.The percentage of secondary abnormal sperm, with tail deformities, in the samples of native sperm was significantly higher in the first group, i.e. in the boars younger than two (1.17% -0.44% = 0.73%, t-test, p <0.05;*). Other differences were statistically unimportant.

**The division according to the findings of protoplasmatic droplets (PPD):**

Including the spermatozoa with tail droplets in diluted semen: boars with ≤ 10% of PPD (n = 36) and boars with >10% of PPD (n = 20).

1. The younger animals had the finidings ≤ 10% PPD (678.42 days - 932.20 days = 253.78 days, t-test, p <0.01;*). The average age was 1.86 year and more than 2.56; the difference was 8.46 months.
2. Number of live born piglets per litter was lower in boars with the finding ≤10%PPD (9,88–10,41=–0,53, t-test, p<0,05; *).
3. The findings of native sperm: progressive sperm motility (active movements):
   (AK-nat): 80.56–68.75=11.81%, t-test, p<0.05;
   SIA%: 54.6136.35=18.26%, t-test, p<0.001;
   PPD%: 4.38–25.60=21.21%, t-test, p<0.001; ***
   I ABN%: 5.9–-11.80=5.86%, t-test, P<0.05; *
   II ABN%: 0.38-1.40=1.01%, t-test, p<0.01; **
   PAT%: 6.33–12.85=6.52%, t-test, p<0.5; *
4. The findings of diluted sperm:
   SIA%: 48.19–27.90=20.29%, t-test, p<0.001; ***
   PPD%: 3.1924.50=–21.31%, t-test, p<0.001; ***
   I ABN%: 5.44–14.20=8.76%, t-test, P<0.01; **
   II ABN%: 0.39–1.35=0,96%, t-test, p<0.01; **
   PAT%: 5.83–15.55=9.72%, t-test, p<0.01; **
   Other differences were statistically insignificant.

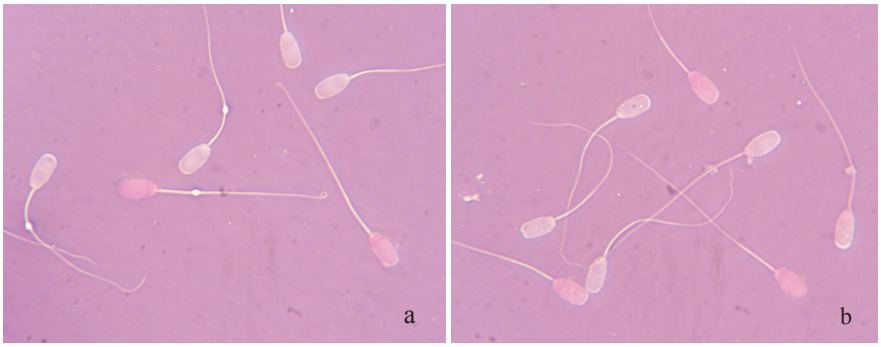
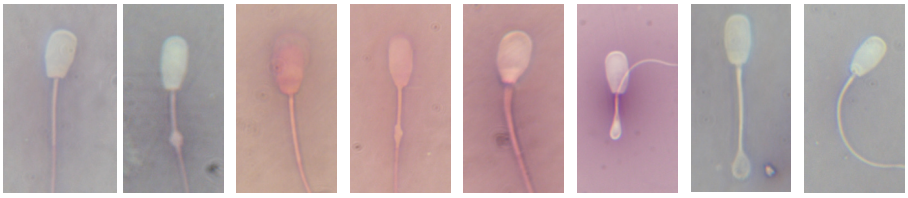



Fig. 1. Supravital colored sperm smear of boar. a) The above are the four living (not stained) below are the two-colored (eosinophilic) spermatozoa, three spermatozoas with bright protoplasmatic droplets on the tail; b) Five of live spermatozoas, a contrasting with the head, two in the middle of the picture right, have protoplasmatic drop on the tail, three are dead, with shady and unclear contours of the head. Staining with eosin / nigrosine, 400 × magnification

*Slika 1. Razmaz supravitalno obojene sperme nerasta. a) gore su četiri živa (neobojena); dole su dva obojena (eozinofilna) spermatozoida; tri spermatozoida su sa svetlim protoplazmatičnim kapljicama na repu; b) pet živih spermatozoida, sa kontrastnom glavom; dva u sredini slike desno, imaju protoplazmatičnu kapljicu na repu; tri su mrtva, sa mutnim i nejasnim konturama glave. Bojenje eozin/nigrozinom, uvećanje 400×*



a b c d e f g h

Picture 2. Supravital colored sperm smear of boar. a) live normal sperm forms with intact edge of acrosom (LIA) ; b) live sperm with protoplasmatic drop (PPD); c) dead sperm with swollen - damaged acrosom (DA); d) live sperm with damaged acrosom (LDA) and protoplasmatic drop; e) live sperm with primary abnormality of the head and a connecting part (I ABN); f) live sperm with secondary abnormality of the tail (II ABN); g) live sperm with tail curved into a loop (II ABN); h) live sperm with simple curved tail (II ABN ). Staining with eosin / nigrosine, 1000 × magnification

*Slika 2. Razmaz supravitalno obojenih spermatozoida nerasta. a) živ spermatozoid normalne forme sa intaktnim rubom akrozoma (ŽIA); b) živ spermatozoid sa protoplazmatičnim kapljicom (PPK); c) mrtav spermatozoid sa nabubrelim – oštećenim akrozomom (OA); d) živ spermatozoid sa oštećenim akrozomom (ŽOA) i protoplazmatičnom kapljicom; e) živ spermatozoid sa primarnom abnormalnošću glave i spojnog dela (I ABN); f) živ spermatozoid sa sekundarnom abnormalnošću repa (II ABN); g) živ spermatozoid sa repom savijenim u petlju (II ABN); h) živ spermatozoid sa jednostavno savijenim repom (II ABN). Bojenje eozin/nigrozinom, uvećanje 1000×.*

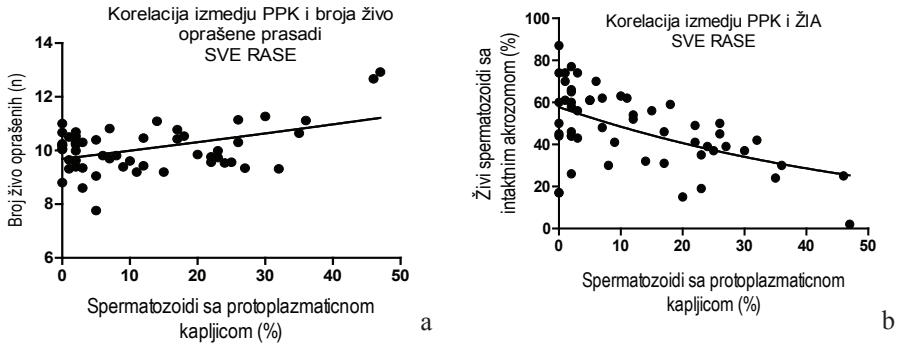



Fig. 1. a) influence of findings protoplasmatic droplets on boar sperm tail to the number of live born piglets per litter (p<0,05). b) mutual influence of the total findings of protoplasmatic drops on tail and intactness akrozoma in living boar sperm (n=56, p<0,0001)

*Grafikon 1. a) uticaj nalaza protoplazmatičnih kapljica na repu spermatozoida nerastova (n=56) na broj živo oprašene prasadi u leglu (p<0,05). b) međusobni uticaj ukupnog nalaza protoplazmatičnih kapljica na repu i intaktnosti akrozoma kod živih spermatozoida nerastova (n=56, p<0,0001)*

**The division according to boars breed:**

The variance analysis showed statistically no significant differences in the examined fertility parameters in the morphology of spermatozoa, neither in native nor in diluted sperm.

Statistically significant correlations were detected between the findings of protoplasmatic droplets on spermatozoa tail in native sperm and the number of newborn piglets. In the Large White breed the correlation was moderate ($r=-0.57$; $p<0.05$; $n=18$), in Duroc breed the correlation was moderate ($r=0.68$; $p<0.05$; $n=12$), and in other breed no significant correlation was detected. In all 56 boars moderate correlation was detected ($r=0.44$; $p=0.001$; $n=56$).

Highly significant positive correlations were found between native protoplasmatic droplets in diluted sperm: the Large White breed =0.73; $p<0.001$; $n=18$; in Swedish Landrace $r=0.95$; $p<0.0001$; $n=11$; in Duroc $r=0.82$; $p<0.01$; $n=12$; in German Landrace $r=0.96$; $p<0.01$; $n=6$; in crossbreeds $r=0.95$; $p<0.001$; $n=9$. In all 56 boars the correlation was $r=0.85$; $p<0.001$; $n=56$.

## DISCUSSION

The volume of native sperm without gel fraction was 258.84 ± 103.41 mL, while the spermatozoa count was 251.66 ± 125.21 × $10^6$/mL. The volume of ejaculate and the spermatozoa count determined in our experiment were suitable in the production of boars in conditions of intensive exploitation (Hafez and Hafez, 2000). Examining the effect of the breed on the sperm characteristics, some authors have found that the volume of ejaculate in Large Yorkshire was on average 238.0 ± 110.2 mL, which is in agreement with our findings (Gerfen et al., 1994).

Progressive spermatozoa motility in native semen was 76.34% ± 18.79%. In diluted sperm progressive motility was 71.54 ± 18.49%. Gerfen et al. (1994) had similar

results. They detected progressive mobility of 74.3 ± 1.8% in Large White breed.

The ability to inseminate was statistically significant in the boars up to at the age of two, i.e. comparing to the boars >2 years ($p<0.05$). Younger boars were with the findings ≤10% PPD ($p<0.01$). The number of live born piglets per litter was smaller in the boars witht the findings ≤10% PPD ($p<0.05$). The percent of secondary abnormal spermatozoide, with tail defomities, in the samples of native sperm were significanlty higher in the first group, i.e. in the boars younger than two ($p<0.05$).

Sonderman et al. (2008) compared the effects of breed and genetic lines of boars on sperm production and fertility. They noted that the ejaculates of genetically pure boars produce ejaculate with less spermatosoide (8 to 15 doses per ejaculate, ie. in a week) compared to the crossbreeds of same age. The aging increases semen volume of pure breed boars, but does not increase the number of sperm.

Comparing the progressive motility of shortly preserved semen (Sonderman et al., 2008) 5% better progressive sperm motility was found in Landrace breed after storage of 1 day compared to the crossbreed and other breeds.

Statistically significant correlations were found between the findings of protoplasmatic droplets on the tail of spermatozoa in native sperm and the number of live born piglets per litter: in Large White breed the mean correlation was ($r=-0.57$; $p<0.05$; $n=18$), in Duroc the average corellation was ($r=0.68$; $p<0.05$; $n=12$), and in other breeds no significant correlation was noted. In all 56 boars mean correlation was ($r=0.44$; $p=0.001$; $n=56$).

Highly significant positive correlations were found between the findings in native protoplazmatskih droplets in diluted semen: the Large White breed $r=0.73$; $p<0.001$; $n=18$; in Swedish Landrace $r=0.95$; $p<0.0001$; $n=11$; in Duroc $r=0.82$; $p<0.01$; $n=12$; in German Landrace $r=0.96$; $p<0.01$; $n=6$; in crossbreeds $r=0.95$; $p<0.001$; $n=9$. In all 56 boars the correlation was $r=0.85$; $p<0.001$; $n=56$.

Shortly after the first sperm is collected, terminal boar lines (hybrids and purebreds) usually produce usable ejaculate. They are usually produce weak sperm - 8 and 15 doses (motile sperm per dose - $2.5 \times 10^9$) are produced, but they meet the minimum criteria regarding the concentration and quantity. The maternal line boars seem to lag for about 2-8 weeks, before they begin to meet the minimum criteria of concentration and sperm count. The factors regarding the differences between the breeds should be taken into accounts in the centers in their decision-making processes, in order to ensure adequate quality of boars, and to meet the satisfaction of customers (Sonderman et al., 2008).

A disparity in the ability to inseminate was noted and it ranged from 29.05% to 91.94%. The differences were also apparent in the average number of farrowed and weaned pigs.

The sperm of "stable boars", containing up to 10% of abnormal sperm (AS) should be used, especially for the elite purebred herds. The boars with high AS level should be timely excluded, as recommended by Cevoski (Cerovski et al., 2005). Prolonged maintainance of initial AS content probably is an evidence of hereditary phenomenon. Co-existence of different sperm subpopulations within the mammalian ejaculate is nowadays widely accepted by the scientists (Peña et al., 2005).

Sperm with morphological abnormalities are an indication of a disorder in spermatogenesis and maturation,but they may also occur during the handling. When a larger number of spermatozoa in the ejaculate show abnormal morphology, sperm vitality,

which is considered normal, comes into question, because both normal and abnormal spermatozoids, present in the given ejaculate, undergo spermatogenesis, maturation, and are handled at the same time. The evaluation of sperm morphology, therefore, should be primarily used as a parameter in the control of ejaculate quality. Having this in mind, the author recommends using only that ejaculate that has <15% proximal and distal cytoplasmic droplets. The sperm from boars that produce unacceptable ejaculate should be taken once a week. If there is no improvement in the quality of the ejaculate within 3 months, the animal should be excluded from the breeding program (Althouse, 1998).

Waberski et al. (1994) recommend that the content of spermatozoa with protoplasmic drop should not exceed 15%, especially when for insemination diluted and preserved semen is used, which is a common practice nowadays. In the Czech Republic the applicability of boar semen insemination is limited to findingof AS to 25%.

Bonet et al. (1991) found out that the frequency of ejaculation affects sperm quality and fertility of boars. Sperm motility decreased 76.3% in the first group of boars, and 35.2% in the boars from the second II ($p<0.01$).

1) The percentage of spermatozoa with proximal droplet increased from 3.5% in the first group of boars to 67.4% for boars in the second group ($p <0.01$).
2) The percentage of sperm with distal droplets decreased from 25.3% in boars in the first group to 1.2% in boars second group ($p <0.01$).
3) The percentage of mature spermatozoa decreased from 68.9% in the first group, to 25.1% for the boars from the second group ($p <0.01$).
4) The percentage of abnormal sperm increased from 1.6%, in the first group of boars, to 6.7% in the second group of boars ($p <0.01$). The number of spermatosoa with twisted or folded tail is much biggee in the ejaculate of the boars in the second group than in the first group. Fertility is reduced from 73.5% in the boars from the first group to 7.7% in the boars from the second group ($p <0.01$).

Eliasson (2010) points out that for the researchers in human medicine, involved in assisted reproductive technology (ART), it is important that the methods are easy to learn and inexpensive to perform. It is stated that spermatozoa morphology may be more sensitive instrument for measuring reproductive health and stress of testis (Menkveld et al., 2011). Measurement and evaluation of spermatozoa morphology remains very important tool in diagnosing of male potential fertility and in making decision for treating the boars with infertility problems, especially if it is known that reduction of dose volume and sperm number per dose, lead to a decrease in sows fertility, both after intracervical, and after intrauterine insemination (Stančić et al., 2010). Genetically determined morphological abnormalities in sperm are caused by stress (physiological, mental, and caused by the conditions in the environment), which are reversible, and it is possibility to stop that stress.

Johnson, et al. (1981) carried out a field survey on 36 farms in the Netherlands to compare the fertilizing capacity of fresh and frozen and thawed boar sperm. Four hundred and fifty-one sows were artificially inseminated with sperm that was frozen and thawed according to the Beltsvils method or diluted in Kiev extender and inseminated on the day of collection. Twelve boars were Dutch Landrace and Dutch Large White. Farrowing rate, total number of piglets per litter and number of live born pigs per litter was higher ($P<0.0001$) for the sows inseminated with fresh sperm, than in the sows inseminated with frozen and thawed sperm (79.1%, 10.6 and 9.9 vs 47.0%, 7.4 and 7.1, respectively)

Rill (1989) states that a combination of these factors, and improvements in application techniques of doses, can be achieved by significant increase in the number of doses produced per ejaculate. Currently 2 to 3 × $10^9$ spz / dose is applied for insemination of sows, but the numer of spermatozoa population must be drastically reduced in the near future.

## CONCLUSION

Based on the results in the present study, it can be concluded:

1) The abbility to inseminate, and the findings of secondary abnormal spermatozoa, was considerably lower in the the boars in the first two years, comparing to boars > 2 years (74.32% - 62.82% = 11.50%, t-test, $p < 0.05$; *)
2) Statistically significant correlations were found between the findings protoplazmatic droplets (PPKD) in the tail of spermatozoa in native semen and the number of piglets born alive per litter, ($r = 0.44$, $p = 0.001$, $n = 56$). In Large White breed there was a medium correlation r=–0,57; p<0,05; n=18), in Duroc it was medium (r=0,68; p<0,05; n=12), but in other breeds there were sno significant corelations.
3) Highly significant positive correlations were found between the findings of protoplazmatic droplets in native and diluted semen ($r = 0.85$, p<0,001; n=56). In Large White r=0,73; p<0,001; n=18; in Swedish Landrace r=0,95; p<0,0001; n=11; in Duroc r=0,82; p<0,01; n=12; in German Landrace r=0,96; p<0,01; n=6; in crossbreed r=0,95; p<0,001; n=9. This was the correlation in all 56 boars.
4) The finding of cytoplasmic droplets on sperm tail was very stable and relatively easy to detect. Control of sperm quality has to be implemented as a tool in selection.

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# MORFOLOŠKA ANALIZA SPERMATOZOIDA NERASTOVA PO UZRASTU I RASAMA

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**Izvod**

Izvršen je citološko-morfološki pregled sperme 56 nerastova, sa 12 imanja, po 3-7 iz svakog gazdinstva. U čistoj rasi su: Veliki jorkšir (VJ, n=18); Švedski landras (ŠL, n=11); Durok (OA, n=12); Nemački landras (NL, n=6); petu grupu su činili melezi (OST, n=9). Sperma je obojena eozin-nigrozinom u jednom koraku. Prema nalazu spermatozoida sa protoplazmatskim kapljicama (PPK), nerastovi su podeljeni na grupu sa ≤ 10% PPK i grupu sa > 10% PPK. Analiziran je uticaj nalaza PPK na broj živo oprašenih prasadi u leglu i međusobna povezanost nalaza PPK i nalaza živih spermatozoida sa intaktnim akrozomom (ŽIA), odnosno normalnim akrozomalnim rubom (NAR). Oprasivost je bila statistički značajno manja kao i nalaz sekundarno abnormalnih spermatozoida, sa deformitetima repa, kod nerastova u uzrastu do dve godine (≤ 2), u odnosu na nerastove sa( >2) godine (74,32% - 62,82%=11,50%, t-test, $p<0,05$). Statistički značajne korelacije su utvrđene između nalaza protoplazmatskih kapljica (PPK) na repu spermatozoida u nativnoj spermi i broja živorođene prasadi u leglu, ($r=0,44$; $p=0,001$; $n=56$). Kod rase Veliki jorkšir je srednja korelacija ($r=-0,57$; $p<0,05$; $n=18$), kod rase Durok srednja korelacija ($r=0,68$; $p<0,05$; $n=12$), a kod ostalih rasa nije bilo signifikantnih korelacija. Nalaz citoplazmatskih kapljica na repu spermatozoida nerasta je vrlo postojan i relativno lako se ustanovljava. Treba da se uvede u praksu kao parameter u kontroli kvaliteta sperme i kao selekcijski parametar.

**Ključne reči:** morfologija, spermatozoid, rasa, uzrast, nerast.

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# BLOOD SELENIUM CONCENTRATION, SOMATIC CELL COUNT AND THEIR CORRELATION AT FIRST AND SIXTH MONTH OF LACTATION IN DAIRY COWS

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*SUMMERY: For the proper functioning of the mammary gland before the sanitation requires is a quality diet based on the presence of macro and micro nutrients. One of the essential and important micro-nutrient is selenium, which became part of the enzyme glutathione peroxidase, and has antioxidant effects. The research was conducted on thirty dairy cows Holstein breed, who received 0,3 mg/kg selenium supplementation in food daily. Samples were colected two times: at first and at sixth lactating months. The mean estimate of selenium blood concentration at first lactating month was 0.536µmol/l and at sixth lactating month was 0.601µmol/l. Average somatic cell count at first lactating month was 450.000/ml of milk and at sixth lactating month was 355.000/ml of milk. According to the analysis of the correlation test, negative correlation within blood selenium concentration and milk somatic cell count has been found . The increased levels of selenium in blood caused a decline in the number of milk somatic cell count. On the basis of these results it could be concluded that selenium is of great importance in the preservation and proper functioning of the mammary glands of cows.*

***Key wards:*** *selenium, somatic cell count, mammary gland, cow.*

## INTRODUCTION

Selenium is an integral component of the enzyme, glutathione peroxidase (Cortinhas et al., 2010; Joksimović-Todorović et al., 2007). This enzyme is also an important part of the cellular antioxidant system, but glutathione peroxidase is water soluble and is found in the cytosol of cell, not in cellular membranes. Selenium as a micronutrient

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Original scientific paper / *Originalni naučni rad*
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is involved in the cellular antioxidant system (Engle, 2001; Spears and Weiss, 2008).

Cell processes, environmental insults and inflammatory responses produce compounds called free radicals. The major free radicals found in biological systems are superoxide, hydrogen peroxide, hydroxyl radical and fatty acid radicals. Free radicals are highly reactive compounds because they are missing an electron. Free radicals can react with nucleic acids causing mutations, with enzymes and render them inactive, and with fatty acids in membranes causing membrane instability. Free radicals can eventually kill cells and damage tissues (Knaapen et al., 1999; Mukherjee, 2008).

For the healthy dairy cow, about one-third of the selenium in whole blood is in serum and two-third is in the red cells. Selenium is incorporated into red cells only when the cell is made (Andrieu, 2008; Engle, 2001). Therefore, selenium content of the red cell reflects selenium intake 1 to 3 months previously. The selenium in serum mainly represents a transport pool and reflects the current status. Plasma or serum selenium will increase shortly after selenium is injected but the selenium content of red cells will not change for several weeks. Whole blood reflects longer term status but is somewhat sensitive to recent changes in selenium nutrition. Recommended level of selenium in blood of dairy cows is 0,6 to 0,9 μmol /l (Erdeljan et al., 2011; Gunter et al., 2003; Juniper et al., 2006).

The dairy cows raised in the soil where concentration of selenium is very low, should be fed with supplemental selenium (Arvidson et al., 2005; Joksimović-Todorović et al., 2007). Potential benefits include reduced clinical mastitis and reduced milk somatic cells (Barbano et al., 2006; Davidov et al., 2011b; Weiss, 2002). Diets for cows should be supplemented with 0,3 ppm of selenium (NRC, 2001). In most situations, feeding 0,3 ppm provides adequate selenium, but occasionally that amount is not adequate. Certain conditions reduce the availability of selenium or increase its requirement.

The dietary selenium requirement is important for livestock health, and has been associated with a reduction in somatic cell count and the incidence of mastitis (Weiss et al., 1990; Weiss, 2002). Selenium supplementation of livestock diets may also enhance the nutritional quality of livestock products. Selenium supplements are in two principal forms, inorganic mineral salts and organic forms such as Se-yeast (Juniper et al., 2006).

Under normal dietary conditions, the majority of endogenous selenium is present in body tissues and fluids (Suzuki and Ogra, 2002). Selenium absorption occurs in the small intestine (Weiss, 2003) and after that, selenium transport to the blood and whole body, including udder.

Concentrations of selenium in serum and whole blood have been used as an index of selenium status because increased concentrations of selenium in serum or whole blood have been related to reduced milk somatic cell count, reduced mastitis and improved neutrophil function (Smith et al., 1984; Erskine et al., 1987; Cebra et al., 2003; Weiss and Hogan, 2005). The positive effect of selenium supplementation on clinical mastitis is probably mediated via effects of selenium on neutrophils and other immune cells.

Davidov et al. (2011) were conducted in two groups of 15 cows, where group I was a control group, and group II received 50 mg/day of selenium. According to the blood test and blood serum analysis, authors noticed that in the group I, selenium levels were below the physiological limits, while in group II the level of selenium was within the margin of physiological values. Also, milk somatic cell count in groups I and II shows

that the majority of cows in the group I had a somatic cell count between 310.000 and 500.000/ ml and in the group II between 210.000 and 300.000/ ml. According to the analysis of the correlation test, a negative correlation was found. The increasing levels of selenium in blood serum cause a decline in the number of milk somatic cells. It has been concluded that selenium is of great importance in the preservation and proper functioning of the mammary glands of cows.

Aim of this investigation is to determine affect of selenium on mammary gland health and production in dairy cows.

## MATERIALS AND METHODS

The study was performed on thirty Holstein cows approximate same body weight, ages 3 to 5 years and in first to third lactation, and they giving approximately the same amount of milk. All cows were stabling with dry straw for bedding and with ad libitum access to potable water, and feed by total mixed ration. The total mixed ration contained maize silage, grass silage, cracked wheat, soyabean meal, rapeseed meal, sugar beet and hay. Before conception and trough all lactating months all cows received 0,3 mg/kg selenium supplementation in food.

Sampling

Blood samples were taken two times: at first and at sixth lactating months. The same sampling procedure was used each time. After the morning milking samples were taken from the caudal vein by applying the principles of asepsis and antisepsis. The blood in tubes was left at room temperature for 24 hours to separate the serum. The level of selenium in blood serum was determined by mineralizing 1g of sample in 4 ml of 16 M $HNO_3$ and 2 ml of 9.8 M $H_2O_2$ within a closed-vessel heating block system. The solution was further diluted with water and selenium was subsequently determined using inductively coupled plasma mass spectrometry (Perkin Elmer Elan 6100 ICPMS, Massachusetts, USA). Milk from all four quarters was taken before morning milking and whole milk samples were taken with milk meter for somatic cell count. When quarter milk samples were taken the teat ends were disinfected. Milk samples for somatic cell count were analyzed by the fluoro-optoelectronic method (Fossomatic; Foss Electric, Hillerod, Denmark).

For statistical analysis we used test of correlation by Microsoft Excel 2007.

## RESULTS AND DISCUSSION

The selenium content of grass and its conserved products and of home-grown feeds may be governed by geological/geographical situations and may be inadequate throughout the whole year.

Selenium blood concentration was measured on 60 samples as well as somatic cell count in milk. The results on selenium blood concentration at first lactating month are in figure 1 and at the sixth lactating month are in figure 2.

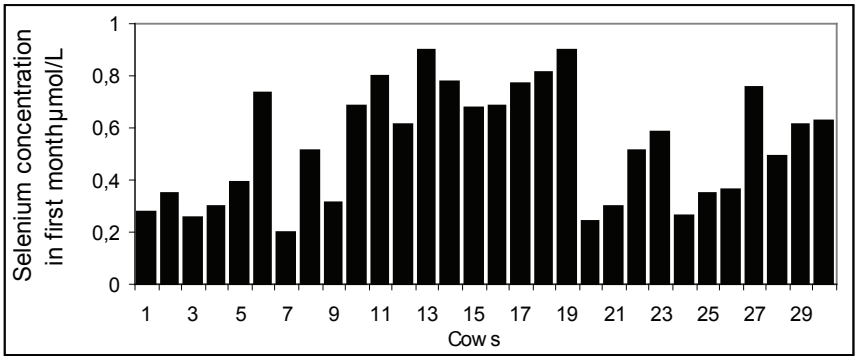


Fig.1. Selenium blood serum concentration at first lactating month of dairy cows
*Graf.1. Vrednosti koncentracije selena u krvnom serumu krava u prvom mesecu laktacije*

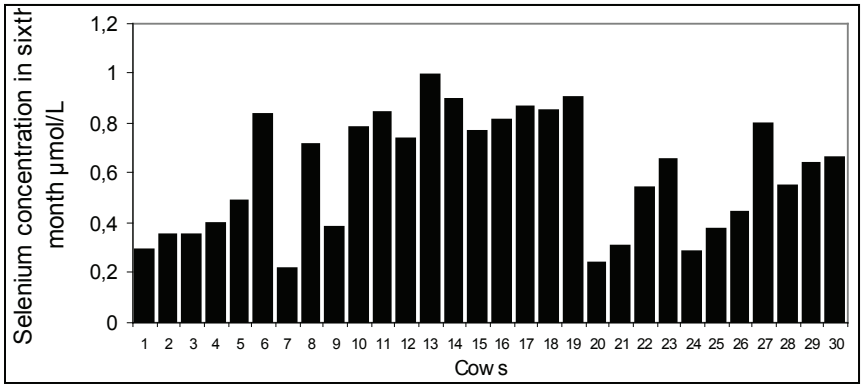


Fig. 2. Selenium blood serum concentration at sixth lactating month of dairy cows
*Graf. 2. Vrednosti koncentracije selena u krvnom serumu krava u šestom mesecu laktacije*

The mean estimate of selenium blood concentration at first lactating month was 0.536μmol/l and at sixth lactating month was 0.601μmol/l. Mean selenium concentrations were found to be lower within first lactating month, and then increased in the sixth lactating month. Results indicates that cows had a level of selenium in blood below the physiological limits, need to be fed with supplement of selenium. These results are similar to authors Arvidson et al. (2005) and Joksimović-Todorović et al. (2007).

Erdeljan et al. (2011), Gunter et al. (2003) and Juniper et al. (2006) reported that the recommended level of selenium in blood serum of dairy cows is 0,6 to 0,9 μmol /l. In this examination, cows at first lactating month had an average value of selenium in the blood 0.536μmol/l, and at sixth lactating month was 0.601μmol/l.

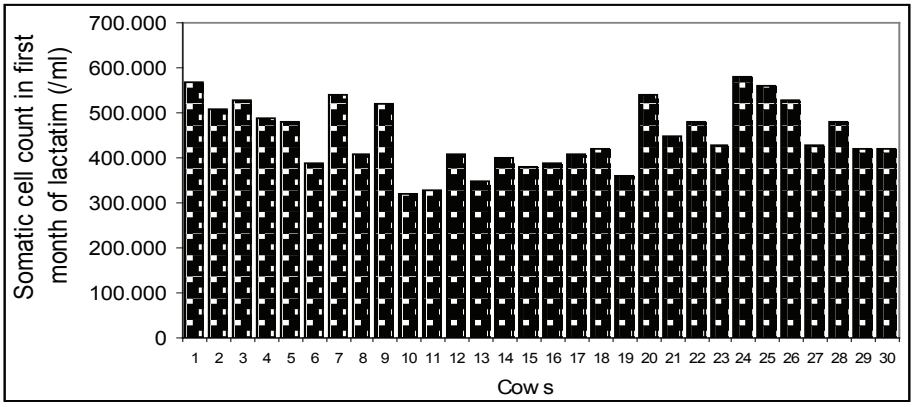


Fig. 3. Milk somatic cell count at first lactating month
*Graf. 3. Broj somatskih ćelija u mleku u prvom mesecu laktacije*

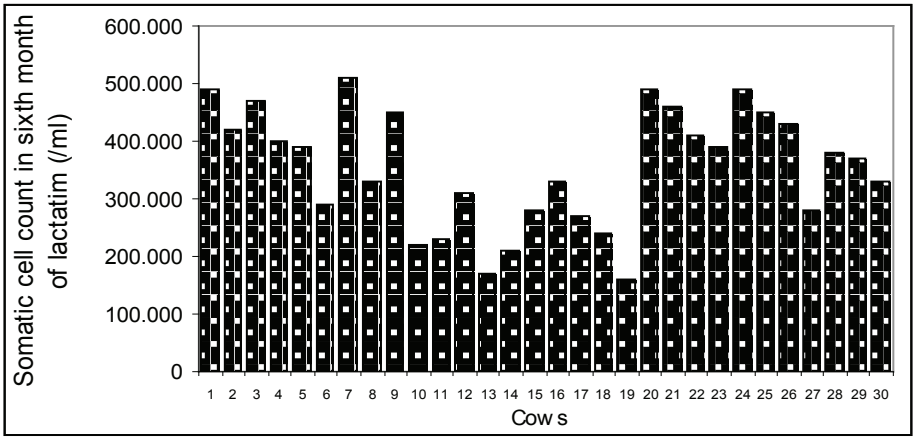


Fig. 4. Milk somatic cell count at sixth lactating month
*Graf. 4. Broj somatskih ćelija u mleku u šestom mesecu laktacije*

In figure 3 is value of milk somatic cell count at first lactating month, and in figure 4 is value of milk somatic cell count at sixth lactating month. Within first lactating month the somatic cell count of 43.33% (13/30) of the cows was over 450.000/ml. In the sixth month of lactating, 26.67% (8/30) of the cows had a somatic cell count over 450.000/ml. Average somatic cell count at first lactating month was 450.000/ml of milk and at sixth lactating month was 355.000/ml of milk.

A reduction in somatic cell count and the low incidence of mastitis are present with blood selenium concentration with estimate value 0.601µmol/l. This results are matched with group of authors Weiss et al. (1990), Weiss, (2002), Juniper et al. (2006), Phipps et al. (2008), Davidov et al. (2011), who claim that selenium has a important influence in reducing somatic cell count and incidence of mastitis.

Davidov et al. (2011b), Barbano et al. (2006) and Weiss (2002) reported reducing subclinical mastitis and milk somatic cell count in cows who received in food supple-

ment of selenium. That fact was noticed in this examination also, because the group of cow who had an average value of selenium in the blood 0.601µmol/l have milk somatic cell count between 150.000-240.000/ ml.

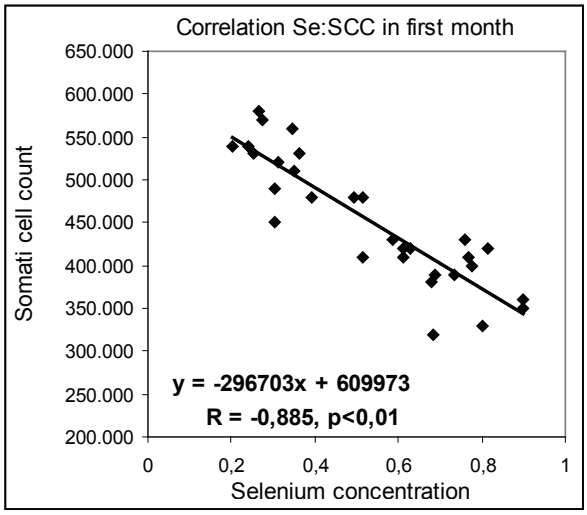


Fig. 5. Correlation test between selenium blood concentration and milk somatic cell count at first lactating month

*Graf. 5. Test korelacije između koncentracije selena u krvnom serumu i broja somatskih ćelija u mleku u prvom mesecu laktacije*

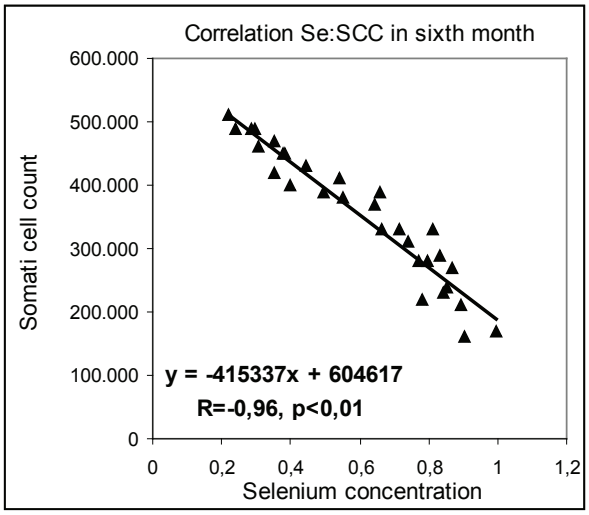


Fig. 6. Correlation between selenium blood concentration and milk somatic cell count at sixth lactating month

*Graf 6. Test korelacije između koncentracije selena u krvnom serumu i broja somatskih ćelija u mleku u šestom mesecu laktacije*

According to the analysis of the correlation test, a negative correlation within blood selenium concentration and milk somatic cell count in first and in sixth lactating month was found. The increased levels of selenium in blood caused a decline in the number of milk somatic cell count.

## CONCLUSION

There is strong relationship between blood serum concentration of selenium and immune function. There are many interrelations of the nutrients and effects of supplementing selenium in daily food of cows. The cow`s uptake and requirement of selenium vary due to the lactating months and health status. On the basis of the results it can be concluded that selenium have affect in proper functioning of the mammary glands of cows, and in reducing somatic cell count and in improving milk quality.

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# ODNOS KONCENTRACIJE SELENA U KRVI I BROJA SOMATSKIH ĆELIJA U MLEKU KRAVA U PRVOM I ŠESTOM MESECU LAKTACIJE

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**Izvod**

Za pravilno funkcionisanje mlečne žlezde pored pravilne primene zoohigijenskih mera, neophodna je i pravilna ishrana makro i mikroelementima. Jedan od esencijalnih mikroelemenata je selen, koji ulazi u sastav enzima glutation peroksidaze i ima antioksidativni efekat. U istaživanju je kao dodatak u hrani dodavan selen u količini od 0,3 mg/kg dnevno. Uzorkovanje krvi i mleka je sprovedeno u prvom i šestom mesecu laktacionog perioda. Srednja vrednost koncnentracije selena u krvi u prvom mesecu laktacije je bila 0.536 µmol/l, dok je u šestom iznosila 0.601 µmol/l. Prosečan broj somatskih ćelija u mleku u prvom mesecu laktacije je bio 450.000/ml, a u šestom mesecu je iznosio 355.000/ml. Na osnovu testa korelacije, uočena je negativna korelacija između koncentracije selena u krvi i broja somatskih ćelija u mleku. Ova negativna korelacija ukazuje da sa povećanjem koncentracije selena u krvi dolazi do pada broja somatskih ćelija u mleku. Dobijeni rezultati nam ukazuju na veliki značaj selena u funkcionisanju mlečne žlezde krava.

**Ključne reči:** selen, somatske ćelije, mlečna žlezda, krava.

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# HEALTH AND PRODUCTIVE CHARACTERISTICS OF COWS ON FARMS WITH DIFFERENT WELFARE SCORE*

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*SUMMARY: Measuring the welfare of cows on farms was carried out according to the protocol of Welfare Quality ® scoring system. On farms with lower level of welfare as well as the poorer implementation of principles of good nutrition, significantly lower milk production was found, as well as a greater percentage of cows with a long service period, lameness and a higher percentage of very thin cows. Providing good accommodation principles will significantly improve milk production, reduce the number of cows with a long service period as well as the ones with lameness, and it is particularly interesting that obliging this principle will provide less subclinical mastitis on a farm and less cows with dystocia and skin lesions. The most important principle, which has demonstrated a significant effect on the values of all parameters, is the principle of good health. Providing the implementation of this principle on farms will result in significant improvement of all parameters of production and health. In addition to medical syndromes such as lameness or dystocia, the score of good health depends on the occurrence of ocular, nasal and vaginal discharge, which supports the infection of the corresponding organs. Ultimately, changes in the behavior of the cows do not show significant effects on the health and productive characteristics. Poor interaction with people and other animals, poor response to food or fear and aggressive behavior can be correlated with the occurrence of higher rate of lameness and increased number of skin lesions on farms. The link between the tested criteria of welfare and health and productive characteristics of cows indicate that there is the strongest correlation with the criterion of good health on farms, then with the criterion of good nutrition and at the end with the criterion of good housing. Productive characteristics such as milk production and length*

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*of service period significantly correlate with the factors of health, food and accommodation. Ensuring good health of farm animals is the most important task in the process of securing the welfare and sustainable production on cow farms.*

***Key words:*** *cows, welfare, productivity, health.*

## INTRODUCTION

Animal welfare is a degree of its adaptation to environmental conditions. The environment should have such characteristics that provide quality of life in terms of nutrition and supply, accommodation, physical, mental and thermal comfort, safety, expressing basic forms of behavior, social contact with animals of the same species, absence of unpleasant emotional and physical experiences such as pain, suffering, fear, stress, boredom, illness, injury, etc. (Vučinić, 2006). Testing welfare was, at first, based on an analysis of living conditions which should correspond to the type, race, gender, age category and other characteristics of animals. Modern research in the area of animal welfare includes an analysis of parameters obtained from the animals themselves on farms (Whayet al., 2003b). Thus, the benefit may be classified as: functional (clinical examination and determination of the medical status of animals), behavioral (testing the presence of physiological forms of behavior and means of satisfying the innate basic needs) and emotional (testing the presence of positive emotions and the absence of negative emotions in animals) (Hristov et al ., 2007). To provide all criteria of animal welfare as well as to eliminate stressors on farms means to enable the sustainable production and stability (Belić and Cincović, 2010).

The aim of this study was to investigate the effect of the levels of animal welfare on milk production, reproductive efficiency and the emergence of major medical disorders of cows on farms.

## MATERIAL AND METHODS

Measuring the welfare on cow farms was conducted according to the protocol of Welfare Quality ® scoring system. This system provides the assessment of the welfare of cows on the basis of four principles alongside the associated criteria, the principle of good housing, good nutrition principle, the principle of good health and the principle of good behavior of cows. Evaluation of farms was performed based on four principles of welfare. We used statistical software that has been formed based on standards set out in the Welfare Quality® Assessment Protocol for Cattle, 2009, ISBN/EAN978-90-78240-04-4. According to the scores of welfare, farms were divided into three groups: a group of cows with low scores (score0-30), a group of cows with medium scores (30.1-70) and a group of cows with good scores (70.1-100). Score was calculated for each of the four principles. 19 dairy farm on the territory of AP Vojvodina participated in this experiment. The amount of milk produced per cow, the percentage of cows with a service period of over a hundred days, % of cows with laminitis, mastitis, %cows with vaginal, ocular or nasal discharge, % of cows with dystocia, %of cows with skin lesions and percentage of very thin cows was examined on each farm. ANOVA analysis was used

for studying the influence of welfare scores on the value of examined characteristics of cows. The correlation coefficient was calculated among the scores of the welfare and the studied parameters along with the testing of significance of the correlation coefficient.

## RESULTS AND DISCUSSION

Results of the research presented in Tables 1 to 4 indicate that providing factors that encourage the higher score of welfare could significantly influence on the improvement of health and productive performance of dairy cows. Thus, on farms with lower score of animal welfare and poorer implementation of good nutrition principle, there is a significantly lower milk production, a greater percentage of cows with a long service period, a greater percentage of cows with lameness and a higher percentage of lean cows. Providing good housing principle will significantly improve milk production, reduce the number of cows with a long service period and lameness, less subclinical mastitis in cows will be recorded as well as fewer animals with dystocia and skin lesions. The most important principle, which has demonstrated a significant effect on the values of all studied parameters, is the principle of good health. Providing a high score of this principle on farms will result in significant improvement of all parameters of production and health. In addition to medical syndromes such as lameness or dystocia, the score of good health depends on the occurrence of ocular, nasal and vaginal discharge, which supports the infection of related organs. Ultimately, changes in the behavior of the cows do not show significant effects on the health and productivity-boosting features. Poor interaction with people and other animals, poor response to food or fear and aggressive behavior can be correlated with the emergence of greater share of lameness and increased number of skin lesions on farms.

The connection between the examined welfare criteria and health and productive characteristics of cows (Table 5) shows that there is the strongest correlation with the criterion of good health on the farm, then the criterion of good nutrition and at the end with the criterion of good housing. Productive characteristics such as milk production and length of service period significantly correlate with the factors of health, food and accommodation.

The frequency of cows with various health and other problems are consistent with previous results by Ostojić-Andrićet al. (2011). Milk production and reproductive efficiency are polygenic characteristics (Vidović, 1993). This means that more minor genes influence the expression of these characteristics. In order for these genes to fully express their effects, appropriate environmental conditions and care are required. Therefore, providing the principles of good nutrition, good housing and health is important for maintaining these features. Another important issue on farms is the unevenness of body weight as well as the percentage of very lean cows. Thinness in cows is the result of negative energy balance and expressed catabolism. Physiologically, catabolic axis in cows dominates in the period around calving, when a large number of metabolic changes occur (Cincović et al., 2011). Prolonged negative energy balance, with a loss of body depots, the increased accumulation of ketones in cows adversely affects milk production and reproduction (Cincović et al., 2011).The significant presence of lean cows shows a negative impact on the welfare and health of cattle (Popescu et al., 2010). In addition to the thinness and metabolic disorders, the next major problem, which can be influenced on by providing the principles of welfare, is lameness in cows. The level of lameness in

cows depends on the disease that was diagnosed on the hoofs, so there is the maximum lameness in case of the hoof ulcer, interdigital dermatitis and arthritis, which is consistent with our previously obtained results (Stevančević et al., 2009). Lameness in cows is the result of poor environmental factors, poor hoof care and poor conditions of nutrition (Stevančević et al., 2011). Reproductive disorders of cows with dystocia and vaginal discharge occur as a consequence of different diseases and the most common are placental retention and endometritis (60%) (Stančić et al., 2011). Mastitis occurs as a consequence of environmental factors, local immunity of the mammary gland and altered health status of cows (Blowey and Edmondson, 2010). Ocular and nasal discharges in cows occur as a consequence of poor microclimatic conditions, and as a consequence of serious infections in the body. Respiratory illness with nasal and eye discharge proved to be significant, especially in younger categories, and the isolated causes were BHV-1, Pi-3, BRSV and BVDV, and the bacteria Pasteurella multocida, Mannhemia haemolytica, Arcanobacterium pyogenes, Haemophilus Somnus and Mycoplasma hyopneuminiae. Younger categories of cows as well as calves experienced signs of enterotoxaemia from Clostridium sp. (Bojkovski et al., 2011).Skin lesions occur as a result of inadequate accommodation (negative coefficient of correlation). So there is a higher incidence of lesions in cowskept intied up housing (Ostojić-Andrićetal., 2011), but the standard deviation is very high. Appropriate behavior was analized by detection and percent calculation of cows in function of: tendency to be connect, tendency to be indifferent, tendency to be friendly, tendency to be irritable, tendency to be uneasy, tendency to be sociable, tendency to be distressed and so on. There were rather inconsistent results, which varied significantly depending on whether the cows were kept tied up or in the free system. Cows in the free housing system express better behavioral characteristics and strategies. These results are consistent with the results of Hristov et al. (2010) who found that the behavioral welfare is evaluated the best in cows bred in the free system with outlet and the worst in tied up cows in a facility without outlets. The same author (Hristovet al., 2011) based on the same methodology that we applied in this study, concluded that there are many forms of unsatisfactory behavior of cows on farms.

Ensuring a high level of welfare on farms is an important goal of farmers, technologists and veterinarians in particular (because everything depends on the health). The significance is even greater because when choosing products of animal origin, consumers pay much more attention to the welfare on farms (Prickett et al., 2010).

Table 1: Influence of principle of good feeding to health and productive characteristic of cows

| Interval of welfare quality score | 0-30 | 30.1-70 | 70.1-100 | p | LSD |
|---|---|---|---|---|---|
| Milk production /cow/day (L) | 22.25±2.54 | 25.55±2.81 | 29.95±3.1 | 0.01 | 1:2, 1:3, 2:3 |
| % cows service period > 100days | 38±5.1 | 34.4±3.9 | 30.12±4.6 | 0.01 | 1:2, 1:3, 2:3 |
| % cows with lameness | 7.55±2.06 | 5.99±3.5 | 5.48±1.82 | 0.05 | 1:3 |
| % cows with mastitis | 2.05±0.43 | 1.92±0.52 | 1.93±0.31 | NS | / |
| % cows with vulvar discharge | 2.55±1.02 | 3.01±0.87 | 3±1.11 | NS | / |
| % cows with ocular discharge | 6.03±2.02 | 5.2±1.66 | 6.5±2.2 | NS | / |
| % cows with nasal discharge | 8.1±0.45 | 8±0.9 | 8±0.74 | NS | / |
| % dystocia | 13.22±2.5 | 12.12±2.44 | 14.3±1.87 | NS | / |
| % of thin cows | 11.1±2.65 | 5.11±2.2 | 3.67±1.79 | 0.01 | 1:2, 1:3, 2:3 |
| % of cows with skin lesion | 6.8±1.11 | 5.42±1.49 | 4.44±1.5 | NS | / |

Table 2: Influence of principle of good housing to health and productive characteristic of cows

| Interval of welfare quality score | 0-30 | 30.1-70 | 70.1-100 | p | LSD |
|---|---|---|---|---|---|
| Milk production /cow/day (L) | 24.45±3.01 | 26.89±3.2 | 28.18±2.25 | 0.05 | 1:3 |
| % cows service period > 100days | 30.77±4.9 | 24.45±5.5 | 22.98±3.91 | 0.05 | 1:3, 1:2 |
| % cows with lameness | 8.11±3.5 | 7.45±3.05 | 5.2±2.11 | 0.01 | 1:3, 2:3 |
| % cows with mastitis | 3.02±0.84 | 2.4±0.96 | 1.88±0.9 | 0.01 | 1:3, 2:3 |
| % cows with vulvar discharge | 2.05±1.14 | 3.4 1±1.12 | 3.2±1.11 | NS | / |
| % cows with ocular discharge | 5.99±2.14 | 5.8±1.99 | 6.4±2.3 | NS | / |
| % cows with nasal discharge | 8.1±1.87 | 8.14±2.82 | 7.72±3.74 | NS | / |
| % dystocia | 12±1.99 | 11±2.2 | 9±2.21 | 0.05 | 1:3, 2:3 |
| % of thin cows | 5.12±2.25 | 4.91±1.9 | 4.23±2.14 | NS | / |
| % of cows with skin lesion | 7.12±1.79 | 4.99±2.05 | 5.02±2.13 | 0.01 | 1:3, 1:2 |

Table 3: Influence of principle of good health to health and productive characteristic of cows

| Interval of welfare quality score | 0-30 | 30.1-70 | 70.1-100 | p | LSD |
|---|---|---|---|---|---|
| Milk production /cow/day (L) | 21.12±3.45 | 25.55±3.78 | 29.19±2.11 | 0.01 | 1:2, 1:3, 2:3 |
| % cows service period > 100days | 42.17±4.15 | 34.36±3.35 | 24.12±5.89 | 0.01 | 1:2, 1:3, 2:3 |
| % cows with lameness | 9.52±2.12 | 6.45±2.29 | 2.35±1.19 | 0.01 | 1:2, 1:3, 2:3 |
| % cows with mastitis | 2.99±0.54 | 2.05±0.57 | 1.78±0.66 | 0.05 | 1:3, 1:3 |
| % cows with vulvar discharge | 4.12±0.95 | 3.41±0.78 | 1.56±.049 | 0.01 | 1:3, 2:3 |
| % cows with ocular discharge | 8.12±2.6 | 6.44±1.99 | 3.21±0.99 | 0.01 | 1:2, 1:3, 2:3 |
| % cows with nasal discharge | 10.09±2.14 | 8.73±2.22 | 6.54±1.71 | 0.01 | 1:2, 1:3, 2:3 |
| % dystocia | 14.49±2.82 | 9.13±3.01 | 5.42±2.11 | 0.01 | 1:2, 1:3, 2:3 |
| % of thin cows | 5.66±1.11 | 4.14±1.14 | 2.28±0.97 | 0.05 | 1:2, 1:3, 2:3 |
| % of cows with skin lesion | 6.89±1.11 | 5.12±1.79 | 4.71±2.01 | 0.05 | 1:3, 1:2 |

Table 4: Influence of principle of apropriate behaviour to health and productive characteristic of cows

| Interval of welfare quality score | 0-30 | 30.1-70 | 70.1-100 | p | LSD |
|---|---|---|---|---|---|
| Milk production /cow/day (L) | 25.11±3.78 | 24.16±4.05 | 26.66±3.11 | NS | / |
| % cows service period > 100days | 35.21±5.12 | 38.11±3.69 | 39.7±4.78 | NS | / |
| % cows with lameness | 5.71±1.15 | 5.14±1.11 | 3.28±2.02 | 0.05 | 1:3, 2:3 |
| % cows with mastitis | 2.5±1.1 | 3±0.79 | 2.1±0.99 | NS | / |
| % cows with vulvar discharge | 2.78±0.64 | 3.13±0.78 | 2.85±0.66 | NS | / |
| % cows with ocular discharge | 7.14±1.19 | 6.98±2.21 | 8.88±2.06 | NS | / |
| % cows with nasal discharge | 6.16±2.21 | 8.06±3.33 | 7.14±2.99 | NS | / |
| % dystocia | 80.6±2.11 | 8.91±1.71 | 8.12±1.46 | NS | / |
| % of thin cows | 4.14±0.99 | 3.72±1.26 | 3.79±1.68 | NS | / |
| % of cows with skin lesion | 8.18±1.48 | 6.55±2.16 | 6.39±1.79 | 0.05 | 1:2, 1:3 |

Table 5: Significance of coefficient correlation between value of welfare criteria and value of health and productivity in cows.

| | Good feeding | Good housing | Good health | Appropriate behaviour |
|---|---|---|---|---|
| Milk production /cow/day (L) | 0.86** | 0.68* | 0.9** | 0.27 |
| % cows service period > 100days | -0.78* | -0.65* | -0.76** | 0.24 |
| % cows with lameness | -0.71** | -0.55* | -0.62** | 0.29 |
| % cows with mastitis | -0.29 | -0.71** | -0.84** | 0.26 |
| % cows with vulvar discharge | -0.36 | -0.26 | -0.85** | 0.25 |
| % cows with ocular discharge | -0.27 | -0.14 | -0.82** | 0.23 |
| % cows with nasal discharge | -0.2 | -0.32 | -0.73** | 0.14 |
| % dystocia | -0.11 | -0.33 | -0.84** | 0.17 |
| % of thin cows | -0.69* | -0.33 | -0.74** | 0.11 |
| % of cows with skin lesion | -0.09 | -0.85** | -0.79** | 0.2 |

*p<0.05 **p<0.01

## CONCLUSION

The health and productive characteristics of cows depend on providing the principle of welfare on farms. The most important principle, which significantly affects these characteristics, is the principle of good health. Milk production and reproductive characteristics depend on the principles of good nutrition, good housing and good health. Good production is determined by many factors on the farm, so ensuring a quality veterinary supervision (the principle of health) must be a priority.

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# ZDRAVSTVENA I PRODUKTIVNA SVOJSTVA KRAVA NA FARMAMA SA RAZLIČITIM SKOROM DOBROBITI

MARKO R. CINCOVIĆ, BRANISLAVA BELIĆ, MILENKO STEVANČEVIĆ, BOJAN TOHOLJ, MIHAJLO ERDELJAN, JOVAN SPASOJEVIĆ

**Izvod**

Merenje dobrobiti na farmama vršeno je na pomoću Welfare Quality ® scoring sistema, koji omogućuje procenu dobrobiti na osnovu principa i kriterijuma dobrog smeštaja, dobre ishrane, dobrog zdravlja i adekvatnog ponašanja krava na farmama. Na farmama sa nižom ocenom dobrobiti i slabijom ispunjenošću principa dobre ishrane postoji značajno niža proizvodnja mleka, veći procenat krava sa dugim servis periodom, veći procenat krava sa šepavošću i veći procenat vrlo mršavih krava. Obezbeđivanjem principa dobrog smeštaja značajno će se unaprediti proizvodnja mleka, smanjiti broj krava sa dugim servis periodom i šepavošću, a posebno je interesantno da će obezbeđivanjem ovog principa postojati manje subkliničkog mastitisa na farmi i manje krava sa distokijama i kožnim lezijama. Najznačajniji princip koji je pokazao značajan uticaj na sve ispitivane parametre je princip dobrog zdravlja. Obezbeđivanjem visokog skora ovog principa na farmama doći će do značajnog unapređenja svih produktivnih parametara i zdravlja. Pored zdravstvenih sindroma kao što su šepavost ili distokija, skor dobrog zdravlja zavisi i od pojave okularrnog, nazalnog i vaginalnog iscetka, koji govori u prilog infekciji pripadajućih organa. I na posletku, izmene u ponašanju kod krava ne pokazuju signifikantno dejstvo na zdravstvene i produktivne karkateristike. Slaba interakcija sa ljudima i ostalim životinjama, slabo reagovanje na hranu ili prenaglašeno strah i agresivno delovanje može se dovesti u vezu sa pojavom većeg učešća šepavosti na farmama i povećanja broja kožnih lezija. Jačina veze između ispitanih kriterijuma dobrobiti i zdravstvenih i produktivnih svojstava krava pokazuje da postoji najjača korelacija sa kriterijumom dobrog zdravlja na farmi, potom kriterijumom dobre ishrane i na kraju kriterijumom dobrog smeštaja. Produktivne osobine kao što su proizvodnja mleka i dužina servis perioda značajno koreliraju sa faktorima zdravlja, ishrane i smeštaja. Obezbeđivanje dobrog zdravlja životinja na farmama predstavlja najznačajniji zadatak u postupku obezbeđivanja dobrobiti i održive proizvodnje na farmi krava.

**Ključne reči**: krave, dobrobit, produktivnost, zdravlje.

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# PERIPARTURIENT HEMATOLOGICAL FINDING IN DAIRY COWS WITH UTERUS AND UDDER INFLAMMATION*

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*SUMMARY: The aim of this study was to investigate blood pictures and NEFA concentration in healthy cows and cows with mastitis and metritis in week before and week after calving. Calving showed significant influence on the blood parameters in the healthy and the sick cow. So after calving leads to decrease in erythrocyte count, decrease in hemoglobin, decrease in white blood cell count with a relative increase of neutrophils to lymphocytes (stress leukogram). NEFA concentration increases after parturition in both groups of cows. Cows suffering from metritis showed a significantly lower number of erythrocytes in the period before calving, and week after calving, while the hemoglobin concentration was significantly lower in the week after calving compared to control healthy cows. Metritic cows had higher leukocyte number in week before and week after calving. Leukocyte profile of these cows was characterized by increased neutrophile percent in week after calving and decreased monocyte percent in week after and week before calving compared to control group. Cows with mastitis showed higher neutrophile percent in week before and week after calving and lower percent of eosinophils in same period compared to healthy cows. NEFA concentration was significantly higher in diseased cows in week after calving. Prediction of metritis is possible in function of monocyte percent and level of anemia. Percent of eosinophil may indicate the mastitis in dairy cows.*

***Key words:*** *dairy cows, periparturient period, hematological findings, metritis, mastitis.*

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## INTRODUCTION

The transition period of dairy cows is the period of 21 days before and 21 days after calving. In this period begins a state of lactation, which increases the energy needs of the organism in dairy cows. Because of negative energy balance in this period apear many periparturient deseases and disorders such as ketosis, fatty liver syndrome, milk fever, retentia secundina, mastitis, metritis, abomasum displacement and lameness. Uterus and udder inflamation (metritis and mastitis) are very important problem in intensive production. Metritis is inflammation of the uterus, which is most often diagnosed in the first thirty days after birth, and its prevalence varies from 5 to 40% (LeBlanc et al., 2002). Results have shown out that incidence rate of clinical mastitis vary from 3.12 to 8.33%. Comparing to clinical, substantiely greater the incidence rate of subclinical mastitis was found, which ranged from 17.19 to 30.00% (Hristov et al., 2005).

Biochemical signs of metabolic peripartal stress are: hyperketonemia, hypoglycemia, hipocalcemia, reduced reactivity of leukocytes and changes in the activity of antioxidants (Goff i Horst, 1997). Cows in the transition period showed certain changes in hematological findings which including: reduced number of erythrocyte, decreased hemoglobin concentration and reducing the number of leukocytes (Gavan et al., 2010). Peripartal negative balance with hyperketonemia dammaging imune cell function and has negative effect to uterus health (Hammon et al., 2006). Also, negative influence of hyperketonemia to udder disease were showed (Suriyasathaporn et al., 2000).

The aim of our research is to investigate diference in periparturient hematological finding in healthy cows and cows with metritis and mastitis.

## MATERIAL AND METHODS

Experiment involved 60 cows: 20 with clinical mastitis in first 100 day of lactation, 20 with metritis and 20 healthy cows (control group).

Blood samples were taken in the 7-10 days before and 7-10 days after parturient. Blood was taken by venipuncture of jugularis vein. The samples were analyzed immediately after the taking by automatically hematology analyzer. It was examined the number of erythrocytes and leukocytes with differential blood count, and measured the concentration of hemoglobin using hematology analyzer Hemavet 950. NEFA concentration was measured by photometric methodology using a commercial kit (Rayto photometer).

As parameters for the presence of metritis take are: vaginal discharge present for more than three weeks postpartum, open cervix, slow uterus involution and elevated rectal temperatures. Rating the presence of mastitis was based on examination of clinical signs of mastitis and monitoring changes in the milk using the CMT test.

Statistical procedures included t-test for difference between healthy and diseased group for each examined parameters in blood. For proof of connection between specific change in blood and udder and uterus inflammation was used chi-square test.

## RESULTS

All result are presented in Table 1. Calving showed significant influence on the blood parameters in the healthy and the sick cow. So after calving leads to decrease in erythrocyte count, decrease in hemoglobin, decrease in white blood cell count with a relative increase of neutrophils to lymphocytes (stress leukogram).

NEFA concentration increases after parturition in both groups of cows. Cows suffering from metritis showed a significantly lower number of erythrocytes in the period before calving, and week after calving, while the hemoglobin concentration was significantly lower in the week after calving compared to control healthy cows. Metritic cows had higher leukocyte number in week before and week after calving. Leukocyte profile of these cows was characterized by increased neutrophile number in week after calving and decreased monocyte percent in week after and week before calving compared to control group. Cows with mastitis showed higher neutrophile percent in week before and week after calving and lower percent os eosinophils in same period compared to healthy cows. NEFA concentration was significantly higher in diseased cows in week after calving.

Chi-sq test (Table 2) presents significant connection between metritis and decreased monocyte percent and mastitis and decreased eosinophils percent. Cows with decreased monocyte percent had 27 time higher chance to develop metritis. Also, cows with decreased eosinophils percent had 5 time higher chance to develop mastitis.

Table 1: Prepartum and postpartum hematology finding in healthy and diseased cow

| Parameters | Healthy | | Metritis | | Mastitis | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Prepart. | Postpart. | Prepart. | Postpart. | Prepart. | Postpart. | SEM | Calving | Disease |
| Erythrocytes x 1012/ml | 6,85 | 5,87 | 6.22* | 5.23* | 7.01 | 6.38 | 0.42 | 0.01 | 0.01 |
| Hemoglobin g/l | 110 | 86 | 100 | 74* | 105 | 91 | 1.55 | 0.05 | 0.05 |
| Leukocytes x109/ml | 8.9 | 8.1 | 9.2* | 8.7* | 8.7 | 8.4 | 0.12 | 0.01 | 0.05 |
| Lymphocytes % | *52* | *43* | *51* | *47* | 51 | 44 | 2.35 | 0.05 | NS |
| Monocytes % | *3.84* | *5.78* | *1.94** | *0.93** | 2.1 | 3.2 | 0.55 | 0.05 | 0.01 |
| Neutrophils % | *40* | *48.55* | *42* | *53** | 49* | 52* | 0.87 | 0.01 | NS |
| Basophils % | *1* | *0.89* | *1* | *0* | 0 | 0 | 0.11 | NS | NS |
| Eosinophils % | *3.05* | *1.56* | *3.3* | *2.1* | 1.3* | 1* | 0.25 | 0.05 | 0.05 |
| NEFA | *0.45* | *0.55* | *0.48* | *0.71** | 0.5 | 0.64* | 0.07 | 0.01 | 0.01 |

Table 2: Chi sq test - 80 samples (20 healthy and 20 diseased cows in week before and week after partum)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Chi-sq. test Metritis: monocyte | Metritis yes Mono.<2% | Metritis yes Mono.>2% | Metritis no Mono.<2% | Metritis no Mono.>2% | $\chi^2$ | p< | Odds ratio |
| | 30 | 10 | 4 | 36 | 17.29 | 0.001 | 27 |
| Chi-sq. test Mastitis: eosinophils | Mastitis yes Eos.<1.5% | Mastitis yes Eos. >1.5% | Mastitis no Eos. <1.5% | Mastitis no Eos. >1.5% | $\chi^2$ | p< | Odds ratio |
| | *25* | *15* | *10* | *30* | 11.43 | 0.001 | 5 |

## DISCUSSION

Peripartal hematological findings have general characteristics that are typical for that period: reducing the number of red blood cells in postpartal period, decreased hemoglobin concentration and reducing the number of leukocytes and increased number of NEFA (Belić et al., 2011; Cincović et al., 2011). This finding observed both in health and diseased cows because calving was enough stress to start metabolic and hematological changes.

In diseased cows was found higher NEFA concentration. Elevated NEFA concentrations in cows may be associated with reduced capacity to adapt and increased stress sensitivity (Hachenberg et al., 2007). Disease is sign of decompensation and higher stress sensitivity. Cows that have excessive lipid mobilization and ketogenesis as well as reduced concentration of glucose have a significantly higher ratio of neutrophils and lymphocytes (Belić et al., 2011a). This previous results are in relation to results in this paper.

Increase the number of neutrophils, reducing the number of lymphocytes with signs of monocytosis and eosinopenia indicate birth stress because glucocorticoids alters the differential white blood cell lineage (Weiss and Wardrop, 2010). In cows with metritis increase the number of leukocytes was occurred on account of increase in neutrophils, but the number of monocytes was decreased. Reducing the number of monocytes was observed by other authors (Chassagne et al, 1998) and characterized as a risk factor for the development of metritis. Galvão et al. (2012) concluded that altered levels of expression and production of pro-inflammatory cytokines by postpartum monocytes could contribute to impaired inflammatory response and predispose cows to development of metritis. Whereas neutrophils are the main phagocytic leukocytes, monocytes and macrophages are actively involved in immunomodulation after infection. Increased number of neutrophils can be explained by the development of inflammation, but it comes as a response to increased concentrations of cortisol during calving. Monocytes migrate from the blood into peripheral tissues, and they are forming macrophages which are very important in the protection from infection (Weiss and Wardrop, 2010). It is important to note that the functionality of neutrophils decreases during peripartal metabolic stress, which predisposes cows to metritis (Hammon et al, 2006). Reduced transcription of genes for prostaglandin E2 that is essential in the protection of metritis is linked with an increase in the number of leukocytes and neutrophils, although this

finding the authors was characterized as a random (Silva et al., 2008). Reduced number of red blood cells and reduced the concentration of hemoglobin, which is dominated by cows prone to metritis ($p<0.05$) may be explanations to bleeding from uterine inflammatory injury. Placenta retention is an important cause of metritis. Blood finding of this disease is characterized by anemia and leucocytosis (Ahmed et al., 2009), which is consistent with our findings. Blood samples obtained from cows suffering from endometritis show characteristics similar to peripartum hematological findings but with monocytosis (Hanafi et al, 2008). Peripartum hematological findings may be useful in assessing the occurrence of metritis. Peripartum increase the number of leukocytes in dairy cows, suffering from mastitis, was in line with previously obtained results (Barnouin and Chassagne, 2000). Cows that showed signs of mastitis in the first third of lactation have shown a tendency to eosinopenia. Previously was determined that the reduced number of eosinophils found on farms with a higher prevalence of mastitis (Holtenius et al., 2004). The relationship between eosinophils and udder infection is demonstrated by prostaglandin E (Atroshi et al., 1996), and the pathogenesis is similar to that described by metritis.

## CONCLUSION

Increase the number of leukocytes in samples of peripheral blood, during the peripartum period and tendency to anemia may be an indicator of future udder and uterus inflammation. Decreased monocyte percent exposed cows to higher risk for uterus infection, while decreased eosinophils percent increases the risk for udder inflammation. This finding could help veterinarian to predict periparturient udder and uterus infection.

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# PERIPARTALNI HEMATOLOŠKI NALAZ KOD MLEČNIH KRAVA SA UPALOM MLEČNE ŽLEZDE I UTERUSA

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**Izvod**

Cilj ovog istraživanja bio je da se ispita krvna slika i koncentracija NEFA kod zdravih krava i krava sa mastitisom i metritisom u nedelji pre i posle partusa. Teljenje je pokazalo signifikantan uticaj na vrednosti ispitivanih parametara, tako da posle teljenja postoji sledeći nalaz: smanjen broj eritrocita, snižena koncentracija hemoglobina, smanjen broj leukocita sa relativnim porastom procenta neutrofila i sniženim procentom limfocita (stresni leukogram) i povećanje koncentracije NEFA. Ovakav nalaz je postojao i kod zdravih i kod bolesnih krava. Krave sa upalom materice (u odnosu na kontrolu) pokazuju značajno niži broj eritrocita kako u periodu pre teljenja tako i posle, dok je značajno niža koncentracija hemoglobina postojala samo u nedelji posle teljenja. Leukocitni profil ovih krava se karakterisao povišenim procentom neutrofila u nedelji posle teljenja i snižen procenat monocita u nedelji pre i posle teljenja poredeći sa zdravom kontrolom. Krave sa upalom mlečne žlezde pokazuju takođe povišen procenat neutrofila u nedelji pre i posle teljenja i snižen procenat eozinofila u poređenju sa zdravom kontrolom. Koncentracija NEFA je značajno viša kod obolelih krava u nedelji posle partusa. Predviđanje nastanka metritisa je moguća i u vezi je sa procentom monocita u diferencijalnoj beloj lozi i stepenom anemije, dok je nastanak mastitisa moguće predvideti u vezi sa procentom eozinofila u diferencijalnoj beloj lozi u periodu oko teljenja.

**Ključne reči:** mlečne krave, peripartalni period, krvna slika, mastitis, metritis.

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# FATTY ACIDS CONTENT IN CATTLE MEAT (A REVIEW)

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*SUMMARY: In this paper, an attempt was made to review the meat fatty acid content in cattle breeds. Cattle meat contain high proportions of saturated fatty acids, that influence cardiovascular diseases in human population. The content of polyunsaturated fatty acids (PUFA) and PUFA/SFA ratio in the beef meat depends on the breed and is higher in autothonous (old) breeds in Europe (Italian Podolian, Greek Katherini) and Asia (Japanese Black, Brahman, Hanwoo), compared with modern intesinve meat production breeds (Charolaise, Limousine and Hereford). Stearoyl-CoA desaturase plays a key metabolic role by changing the saturated FA content of ruminant meat. The enzyme responsible for the conversion of all saturated fatty acids (SFA) to their respective monounsaturated fatty acids (MUFA) is Δ9 desaturase. This enzyme is encoded by the stearoyl coenzyme A desaturase (SCD) gene. The polymorphisms in fatty acids content could be potential useful genetic method to improve the nutritional quality of the cattle meat.*

***Key words:*** *meat, fatty acids, content, breed, cattle.*

## INTRODUCTION

In the recent years, the consumer's decision to purchase a specific food, especially in developed countries, is greatly influenced by the perception of the food 'healthiness', which in the case of meat is largely related to its fat content and its fatty acid composition, namely the polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and the saturated fatty acids (SFA) contents (Hermansen, 2003; Dias et al., 2008). An imbalance of dietary cholesterol and fats are the primary cause of atherosclerosis and cardiovascular disease (Griel et al., 2006). Many studies demonstrate strong positive correlations between intake of SFA and the incidence of cardiovascular

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disease, a condition believed to result from the concomitant rise in serum low-density-lipoprotein (LDL) cholesterol as SFA intake increases (Daleu et al., 2010). According to these findings, health professionals world-wide recommend a reduction in the overall consumption of saturated fatty acids (SFA), trans-fatty acids (TA) and cholesterol, while emphasizing the need to increase intake of polyunsaturated fatty acids (PUFA). Until recently, dietary recommendations for humans promoting the consumption of less saturated fat have led to an increased interest in meats containing more unsaturated and polyunsaturated (PUFA) fatty acids and with a satisfactory PUFA/SFA ratio (Griel et al., 2006; Kris-Etherton and Innis, 2007).

Genetic variations among the cattle breeds, nutrition, housing systems, gender, age and climatic ambient are the major factors that change carcass characteristics, chemical composition and fatty acid profile (Čobić et al., 1988; Antov et al., 1995; De Smet et al., 2000; De Smet et al., 2004; Dias et al., 2008; Plavšić et al., 2008; Rotta et al., 2009; Daley et al., 2010). Breeds genetic variations is one of the most important factors for fat deposition, composition an fatty acids profile, which needs to be understood because of its genetic transmission (Perotto et al., 2000; Rotta et al., 2009). It was frequently demonstrated that content of PUFA and PUFA/SFA ratio in the beef meat is higher in autothonous (old) breeds in Europe (Italian Podolian, Greek Katherini) and Asia (Japanese Black, Brahman, Hanwoo), compared with modern intesinve meat production breeds (Charolaise, Limousine and Hereford) (Braghieri et al., 2000; Carnovala et al., 2000; Dymicka et al., 2004; Smith et al., 2006; Smith et al., 2009; Rotta et al., 2009; Dinh et al., 2010; Karatosidi et al., 2010). However, the detailed mechanisms of this variation, and whether or how they can be manipulated are not clearly known.

The aim of present paper is to review some recent results about fatty acids content variations in cattle breeds and possible mechanisms that influence to this phenomenon.

## FIELD EXPERIMENTS RESULTS

The consumer market for beef has become increasingly demanding as a result of negative factors associated with meat quality. Among these factors is the relationship between beef consumption and heart disease, atherosclerosis, intestinal cancer and obesity, among other diseases. These desease is mainly influenced by the content of total lipids and total cholesterol in cattle meat (Rotta et al., 2009). Reductions in total fat and in saturated fatty acid (SFA) intake decrease the prevalence of coronary heart disease. Consequently, if the saturated fatty acids can be reduced and replaced with polyunsaturated fatty acids (PUFA) of known health benefits, then it could be expected that consumers would look more favorably on cattle meat (Williams, 2000).

It hase been frequently demonstrated the significant variation among cattle breed in total content of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and the saturated fatty acids (SFA) contents, as well as PUFA/SPA and MUFA/SFA ratio in meat (Braghieri et al., 2005; Smith et al., 2006; Bureš et al., 2006; Dias et al., 2008; Smith et al., 2009; Karatosidi et al., 2010) (Table 1).

Table 1. Fatty acid composition in cattle breeds meat

| Breed | Fatty acids (% total fatty acid) | | | Fatty acids ration | | Source, Country/Authors |
|---|---|---|---|---|---|---|
| | SFA | MUFA | PUFA | PUFA/SPA | MUFA/ SFA | |
| Podolian | 46.98 | 36.72 | 16.30 | 0.35 | - | Italy Braghieri et al., 2005. |
| Katerini | 46.42 | 28.32 | 16.16 | 0.32 | - | Greece Karatosidi et al., 2010. |
| Podolian x Limousine | 46.16 | 37.08 | 13.44 | 0.28 | - | |
| Mirandesa | 52.56 | 32.37 | 13.19 | 0.25 | - | Portugal Dias et al., 2008. |
| Barrosa | 52.93 | 34.50 | 11.80 | 0.22 | - | |
| A. Angus | - | - | - | - | 0.66 | USA (Texas), Japan, Korea Smith et al., 2006. Smith et al., 2009. |
| Australian | - | - | - | - | 0.77 | |
| Japanese Black | - | - | - | - | 1.86 | |
| Hanwoo | - | - | - | - | 1.28 | |
| Brahman | - | - | - | - | 1.85 | |
| Hereford | - | - | - | - | 1.59 | |
| A. Angus | 51.43 | 38.53 | 7.39 | 0.15 | - | Czeh Republik Bureš et al., 2006. |
| Charolais | 53.30 | 35.29 | 8.31 | 0.16 | - | |
| Simental | 48.87 | 39.67 | 8.73 | 0.18 | - | |
| Hereford | 50.58 | 39.56 | 7.23 | 0.14 | - | |

The content of PUFA and PUFA/SFA ratio in the beef meat depends on the breed and is higher in autothonous (old) breeds in Europe (Italian Podolian, Greek Katherini) and Asia (Japanese Black, Brahman, Hanwoo), compared with modern intesinve meat production breeds (Charolaise, Limousine and Hereford). The meat produced by Podolian and other Italian autothonous breeds showed a higher percentage content of polyunsaturated fatty acids and a beneficial PUFA/SFA ratio (Carnovale and Nicoli, 2000; Cifuni et al., 2004). A detrimental effect of crossbreeding (autohtonous x modern intensive breeds) on the PUFA/SFA ratio was observed as the meat from autothonous breeds showed higher and more beneficial levels of PUFA (Braghieri et al., 2005; Rotta et al., 2009).

Ruminant-derived foods contain high proportions of saturated fats, a result of microbial biohydrogenation within the rumen which rapidly saturates and thus limits the availability of free unsaturated fatty acids for absorption and assimilation (Anderson et al., 2011). Many attempts have been made in order to increase the PUFA content of meat, such as administration of fish oil or vegetable oils rich in PUFAs (coconut, olive, canola, sunflower, etc.). But, unfortunately most of the methods adopted are uneffective, since PUFAs administered by the diet undergo bio-hydrogenation inside the rumen. As a consequence, the fatty acids leaving the rumen and hence absorbed through the intestine and eventually incorporated into muscles are quite different from those introduced with the diet (Gonzàlez and Andrès, 2003; Jenkins and Bridges, 2007; Vicenti et al., 2009).

## PHYSIOLOGICAL MECHANISME OF MEAT FATTY ACIDS PROFILE

Meat fatty acid composition is influenced by genetic factors, although to a lower extent than dietary factors. The species is the major source of variation in fatty acid composition with ruminant meats being more saturated as a result of biohydrogenation

in the rumen compared to the meat of monogastric animals. The level of fatness also has an effect on the meat fatty acid composition. The contents of saturated (SFA) and monounsaturated (MUFA) fatty acids increase faster with increasing fatness than does the content of PUFA, resulting in a decrease in the relative proportion of PUFA and consequently in the polyunsaturated/saturated fatty acids (P/S) ratio (De Smet et al., 2004).

Some authors determined enzyme activities in subcutaneous adipose tissue for enzyme activities from fatty acid (FA) composition data in order to explain the observed cattle breeds variability in fatty acid composition (Cameron et al., 1994). Stearoyl-CoA desaturase (SCD) plays a key metabolic role by changing the saturated FA content of ruminant meat. The enzyme responsible for the conversion of all saturated fatty acids (SFA) to their respective monounsaturated fatty acids (MUFA) is Δ9 desaturase. This enzyme is encoded by the stearoyl coenzyme A desaturase (SCD) gene. Tissue accumulates monounsaturated fatty acids, coincides with an increase in Δ9 desaturase gene expression and catalytic activity. Although differences in SCD gene expression may contribute to the meat fatty acid compositional differences between cattle breeds, biochemical and molecular genetic studies should be encouraged to unravel the mechanisms responsible for differences in the metabolism and incorporation of specific fatty acids in meat (Dance etr al., 2009; Pauciullo et al., 2010). Such polymorphisms in fatty acids content could be potential useful genetic method to improve the nutritional quality of the cattle meat.

## CONCLUSION

According to results of the present investigations, it is possible to concluded:

1) Diets high in saturated fats are associated with certain negative-health effects such as coronary heart disease.
2) Breeds genetic variations is one of the most important factors for fat deposition, composition an fatty acids profile. Rearing sistem (pasture vs. indoors) and diet (grass, hay, sillage, corn, concentrate) are factors that can influence beef total fat and fatty acid composition, too.
3) The content of PUFA and PUFA:SFA ratio in the beef meat depends on the breed and is higher in autothonous (old) breeds in Europe and Asia, compared with modern intesinve meat production breeds.
4) Stearoyl-CoA desaturase plays a key metabolic role by changing the saturated FA content of ruminant milk and meat. The enzyme responsible for the conversion of all saturated fatty acids (SFA) to their respective monounsaturated fatty acids (MUFA) is Δ9 desaturase. This enzyme is encoded by the stearoyl coenzyme A desaturase (SCD) gene. Tissue accumulates monounsaturated fatty acids, coincides with an increase in Δ9 desaturase gene expression and catalytic activity.
5) Research carried out on Podolian cattle reared in Italy has reported the good nutritional value of meat, characterized by a lower concentration of saturated fatty acids as compared with other beef breeds, in turn of a better content of unsaturated and polyunsaturated fatty acids. This makes Podolian meat a valuable food since saturated fatty acids are held responsible for coronary diseases and cancer.
6) There is no evidence about PUFA and PUFA:SFA ratio in the Serbian Podolian breed.

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# SADRŽAJ MASNIH KISELINA U MESU GOVEDA (PREGLED)

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**Izvod**

U radu je pokušano da se napravi pregled sadržaja masnih kiselina u mesu različitih rasa goveda. Meso goveda sadrži visok nivo zasićenih masnih kiselina (ZMK), što dovodi do pojave kardiovaskularnih obolenja kod ljudi. Sadržaj polinezasićenih masnih kiselina (PNMK) i odnos PNMK/ZMK u goveđem mesu zavisi od rase i veći je kod primitivnih evropskih i azijskih rasa, u poređenju sa modernim rasama za intenzivnu proizvodnju mesa. Stearoyl-CoA desaturaza ima ključnu metaboličku ulogu kod promene sadržaja ZMK u mesu goveda. Enzim Δ9 desaturaza je odgovoran za konverziju svih ZMK u odgovarajuće mononezasićene masne kiseline. Sintezu ovog enzima kontoliše stearoyl coenzyme A desaturaza gen. Polimorfizam sadržaja masnih kiselina može biti potencijalno upotrebljiv metod poboljšanja nutritivnog kvaliteta goveđeg mesa.

**Ključne reči:** meso, masne kiseline, sadržaj, rasa, goveda.

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# UPDATE ON SYNDROMES AND CLINICAL PROBLEMS ASSOCIATED WITH PORCINE CIRCOVIRUS TYPE 2 INFECTION (A REVIEW)

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*SUMMARY: Porcine circovirus type 2 is considered as one of the most important pathogens of swine. The identification and correlation of PCV2 with PMWS, was followed by an increasing isolation of the virus from pigs suffering from various syndromes and disease problems. Infection by PCV2 has been associated with systemic disease, porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex and reproductive disorders. Moreover, PCV2 has been associated with congenital muscular tremor type AII, proliferative and necrotizing pneumonia, acute pulmonary edema, granulomatous enteritis and necrotizing lymphadenitis. Despite the evidence of PCV2 association with all these syndromes and disorders, still there is a lack of knowledge concerning the relative pathogenic mechanisms.*

***Key words**: porcine circovirus type 2, syndromes, clinical problems.*

## PORCINE CIRCOVIRUS TYPE 2 (PCV2) AND ASSOCIATED DISEASES

Porcine circoviruses (PCV), members of the genera Circovirus, family Circoviridae, are small non-enveloped viruses that contain a single-stranded circular DNA of about 1.76 kb. Two types of circoviruses have been isolated and identified, PCV type 1 and PCV type 2 (Meehan et al., 1998). Initially PCV1 was a common finding in porcine kidney PK-15 cell lines (Tischer et al. 1974). PCV1 is considering as non pathogenic to pig (Allan et al., 1995, Tischer et al. 1986). However, Saha et al (2011) demonstrated that under experimental infection of porcine foetuses inoculated at 55 days of foetal life with PCV1, the virus can replicate and produce pathology in the lungs of foetuses. Still, more research is needed on this area. Regarding PCV2, the virus was initially associated with Postweaning Multisystemic Wasting Syndrome (PMWS) (Harding and Clark,

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1997, Ellis et al., 1998) and shortly, more scientific works followed revealing a link between PCV2 and other clinical features and disease forms differentiated from PMWS. Under this scope, Allan et al. (2002) proposed as a new terminology able to describe the new situation replacement of PMWS Porcine Circovirus Disease (PCVD). Respectively, on March 2006 the Board of American Association of Swine Veterinarians (AASV) adopted the term Porcine Circovirus–Associated Disease (PCVAD).

PCVD attract the relevant research interest worldwide, resulting in a continuous flow of new information. As a consequence, several terminologies referring to PCVD have been proposed. Thus, according to AASV (2006), PCVD may be subclinical or include one or more of the following clinical manifestations: Multisystemic disease with weight loss (formerly known as PMWS), high mortality, respiratory signs including pneumonia, Porcine Dermatitis and Nephropathy Syndrome (PDNS), enteric signs, reproductive disorders. Likewise, the Veterinary Diagnostic Laboratory at Iowa State University, Ames, Iowa, USA, summarizes PCVAD as follows: Severe Systemic PCV2 Infection (former PMWS), PCV2-Associated Pneumonia, PCV2-Associated Lymphoid Depletion, PCV2-Associated Abortions and Reproductive Failure, PCV2-Associated Myocarditis and Vasculitis in Growing Pigs, PDNS, PCV2-Associated Enteritis, PCV2-Associated Hepatitis, PCV2-Associated CNS Disease, PCV2-Associated Exudative Epidermitis.

The most recent proposed change in current terminology derives by Joakim Segales (2012) and in accordance with, PCVD includes: PCV2 subclinical infection (PCV2-SI), PCV2 systemic disease (PCV2-SD) (former PMWS), PCV2 lung disease (PCV2-LD) instead of PCV2-associated respiratory disease and proliferative and necrotizing pneumonia, PCV2 enteric disease (PCV2-ED) instead of PCV2-associated enteritis, PCV2 reproductive disease (PCV2-RD) instead of PCV2-associated reproductive failure and PDNS.

Many research efforts focus towards understanding the pathogenic mechanisms and identification of factors needed for PCVD modulation. Probably more than one factor involve in PCVD etiology and these can be viral depentent, host dependent, factors that can cause immunomodulation and coinfections (Opriessnig et al., 2007, 2011).

## PCV2 SUBCLINICAL INFECTION

PCV2 infection is ubiquitous, while the prevalence of PCVD is not commensurate regarding to PCV2 worldwide distribution. Thus it can be postulated that the most common form of PCV2 infection is the subclinical one (Segales, 2012). In this form, PCV2 infection may be limited to some lymph nodes with no clinical disease (Gillespie 2009). In these cases a necrotizing lymphadenitis may be revealed in clinically healthy pigs (Opriessnig et al 2007). On the other hand, there are field evidences that PCV2 subclinical infection may have a negative impact on vaccine efficacy (Opriessnig et al 2006), while there is indication that PCV2 vaccination may have a positive impact in improving productive parameters (average daily gain, percentage of runts, body condition and carcass weight) in PCV2 subclinical infected pigs (Young et al. 2011).

## PCV2 SYSTEMIC DISEASE (PCV2-SD)

PCV2-SD is a relative recent described syndrome having a significant economic impact on global pig industry (Allan and Ellis 2000; Clark, 1997; Segales et al., 1997). PCV2 is considered as the main causative agent of the disease (Allan and Ellis 2000; Allan et al., 1998; Ellis et al., 1998; Hamel et al., 1998; Meehan et al., 1998). PCV2-SD has been reproduced experimentally by inoculation of PCV2 alone and in combination with other pathogens, such as porcine parvovirus or Reproductive and Respiratory Syndrome (PRRS) virus. The combined experimental infection with parvovirus and PCV2 found to lead to increased replication of PCV2, possibly due to synergistic mechanisms, while large PCV2 antigen quantities and little or no antigens of parvovirus were detected at lesions. Additionally, simultaneous infection with PCV2 and PRRS virus is common in farm level and according to Allan et al. (2000) in these cases replication of PCV2 is reinforced. Therefore, PRRS virus probably plays an important role at PCV2-SD pathogenesis not only under experimental conditions but also in field. Furthermore, simultaneous infection by *Mycoplasma hyopneumoniae*, a frequent finding in PCV2-SD cases probably increases the incidence and severity of disease (Opriessnig et al., 2004; Kim et al., 2003a; Stockhofe-Zurwieden et al., 2003; Rovira et al., 2002; Bolin et al., 2001<, Harms et al., 2001; Kennedy et al., 2000; Krakowka et al., 2000; Allan et al., 2000; Choi and Chae, 2000). Studies with experimental PCV2 infections of pigs indicate that there is a long incubation period and the impact of various stress factors is needed for PCV2-SD manifestation to be expressed (Wellenberg et al., 2004; Fenaux et al., 2002; Krakowka et al., 2001; Allan et al., 2000).

The syndrome is characterized by low morbidity while mortality varies from 1-2% up to more than 40% and most often affects animals aged 5-12 weeks. Clinical manifestations are progressive weight loss, dyspnea, lymphadenopathy, muscle weakness, lethargy, dark-colored diarrhea, paleness and sometimes jaundice. Moreover, findings such as cough, pyrexia, gastric ulcer, meningitis and sudden death may be present (Gillespie, 2009; Segales and Domingo, 2002; Allan and Ellis, 2000; Harding and Clark, 1997).

At postmortem examination, lymph nodes, especially mesenteric, inguinal and submandibular, are usually finding enlarged. Lungs are discolored, spleen is swollen and proliferative, while kidneys may be swollen and discolored. Wasting, pale skin, jaundice maybe reveals. However, these findings are not characteristic (Segales et al., 2004; Allan and Ellis, 2000; Rosell et al., 1999; Harding and Clark, 1997).

The main histopathological findings include lymphoid depletion together with histiocyte replacement in lymphoid tissues, and intracytoplasmic inclusion bodies (Segales et al., 2004; Chianini et al., 2003; Kennedy et al., 2000; Krakowka et al., 2000; Rosell et al., 1999; Allan et al., 1998; Harding and Clark, 1997). In the lungs, liver, kidney, heart and intestines it is possible to have granulomatous lesions (Opriessnig et al., 2007).

Although PCV2 is omnipresent in pig populations, the prevalence of PCV2-SD is not relevant. Thus, Sorden (2000) proposed that diagnosis of PCV2-SD should be based on certain criteria: 1) relevant clinical symptoms, such as wasting, weight loss, and respiratory disease, 2) presence of PCV2-associated microscopic lesions (lymphoid depletion and/or histiocytic replacement of follicles in lymphoid tissues), and 3) detection of PCV2 antigen or nucleic acids in microscopic lesions using immunohistochemistry or *in situ* hybridization. According to Segales (2012) these diagnostic criteria should be: 1) Weight loss and paleness of skin (respiratory and/or digestive clinical signs may

be present as well) 2) Moderate to severe lymphocyte depletion with granulomatous inflammation of lymphoid tissues (plus granulomatous inflammation in a number of other tissues) 3) Moderate to high amount of PCV2 in damaged tissue (Segales et al, 2012). Moreover, Opriessnig et al. (2007) proposed that in order to put PCV2-SD diagnosis, PCV2 antigen must be revealed in more than 1 lymphoid tissue (lymph node, tonsil, spleen) or in 1 lymphoid tissue (lymph node, tonsil, spleen) and at least 1 other organ system (i.e., lung, liver, kidney, intestines) or in 2 two organ systems such as lung, liver, kidney, intestines. In case that PCV2 antigen is associated with only 1 specific organ system, diagnosis should be referred to another PCVD, while existence of severe lesions with a limited amount of PCV2 antigen present is consistent with severe chronic PCVAD.

## DERMATITIS AND NEPHROPATHY SYNDROME (PDNS)

PDNS was firstly reported in 1993 in Great Britain while the association of this syndrome with PCV2 infection was suggested at 2000 (Rosell et al.. 2000; Smith et al., 1993). The syndrome occurs at weaning, growing and adult pigs as well (Drolet et al., 1999). Affected animals are lethargic with little or no pyrexia, but the most characteristic symptom is the appearance of raised red to purple skin lesions, most prominent on the hind limbs and perineal area, although these lesions might be extended also in other body areas. With time, lesions covered by dark crusts, cutaneous lesions gradually fade, sometimes leaving scars (Drolet et al., 1999; Chae, 2005; Segales et al., 2004). Although, morbidity is low (~ 1%), the syndrome is often fatal, especially in pigs aged more that 3 months old, while in younger animals mortality rates decrease (Chae, 2005; Done et al., 2001; Duran et al., 1997; Ramos-Vara et al., 1997). Pigs that survive will recover and gain weight within 7 to 10 days (Segalés et al., 1998).

Gross lesions, apart these of skin, include enlarged, tan and waxy kidneys with petechial hemorrhages (Segales et al., 2004; Harding, 2004; Ramos-Vara et al., 1997). Histopathology examination reveals systemic necrotizing vasculitis (particularly in skin, kidney, lymph nodes, stomach, spleen and liver) and fibrino-necrotizing glomerulonephritis, while there is an absence or just a mild depletion of lymphocyte with mild granulomatous inflammation of lymphoid tissue. (Segales, 2012; Segales et al., 2004; Duran et al., 1997; Ramos-Vara et al., 1997; Thibault et al., 1998). Kidney lesions are suggestive of a type 3 hypersensitivity reaction, which is characterized by deposition of antigen-antibody aggregates or immune complexes (Rosell et al., 2000; Thibault et al., 1998), although the pathogenesis of the syndrome has not yet been fully elucidated.

Coinfections with other pathogens, eg *Pasteurela multocida* (Lainson et al., 2002; Thomson et al., 2001) or combinations of pathogens such as PCV2 and PRRS virus (Rosell et al., 2000b; Thibault et al., 1998) supported that are involved in PDNS pathogenesis. Moreover, Krakowka et al. (2008) was experimentally reproduced PDNS with PRRSV and terqo teno virus in PCV2-free pigs, assuming that PDNS is not always associated with PCV2.

## PCV2 LUNG DISEASE (PCV2-LD)

Porcine Respiratory Disease Complex (PRDC) has an important impact on health and productivity of pigs aged 6-22 weeks. Clinically, PRDC is characterized by decreased growth, increased feed conversion ratio, lethargy, anorexia, cough, fever and dyspnea. The intensity of symptoms and the mortality rate of the syndrome vary and depend on the combinations of pathogens involved each time (Segales et al., 2004; Kim et al., 2003b; Thacker, 2001). Several viruses and bacteria can be involved in the etiology of PRDC such as PRRSv, influenza virus, Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Pasteurella multocida (Thacker, 2001).

Ellis et al. (1999) after the detection of PCV2 in pigs suffering from proliferative necrotizing pneumonia, speculated that PCV2 may be also involved in the PRDC etiology. This aspect was supported by Kim et al. (2003b) following the increased frequency of PCV2 detection in PRDC cases. This specific role of PCV2 is possibly related to interaction or synergy with other viral respiratory pathogens, such as PRRS virus, which has been found to increase the activity of PCV2 (Harms et al., 2001; Allan et al., 2000; Ellis et al., 1999). Indeed, experimental studies have shown that mixed infection of pigs with both viruses respiratory symptoms and lung lesions were particularly severe (Chae, 2005; Harms et al., 2001). Simultaneous infection with PCV2 and bacteria has also been reported in cases of PRDC (Kim et al. 2003b), while of Opriessnig et al. (2004) indicated that infection by *M. hyopneumoniae* increases the severity of lung lesions caused by PCV2.

Symptoms of PCV2-LD and PCV2-SD are quite similar, thus differential diagnosis is important, which is based mainly on histopathological findings. Microscopic lesions include granulomatous bronchointerstitial pneumonia with mild to severe necrotizing and ulcerative bronchiolitis and bronchiolar fibrosis, while there is an absence of PCV2-SD lesions in lymphoid tissues. However, lesions of bronchiolitis should be differentiate from these that can be caused by swine influenza or porcine respiratory coronavirus infections (Oppriesnig et al, 2007; Gillespie et al, 2009; Segales, 2012). According to Chae (2005) four diagnostic criteria should be satisfied in PCV2-RD cases: (a) existence of respiratory symptoms such as severe breathing, not responding to antibiotic treatment, (b) presence of microscopic lung lesions compatible with infection with PCV2, (c) detection of PCV2 in these lesions and (d) absence of the characteristic microscopic lesions in other tissues, particularly lymphoid.

## PCV2 REPRODUCTIVE DISEASE (PCV2-RD)

In international literature several reports associate PCV2 infection with reproductive disorders. PCV2-RD main clinical manifestations are abortions, stillbirths, mummified fetuses and increased preweaning mortality (Pensaert et al., 2004; Kim et al., 2004a; Farnham et al., 2003; Sanford, 2002; Meehan et al., 2001). Histopathologic lesions include non-suppurative to necrotizing or fibrosing myocarditis in stillborn and neonatal pigs, chronic, passive, hepatic congestion and mild pneumonia in fetuses (West et al., 1999; Segales et al., 2012). These lesions are not typical in orderto establish an accurate diagnosis (Chae, 2005).

Johnson et al. (2002) experimentally infected embryos of different ages with PCV2 and noted that the virus can affect late-term fetuses causing reproductive failure,

while the time of infection determines the clinical course of the disease (Gillespie et al., 2009). It should be noticed that field cases of PCV2-RD are quite rare, presuming that old adult animals are immune due to high seroprevelence (Pensaert et al., 2004; Oppriesnig et al., 2007). Thus, gilts, breeding populations with a high proportion of gilts or naive herds are susceptible for PCV2-RD. However, there are still several unanswered questions regarding the impact of PCV2 infection on sows and their embryos and further research is needed.

## PCV2 ENTERIC DISEASE (PCV2-ED)

PCV2-ED is considered as another PCVD with increasing trend of prevalence (Opriessnig et al., 2007). On farms facing this PCV2 clinical manifestation 10-20% morbidity and mortality up to 50-60% have been reported. Usually, it occurs in pigs aged 8-16 weeks and the main clinical symptoms are diarrhea and growth retardation (Chae, 2005; Kim et al., 2004b; Oppresing et al., 2007). Both clinical expression and gross lesions (thickened intestinal mucosa and enlargement of mesenteric lymph nodes) are quite similar with these of ileitis due to Lawsonia intracellularis. However, histopathology is instructive since PCV2-ED is characterized by granulomatous enteritis and the appearance of lesions at Peyer's patches compatible with PCV2 infection, while there are not lesions in other organs (Chae, 2005; Kim et al., 2004b). However, it is essential to differentiate PCV2-ED from PCV2-SD. According Oppressing et al. (2007) diagnosis of PCV2-ED should be relied on the diarrhoea presence, together with the existence of characteristic lesions in Peyer patches but not in other lymph nodes, while PCV2 antigen or nucleic acids should be present within the lesions.

## OTHER POSSIBLE PCVD

The continued association of PCV2 with various diseases and pathologic conditions has expanded the list of PCVD, although, further research is needed in order to explore the possible impact of the virus on the relative diseases.

***PCV2-Associated Neuropathy:*** The first association of circovirus with congenital tremor type A2 was done by Hines and Lukert (1994), and later this aspect supported by Stevenson et al. (2001) after finding PCV2 nucleic acid in the brain and spinal cord of infected piglets, while Kennedy et al. (2003) reached on different conclusions.

More recently, Correa et al. (2007) associated PCV2 infection with cerebellar lymphohistiocytic vasculitis combined with hemorrhages or with lymphohistiocytic meningitis. Moreover, PCV2 antigen was found in the lesions of brain tissue. At the same year, Seeliger et al described a neurologic disease characterized by opisthotonus, nystagmus, and convulsions in pigs 6 - 8 weeks of age in which cerebellar vasculitis was also present, which they associated it with PCV2 infection. Still, it is unclear the potential role of PCV2 in the pathogenesis of this disease and further research is needed

***PCV2-Associated acute pulmonary oedema:*** Recently, a new PCV2 disease syndrome in herds vaccinated against PCV2, under the name acute pulmonary edema (APE). APE has a peracute onset affecting nursery and younger finisher pigs (Cino-Ozuna et al., 2011). Mortality can reach 20%, while main clinical signs are the rapid onset of respiratory distress followed rapidly by death. PCV2 involvement was speculated

since it was the only pathogen detected in the examined tissues. Although, authors mentioned a possible explanation regarding pathogenesis, since there is only one relevant report confirmation is needed.

## CONCLUSIONS

Several reports associate PCV2 with various syndromes and disease problems of pigs. However, even though PCV2 is widespread in pigs, there is not indubitable proofs that PCV2 really induce all these problems, while too many questions exist concerning the contributing factors that involve in PCVD pathogenesis. Thus, the continuation of relative research effort is imperative in order to be able to proceed to effective control and prevention of PCVD.

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# DOKAZIVANJE SINDROMA I KLINIČKIH PROBLEMA POVEZANIH SA CIRKOVIRUS TIP 2 INFEKCIJOM

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**Izvod**

Svinjski cirkovirus tip 2 je jedan od vrlo važnih patogena kod svinja. Identifikacija i korelacija PCV2 sa PMWS je praćena sa povećanjem broja izolacija kod svinja obolelih od različitih sindroma i bolesti. Infekcija sa PCV2 je povezana sa sistemskim obolenjem, svinjskim dermatitisom, sindromom nefropatije, kompleksom respiratornih obolenja svinja i poremećajaima reprodukcije. Šta više, PCV2 uzrokuje i kongenitalni tremor mišića tip AII, proliferativnom i nektotičnom pneumonijom, akutnim pulmonalnim edemom, granulomatoznim enteritisom, i nekrotičnim lifadenitisom. Uprkos dokazianoj povezanosti PCV2 sa navedenim obolenjima i poremećajima, još uvek nije potpuno razjašnjen patogeni mehanizam ove infekcije.

**Ključne reči:** svinjski cirkovirus tip 2, sindrom, klinički problemi.

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# EFFECTS OF LAIRAGE CONDITIONS AND TIME ON PORK QUALITY (A REVIEW)

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*SUMMARY: Since pigs spend some time in a lairage, it is necessary to pay attention to the conditions in which animals are, and which affect the welfare and meat quality of pigs. In practice, lairage time differs, but it is recommended to be 2-3 hours. Too short lairage time adversely affects pork quality because meat has a lower pH, higher temperature, lower WHC, brighter color, which is recognized as PSE meat. On the other hand, too long lairage time also is not desirable, due to increased incidence of damaged carcasses and DFD meat. Therefore, it is necessary to know and follow recommendations regarding lairage conditions and time in order to save and enhance welfare and meat quality of pigs.*

***Keywords:*** *pig welfare, pH, WHC, PSE, DFD.*

## INTRODUCTION

Lairage is a part of slaughterhouse where animals temporarily stay prior to slaughter, to recover from transport and other stressors. During lairage pigs rest which improves meat quality. However, lairage is often accompanied by economic losses due to death, skin damages and lower meat quality as a result of inadequate design of pens and corridors, environmental conditions, lairage time, handling procedures and mixing with other pigs in lairage pens (Warriss, 2003; Faucitano and Gavernik, 2008). In addition, lairage may be a reservoir of infections by pathogenic bacteria, so there is an evidence that longer lairage time increases the possibility of carcass contamination (Warriss, 2003). As the lairage conditions and time may have a positive, but also nega-

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Scientific review paper / *Pregledni naučni rad*

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tive impact on pig welfare and pork quality, attention to this segment of pork production gains importance.

## LAIRAGE CONDITIONS

During design, construction, and assessment of lairage conditions it is necessary to pay attention to the lairage capacity, layout, form and size of boxes and passageways, space allowance, floors, efficiency of ventilation, temperature, humidity, intensity of light and noise and availability of drinkers and feeders in lairage pens (Farm Animal Welfare Council, 2003).

The slaughter should be planned depending on lairage capacity, otherwise may occur overcrowding of animals in pens or waiting too long in vehicles to unload. Such events cause poor animal welfare.

Layout, form and size of boxes and corridors in lairage should be in such way designed to facilitate manipulation of animals and to encourage them to move from unload to slaughter area. The passageways between unloading area, pens and stunning boxes should be as short as possible and without sharp corners. Poorly designed lairage makes pig handling difficult, reduces productivity of slaughterhouse and also pig welfare and safety of staff. Lairage should be built from solid and resistant materials, such as concrete and metal, to protect pigs from adverse weather conditions (OIE, 2010). In addition, it is desirable to have flexible railings in order to optionally subdivide pens and avoid mixing of unfamiliar pigs. Mixing of unfamiliar pigs during transport and lairage is not advisable. During life pigs develop social hierarchy, which is disordered if unknown individuals are introduced to a group. Then pigs start to fight in order to establish a new hierarchy. The fighting reaches its peak after 40-60 minutes of lairage and thereafter gradually subsides. However, a high level of injuries on carcasses after longer lairage time indicates that fights last throughout lairage, but only at lower intensity (Gispert et al., 2000). These fights contribute to skin blemishes and if they are severe, reduce carcass value (Faucitano, 2001). Additionally, fights increase lactate and cortisol level in blood, reduce muscle glycogen stores and therefore meat has a higher pH value after 24 hours (Warriss et al., 1998a). Moreover, aggressiveness in pigs is provoked by longer period of fasting (Turgeon, 2003). If it is impossible to avoid mixing of individuals, it is desirable to reduce the size of a group, because it reduces aggressiveness in pigs. Researches of Barton-Gade (1997) indicated that a small group of mixed animals, which consisted of 15 pigs, reduced anxiety and more animals were resting. The former has been confirmed by Rabaste et al. (2007) who observed that groups of 30 pigs spent more time standing and fighting than 10 pigs in the group with the same stocking density (0,59 $m^2$ per pig).

Each animal should have enough place to stand up, lie down and turned around when it is in lairage pen (Farm Animal Welfare Council, 2003). Insufficient space area per animal may be allowed if pigs are for a short time in lairage, but if they are held overnight or a longer period of time, it is necessary to provide more space per pig. Although in practice space allowance for pigs with live weight of 100 kg varies from 0,3 to 2,7 $m^2$, it is recommended to be 0,5 $m^2$ (Chevillon, 2001). In Table 1 are given recommendations for space allowance depending on pig live weight (RSPCA welfare standards for pigs, 2010). However, the size of available area per pig affects their behavior. At high stocking density fighting is limited as pigs mostly interact with few pigs

in proximity. On the contrary, at low stocking density the number of fights has been increased due to more mixing of individuals, although these conditions give more opportunities for subordinate pigs to escape from dominant ones (Weeks, 2008). As much of the fights occurs in the first hour of lairage, Weeks (2008) suggests a higher stocking density (0,42 $m^2$/pig) for a short lairage (< 3 h) and lower stocking density (0,66 $m^2$/pig) for a long lairage (> 3 h).

Table 1. Lying and total area per pig depending on live weight

| Live weight (kg) | Lying area (m2) | Total area (m2) | Live weight (kg) | Lying area (m2) | Total area (m2) |
|---|---|---|---|---|---|
| 10 | 0,10 | 0,15 | 70 | 0,41 | 0,61 |
| 20 | 0,15 | 0,225 | 80 | 0,45 | 0,675 |
| 30 | 0,20 | 0,30 | 90 | 0,475 | 0,715 |
| 40 | 0,26 | 0,40 | 100 | 0,50 | 0,75 |
| 50 | 0,31 | 0,47 | 110 | 0,53 | 0,80 |
| 60 | 0,36 | 0,55 | // | / | / |

Lairage surfaces must not have sharp edges and protruding parts that could injure pigs. The floors of lairage should be designed, constructed and maintained in such way to minimize the risk of slipping and falling (OIE, 2010). Slipping and falling is particularly evident at unloading areas, entrances to the pens and along the corridor that leads to the stunning area, which depends not only on floor surface, but also on lairage design and handling procedures. Conditions of the floor can be assessed by scoring the number of animals that slipped and fell on these critical points. According to this, it is unacceptable if 1% of pigs falls, but if 5 % fall or 15 % slipp, that indicates serious problems in the slaughterhouse (Farm Animal Welfare Council, 2003). In addition, different types of floor are not desirable in lairage, because it may interfere with pig moving.

It is necessary to provide efficient ventilation in lairage in order to control temperature, humidity and concentration of ammonia and other noxious gases. Also, it is recommended that temperature is 15-18 $^0$C and humidity 59-65 % in lairage. When these recommendations are not followed, pigs suffer from cold stress, especially at low stocking density, or from heat stress, at high stocking density, temperature above 30 $^0$C and humidity above 80 %. Efficient ventilation and water spraying can reduce adverse effects of high temperature and humidity.

Adequate lighting should be provided in lairage. However, very intensive light and shadows fear pigs and they become distracted during movement. Pigs readily move from darker to brighter areas, so this principle can be used for easier handling.

Also, during lairage pigs should be protected from excessive noise by avoiding the use of noisy hydraulic or pneumatic equipment, muffling metal equipment or preventing expansion of noise to the area where pigs are (OIE, 2010). The sound level in a lairage varies from 76 to 108 dB on average, with highest levels in stunning area (120 dB) (Rabaste et al., 2007). The sudden and high-pitched sounds can be a source of stress, as shown by high blood lactate, creatine phosphokinase and cortisol level, increased heart rate (Kanitz and Tuchscherer, 2005), while pigs huddle together or escape from sound sources. There was a relationship between the intensity of noise in lairage and the degree of pH fall in meat as a response to the stress (van de Perre et al., 2010).

Pigs in lairage should have permanent access to the water in order to recover from dehydration caused during transport. Food should be provided if pigs stay in a lairage more than 12 hours.

## LAIRAGE TIME

Nanni Costa et al. (2002) found that more than other procedures before slaughter, lairage time has the most significant influence on pork quality. Lairage time affects the level of stress in pigs, because it can compensate negative effects of loading, transporting and unloading. Optimal lairage time for pigs is 2-3 hours (Warriss, 2003). After 2-3 hours of lairage a level of blood cortisol decreased to basal values in pigs (Perez et al., 2002), indicating a reduction of stress. Also, Warriss et al. (2003) came to similar conclusions and found that cortisol blood concentration in pigs after 2-3 hours of lairage was two times lower than after one hour of lairage. It was observed that pigs were easily handled after rest of 1-3 hours and the incidence of pale, soft and exudative (PSE) meat in these animals was lower (Perez et al., 2002; Warriss, 2003). After two hours of lairage pigs become calmer and fightings stop (van der Wal et al., 1999). In addition, Fortin (2002) found that lairage improved pork quality, regardless of transport duration. On the other hand, the slaughter of pigs immediately after unloading or after short lairage (15-60 minutes) is not recommended, because pigs are exhausted and upset. Then there is an increase in muscle temperature (+1 $^0$C) immediately before slaughter and also an increase of lactic acid in muscles, which contributes to higher incidence of PSE meat (Warriss, 2003; Warriss et al., 1998b; Owen et al., 2000; Shen andal., 2006).

Lairage time affects the initial pH value of meat (Milligan et al., 1998) and in pigs that were not resting pH value of meat (after 30 minutes of slaughter) was significantly lower than after three hours of rest. Also, increasing of pH value with a longer lairage time determined Panella-Riera et al. (2012) and Hoffman and Fisher (2010). This is explained by the fact that the reserves of glycogen are being depleted during lairage and after a longer lairage there is a lower muscle glycogen content and consequently higher pH value of meat. As there is a positive correlation between initial and final pH value of meat, the final pH value of meat was significantly lower after two hours in relation to 22 hours of lairage (Perez et al., 2002; Nanni Costa et al., 2002). In addition, Carr et al. (2008) found that during the first three hours of lairage there is a statistically significant increase in ultimate pH of meat with a lairage time. Researches of Milligan et al. (1998) showed that longer lairage time reduced the temperature of meat after 90 minutes of slaughter, so in pigs that were not rested was 41,5 $^0$C and after three hours of rest 39,0 $^0$C. With longer lairage time increases the water holding capacity (WHC) expressed through drip loss (Warriss et al., 2003). In pigs that stayed two hours in lairage drip loss was significantly higher compared to a longer lairage time (Warriss et al., 2003, Hoffman and Fisher, 2010; Salajpal et al., 2005). This can be explained by the influence of pH value on WHC of meat that is higher when pH value of meat is higher, as it is after a longer lairage. Lairage time affects meat color, so longer lairage depletes glycogen stores, pH value of meat measured 60 minutes and 24 hours after slaughter is higher and meat is darker (Warriss et al., 2003). Nanni Costa et al. (2002) have found significantly darker meat color after a longer lairage. This is confirmed by the results of Hoffman and Fisher (2010) who found a significantly higher ($p<0,05$) L * value after two hours (58,65 ± 0,62) compared to 24 hours of lairage (56,41 ± 0,68).

The percentage of skin blemishes is higher after longer lairage time because it increases the aggressiveness in pigs (Warriss et al., 1998b; Nanni Costa et al., 2002; Perez et al., 2002; Warriss, 2003). In addition, with longer lairage time increases the frequency of carcasses with medium and severe injuries (Warriss et al., 1998b). Risk of

injuries is almost doubled after 15 hours (18%) in relation to three hours of lairage (10%) (Guardia et al., 2009).

Warris et al. (1998b) studied the effect of lairage time on development of meat quality defects and found that longer lairage time reduced the incidence of PSE meat, but increased the incidence of dark, firm and dry (DFD) meat. Accordingly to these findings are results of Perez et al. (2002) and Nanni Costa et al. (2002) who concluded that percentage of PSE meat decreases and DFD meat increases with longer lairage time. However, Guardia et al. (2004) found that the differences in PSE meat risk between short and long lairage time were insignificant. The possibility of developing PSE meat after three hours of lairage was estimated at about 40 %, while lairage time of 10 hours increased risk for only 2 %. On the contrary, significant differences were found in the risk of DFD meat in pigs with different lairage time. After three hours the possibility of developing DFD meat is about 12 %, while after overnight lairage increases to 25 % (Guardia et al., 2005). Increased risk of DFD meat with longer lairage time is a result of glycogen store depletion due to starvation and fightings between pigs (Nanni Costa, 2002).

Regardless of recommendations, the optimal lairage time very depends on conditions in lairage (eg. size of boxes), mixing of unfamiliar individuals and intensity of stress that pigs experienced during transport.

## CONCLUSION

Although the main purpose of lairage is animal rest and recovery from stress, it can be a major cause of pork quality deterioration. Lairage conditions and time can be relatively easily controlled in a slaughterhouse, so it is necessary to know and follow recommendations regarding lairage conditions and time in order to save and enhance pig welfare and pork quality.

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# USLOVI I DUŽINA BORAVKA U STOČNOM DEPOU I NJIHOV ZNAČAJ ZA KVALITET MESA SVINJA

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**Izvod**

Kako svinje borave određeno vreme u stočnom depou, neophodno je obratiti pažnju na uslove u kojima se životinje nalaze, a koji utiču na dobrobit i kvalitet mesa svinja. U praksi se razlikuje vreme boravka svinja u stočnom depou, ali preporučuje se da bude 2-3 sata. Suviše kratko vreme boravka nepovoljno se odražava na kvalitet mesa svinja, zato što takvo meso ima nižu pH vrednost, višu temperaturu, slabiju SVV, svetliju boju, što se prepoznaje kao BMV meso. Sa druge strane, dugo vreme boravka u stočnom depou takođe nije poželjno, jer su trupovi više oštećeni usled ozleda, a meso se često klasifikuje kao TČS. Stoga, neophodno je poznavati i poštovati preporuke vezane za uslove i dužinu boravka u stočnom depou kako bi se očuvali, ali i poboljšali dobrobit i kvalitet mesa svinja.

**Ključne reči:** dobrobit svinja, pH mesa, SVV mesa, BMV meso, TČS meso.

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# UTILIZATION OF BOAR SEMEN BY ALTERNATIVE TECHNIQUES OF SWINE ARTIFICIAL INSEMINATION (A REVIEW)

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*SUMMARY: Conventional artificial insemination (CAI) has been highly contributed in swine global industry development. However, new boar semen technologies have been presented, such as sex sorted, encapsulated and sperm mediated gene transfer spermatozoa. Moreover, the improvement of frozen-thawed boar semen fertilizing ability is a scientific topic under investigation. New technologies must be supported by alternative insemination procedures in order to be economically performed in pig industry. For these purposes, deep intrauterine insemination (DUI), as well as, intra-oviductal insemination (IOI) by laparoscopy, has been applied and their efficiency has been studied. One of the main targets of the aforementioned techniques is to benefit the potential advantages of the high genetic value boars by using the minimal number of spermatozoa needed to achieve a high fertilization rate following artificial insemination. Although CAI is an indispensable method for the commercial pig farms, the application of alternative techniques is feasible to be performed in selected animals of high genetic value. This review discusses the suitability of the available insemination procedures for the efficient of biotechnological applications.*

***Key words:*** *swine, pig, laparoscopic, intra-uterine, artificial insemination, biotechnology.*

## INTRODUCTION

Conventional intra-cervical swine artificial insemination (CAI) is an indispensable method for the fertilization of the sows in commercial pig farms. The use of liquid diluted boar semen by the performance of traditional AI has contributed a lot in the development of swine global industry improvement. It is a simple technique that involves the deposition of high number of spermatozoa within the posterior portion of the cervical canal by means of a catheter that engages with the folds of the cervix, simulat-

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ing the corkscrew tie of the boar's penis. However, in nowadays the demand for semen from genetically superior boars has become bigger and can only be satisfied by using more boars for semen collection. A solution for this requirement could be the decrease of spermatozoa's number per insemination dose, without reduction of fertilization rate. The minimum threshold number of spermatozoa for maximum fertilization depends not only on the boar, but also on the sperm manipulation process applied before insemination. On the other hand, new sperm technologies have already been developed, such as frozen-thawed sperm, or are into the developing process, such as sex-sorted spermatozoa, sperm mediated gene transfer (SMGT), encapsulated spermatozoa or freeze-dried spermatozoa. How quickly these technologies can be utilized in the pig breeding industry depends upon their efficiency after wide application.

After insemination, the terminal stage of spermatozoa's pathway is the oviduct. Spermatozoa migrate through the uterus, the horns and the utero-tubal junction (UTJ) into the oviduct. In the ampulla of the oviduct, sperm cells interact with the oocyte, penetrate the zona pellucida and fertilize the oocyte. However, the volume of the insemination dose is quickly reduced because only a little fraction of the inseminated spermatozoa reach the oviductal ampulla (Scott and Overstreet, 1999). Polymorphonuclear leucocytes eliminate the sperm population within 30 min, up to 60%, by phagocytosis (Woelders and Matthijs, 2001). Further sperm limitation is caused by the adhesion of sperm cells to epithelial cells of endometrium and by migration into uterine glands (Hadjisavas et al. 1994). Consequently, over 90% of the inseminated spermatozoa are eliminated from the reproductive tract of the female pig within 2–3 h after insemination (Roca et al. 2006).

Keeping in mind the physiological events regulating spermatozoa's transport inside female genital tract, we can understand that more sensitive and stressed boar spermatozoa, such as sorted and frozen-thawed, should be depopulated more in the case that they inseminated intra-cervical using CAI. Freezing process of boar sperm has a detrimental effect on spermatozoa membranes (Guthrie and Welch, 2005), reduces motility and viability (Hernandez et al. 2007), impairs sperm chromatin integrity (Fraser and Strzezek, 2005) and induces oxidative stress and production of reactive oxygen species (Chatterjee and Gagnon, 2001). Moreover, sex sorted spermatozoa have short lifespan because sperm membrane is adversely affected by the flow cytometry and sorting processes, which limits the viability, storage capability and fertilization ability of spermatozoa (Parilla et al. 2005). Furthermore, flow cytometric sorting is related to the low number of spermatozoa obtained, since the number of sexed spermatozoa produced per unit time is limited for commercial use by conventional methods of use (Garcia et al. 2007). However, swine biotechnological applications have recently improved and a need for their proper use exists. All the aforementioned comments lead in the requirement for the use of a low dose insemination technique, in relation with semen deposition closer to the oviduct. For this purpose, a non-surgical, deep intra-uterine insemination (DUI), as well as, a surgical, laparoscopic intra-oviductal insemination (IOI), method have been development and successfully used with both frozen/thawed and sex sorted semen.

## DEEP INTRA-UTERINE INSEMINATION (DUI)

Transrectal ultrasonography application, before the performance of DUI is very useful for the determination of sow's ovarian status. A classification of the sows in 3

groups (pre-ovulatory, peri-ovulatory, and post-ovulatory) can be take place. According to Bolarin et al. (2006), pre-ovulatory insemination result in higher pregnancy rate, farrowing rate and litter size. Moreover, in order to avoid the damage of genital tract, DUI must be performed in sows of parity ≥2.

Using DUI, semen should be deposited into the uterine horn. A usual successfully dose for frozen-thawed boar semen is $1000x10^6$ spermatozoa in a volume of 7.5 ml (Vazquez et al. 2008). A flexible catheter with length more that 1.50m (usually 1,80m), external diameter 4 mm, internal diameter 1.80 mm must be inserted in genital tract of the sow through the hole of the classic part of the catheter which must be locked firmly into the cervix. Afterwards, the flexible part of the catheter must be moved slowly and carefully through the cervical canal and be gently pressed forward along the one uterine horn as far as possible. Before and after semen insemination, a little volume of semen-free extender can be administrated to lubricate the catheter and to force any remaining spermatozoa, respectively. Mezalira et al. (2005) reported that 12.6-17.1% of inseminated spermatozoa by DUI can be loss because of the backflow 60 min after insemination. The total time of insemination process is about 4-5 min.

After the removal of the catheter, it must be observed carefully for possible hurt of the genital tract, bleeding, remaining semen etc.; events that could affect the future reproductive efficiency of the inseminated sow. However, the incidence of bleeding in sows after DUI is low (<2%) and similar to that observed in sows inseminated with the traditional AI method (Wongtawan et al. 2006). Bolarin et al. (2006) did not found significant changes of sows' reproductive productivity after the application of DUI in 407 sows. Inserting of the catheter into the left or into the right horn is randomly. However, the fertilization of the oocytes takes place in both of the horns. Martinez et al. (2005) reported that spermatozoa are moving through the uterus and can fertilize oocytes in the contralateral oviduct. Investigating the same issue, Tummaruk et al. (2007) 24 h after DUI application, flushed with BTS extender both of the oviducts and the horns in 5 sows. They found spermatozoa only in one side of the oviducts and/or horns (left side in 3 sows, right side in 2 sows). However, a second experiment of them in 5 sows was carried out. Early embryos were found in both of the horns, 48 up to 72 h after DUI, confirming that the fertilization takes place in both oviducts, independently of the deposition of semen in one horn. Two different pathways of spermatozoa have reported to explain the fertilization in the contralateral oviduct: trans-peritoneal and intrauterine pathway. According to Martinez et al (2005) the trans-peritoneal pathway is effective in a very small percentage of the sows (<5%), while the intrauterine pathway seems be the predominant route (>75% of the sows). However, Brussow et al. (2011) studied sperm migration ways and provided experimental evidence that intrauterine, but not trans-peritoneal, sperm migration occurs after intrauterine insemination with low number of boar spermatozoa. The efficiency of DUI as a low dose insemination technique was studied by Martinez et al. (2002). They reported that DUI comes up to conventional AI results, when $150x10^6$ spermatozoa are inseminated 36 h after the onset of hormonal controlling estrous (table 1).

Table 1. Comparison between CAI and DUI in 519 sows (Martinez et al., 2002)

| Technique | Dose: sptz. x106 | Pregnancy (%)* | Farrowing rate (%) | Litter size | Live born piglets |
|---|---|---|---|---|---|
| DUI Once (1) | 10 | 39,1 a | 39,1 a | 9,44±0,36 | 9,03±0,38 |
| | 25 | 51,7 a | 46,7 a | 9,3±0,35 | 8,75±0,37 |
| | 50 | 77,8 b | 76,2 b | 9,4±0,19 | 8,91±0,20 |
| | 150 | 86,3 b | 82,9 b | 9,7±0,19 | 9,30±0,20 |
| CAI Twice (2) | 6.000 (2x3.000) | 86,4 b | 83,0 b | 9,97±0,17 | 9,4±0,18 |

Dissimilar letters (a, b) denote a significant difference ($p<0.001$). * Detected in 24 - 28 days

## LAPAROSCOPIC INTRA-OVIDUCTAL INSEMINATION (IOI)

Laparoscopic oviductal insemination is a minor surgical procedure that allows semen to be deposited into the site of fertilization, the oviduct, decreasing the loss of spermatozoa by phagocytosis or backflow and increasing the probability of fertilization. Therefore, it is not a widespread method, but it is the best choice when a low number of spermatozoa with high value, short lifespan and special characteristics, such as sex-sorted and SMGT should be used. After the hormonal synchronization of estrus, the ovaries of the sows are scanned by transrectal ultrasonography. Sows showing multiple preovulatory follicles (diameter >6 mm) are selected for insemination. A sedation of the sows is required, while the laparoscopic insemination must be performed under general anesthesia. The animals are placed in supine position in a laparoscopy cradle at an angle of approximately 20°-30° above horizontal. Laparoscopy allows the operator to locate the exact point where the insemination needle should be inserted. Generally, sperm must be deposited into the two oviducts, as close as possible to the utero-tubal junction UTJ without forcing it. The complete minor surgery lasts about 15 min. Fertilization rate higher than 90% have been reported after laparoscopic IOI with low number of boar spermatozoa 10-15x$10^6$ per horn (Table 2), (Fantinati et al., 2005).

Table 2. Fertilization rate in gilts after laparoscopic insemination with different doses of semen (Fantimanti et al., 2005)

| Semen dose: spermatozoa x106 | Number of inseminated gilts | Mean fertilization rate |
|---|---|---|
| 1500 | 4 | 94.5±2.1a |
| 15 | 4 | 91.2±3.2a |
| 10 | 4 | 92.3±2.6a |
| 5 | 6 | 81.9±6.2a |
| 1 | 6 | 50.5±10.1b |

Dissimilar letters (a, b) denote a significant difference ($p<0.05$)

Johnson et al. (1991) demonstrated encouraging results after IOI with 3x$10^5$ sex sorted boar spermatozoa (Table 3).

Table 3. Intra-Oviductal insemination with sex sorted semen (Johnson, 1991)

| Spermatozoa | Number of inseminated sows | Number of farrowings | Live born piglets | Litter size |
|---|---|---|---|---|
| X | 8 | 4 | 37 | 9,3 |
| Y | 10 | 5 | 34 | 6,8 |
| Un-sorted | 11 | 5 | 40 | 8,0 |

More recently Vazquez et al. (2005) achieved notable fertilization percentage and low rate of polyspermy by the performance of IOI with $3x10^5$ or $6x10^5$ sex sorted spermatozoa (Table 4).

Table 4. Intra-Oviductal insemination with sex sorted semen (Vazquez et al., 2005)

| Number of spermatozoa | Fertilization (%) | Polyspermy (%) |
|---|---|---|
| 3x105 sex sorted | 72,6 | 5,1 |
| 3x105 un-sorted | 74,4 | 6,7 |
| 6x105 sex sorted | 72,7 | 7,1 |
| 6x105 un-sorted | 69,2 | 11,1 |

## CONCLUDING REMARKS

The alternative techniques of swine artificial insemination can assist the development of other biotechnological applications. Non-surgical deep intra-uterine and laparoscopic intra-oviductal insemination can optimize the use of boar ejaculates, as well as, be advantageous for the utilization of frozen, sexed and SMGT boar treated spermatozoa. Both methods can be an efficient tool for obtaining a high rate of fertilization using low doses of semen of genetically improvement boars, while the use of the aforementioned sperm products seems to be more feasible. Thus, alternative artificial insemination methods provide more choices in swine industry to increase its productivity.

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# UPOTREBA SPERME NERASTA KOD ALTERNATIVNIH TEHNIKA VEŠTAČKOG OSEMENJAVANJA SVINJA

IOANNIS A. TSAKMAKIDIS, ELENI D. TZIKA

**Izvod**

Konvencionalno veštačko osemenjavanje (CVO) je verlo mnogo korišteno za unapređenje globalne industrije svinja. Sada se koriste nove tehnologije, kao što su sexing, enkapsulacija i transfer gena spermatozoida. Osim toga, unapređenje upotrebe duboko zamrznutog-otopljenog semena nerasta se sve više naučno istražuje. Ove nove tehnologije moraju biti praćene novim, alternativnim, procedurama veštačke inseminacije, radi povećanja ekonomičnosti njihove industrijske primene. Zbog toga se, sve više izačavaju mogućnosti primene novih VO tehnologija, kao što su duboko intrauterino osemenjavanja i depozicija inseminacione doze u ovidukt. Ove tehnologije, naime, pružaju mogućnost upotreba inseminacionih doza znatno manjeg volumena i broja spermatozoida, što značajno povećava efikasnost reproduktivnog iskorištavanja genetski superiornih nerastova. U ovom radu su opisane mogućnosti primene novih tehnologija veštačkog osemenjavanja svinja.

**Ključne reči:** svinje, laparoskopija, intra-uterino, veštačko osemenjavanje, biotehnologija.

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# ACTINOMYCOTIC GRANULOMA IN HIGHLY PREGNANT SOW (CASE REPORT)

TIHOMIR PETRUJKIĆ, VLADIMIR KUKOLJ, BRANKO PETRUJKIĆ, BRANISLAV STANKOVIĆ[1]

*SUMMARY: This paper presents a case of udder actinomicotic granuloma in high-pregnant sows, which was surgically treated. The sow is from a private pig farm in eastern Serbia. Landrace sows, about 3 years old, weighing about 200 kg, it is normal eating and behaving normally. During the first examination, the mammary gland tumor was established, in the size of a fist. In another review, 81 days after mating the sow, the tumor was the size of the balloon of 5 liters. The tumor was surgically removed, and a sample was sent for histopathological diagnosis. The clinical picture and histopathological findings confirmed chronic purulent-granulomatous actinomicotic inflammation of the mammary gland. The postoperative course was uneventful. The sow farroved 6 live piglets, 32 days after surgery (113 days gestation). All 6 piglets are weaned after 6 weeks. After weaning the litter, sows were surgically ovariectomised. The metastatic formations was not observed. Applied surgical procedure and postoperative treatment, show that the surgical treatment of mammary tumors can be successfully applied in practice.*

***Ključne reči**: high pregnant sow, actinomycosis, mammary gland, surgical trtetment.*

## INTRODUCTION

Actinomycosis is described as chronic granulomatous disease by cattle and pigs and rarely other animals and humans (Šamanc, 2001 and 2009). The cause of this disease is sometimes *A.bovis* and *A.isreali*. Acording to the some authors, swine actinomycosis occurs sporadically (Sofrenović et.al., 1979; Naglić et.al., 2005). In the sows, the most common changes are observed on udder, and rarely on the other parts of skin and

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Case report / *Prikaz slučaja*

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ears. Chronic suppurating granulomatous mastitis is usally the primary process occur on the udder. Also, changes have been observed on the skin. It is very rare visceral actimycosis. The sympotom of indigestion in visceral actinmycosis is very similar to the finding in the chronic peritonitis. In our article we present a clinical case of actinmycosis granulomas in high pregnant sow, which was surgically treated.

## MATERIALS AND METHOD

Landrace breed sows, about 3 years of age, and about 200kg body weight, was eating normally, and exhibited no changes in behavior. Sow originated from private pigf farm eastern Serbia. During initial diagnosis was tumor the size fist. During the second diagnosis, 81 days after mating, the tumor was the size of balloon of 5 liters. Preparing for surgery is performed so that meal sow deprived for 24 hours and water for 12 hours before surgery.The further course of preparation for the sow operation consisted in bathing and disinfecting the entire udder. Removed tumor was sent for histopathological diagnosis.The patho-histological laboratory of clip granuloma of the mammary gland is fixed with 10% neutral formalin.Subsenquentlly the tissue processed standard automatic tissue processor (dehydration several time through alchol, polymerization in xylene, parafin, impregnation) and paraffin block. Paraffin thickness of 3- 5 μm were stained with hematoxylin-eozin (HE). The described changes are consistent with chronic-purulent actinomycotic granulomatous inflamation.

## RESULTS AND DISCUSSION

Preparation for surgery sows udder we did Acetpromazina combination (0.22 mg / kg intramuscularly) and ketamine 20 mg / kg intramuscularly. The operation lasted 2 hours. At the moment of separation tumor glanuloma tissue from the udder of a stronger, there was bleeding. In the inguinal region sow blood vessels were very large, about 2 cm in diameter and clearly defined. Cut the udder was over 40 cm long, and during the operation was a problem separation tumors of the udder. During surgery the tumor in sow's ear vein intravenously given 2 liters of infusion (5% glucose, Hartmann's solution, vitamin C and calcium at recommended doses). Closure of the wound edges was performed linen thread.

Histological examination receipt granuloma of the mammary gland was found in central supurous fireplace set consisting of neutrophils affected by degenerative process. In the purulent mass were submerged colonies eozinofline agents radial looks. Around described the formation of specific granulation tissue composed of macrophages, epithelioid cells and lymphocytes. Periphery of the nodules seems poorly developed connective tissue made of fibroblasts and connective-tissue fibers (Fig. 1).

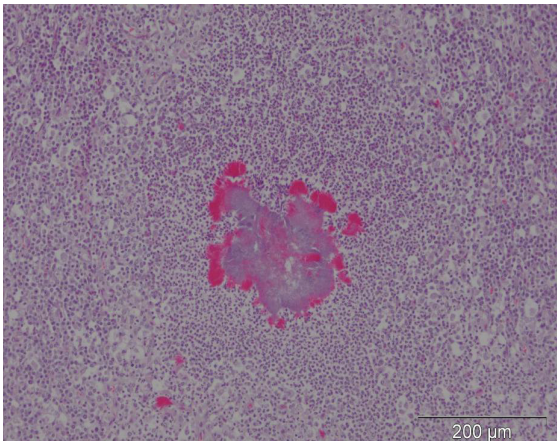


Fig. 1. Sow Mammary gland, actinomicotic granuloma with central druse in pus, HE, 200x
*Slika 1. Mlečna žlezda krmače, aktinomikotični granulom sa centralno postavljenom druzom, u gnoju, HE, 200x*

The sow was treated with oxytetraciclin in recommended doses for seven days after surgery. The wound is protected with an external drain placement. Cutisan Flogocid-fat and Chloramivet spray (twice daily) to the removal of stitches. Sutures were removed 24 days after surgery. Before and after surgery sows were kept in a clean white washed box of straw. Sows farrowed the 6 live piglets, 32.days after surgery (it was 113. day of pregnancy). After farrowing, the sow had no health disorders. Udder is a place for localization of actinomycosis. Leads to the formation of lumps and knots (actinomycoma) of various sizes that have charcterisitc histological structure (Satoshi et al., 1998). According to many authors chronic mastitis in sows is associated with actinomycosis, a rare tuberculosis and brucellosis (Yamini et al., 1988) . It was found that the strep actinomycoma granulomatous tissue is in most cases a mixed microflora, depending on the situation in which a flammable process (Jawetz et al., 1982). Injuries to the skin allows agents to penetrate and form a chronic mastitis actinomycoma character (Bollwain, 1986). Chacterstic that prevails in sows findings knot or thickening granuloma island with abscess or necrosis induaration in one or more mammary complexes, which typically leads to a large increase of the udder. Changes occur on the teats are enlarged hard nodular relaxed and more at the mammary complex. Secretion of milk is negligible or does not exist in general. Health status of sows has not changed. In older sows are formed actinomycotic changes. In the parenchyma of one or more of the mammary glandular complexes are formed nodular formation, which consists of granulation tissue containing radiating formations, so called "comrade." On the industrial swine farms were recorded in the presence of granuloma actinomycotic sows. (Valcea, 2011). It is recommended that funeral prophylactic measures aimed at combating appearance this conquer technique. Monitoring the health of the sow, regulation and hygienic measures biosicurity on the farm. Monitoring indicators biosicurity farm (Stanković et al., 2009) suggest special attention to hygiene at farrowing and adequate preparation for farrowing sow also be disable presence of infection at farrowing during the puerperium. Conduct corresponding diet sows. Reports of the World Technical Committee animal health and food hygiene, it is said that Actinomycosa diagnosed in cattle herds in cer-

tain areas, and pigs are not mentioned. General recommendations in all countries is that their veterinary services and other universities to examine the institution's animal health and monitor the occurrence of the disease. Such reports should publish and make annual reports on these investigations. In the current practice of treatment mastitis occurring in sows usually related to the formation of MMA syndrome (mastitis, metritis, agalactia syndrome) in swine farms have not noticed or found formation actinomycotic granuloma (tumor) of the udder in sows, although there were tumorous formations of mastitis and cured exclusion of certain dairy complex in older sows (six or more parities) but not by the appearance of large tumors in pregnancy in sows.

## CONCLUSION

Clinicaly, this case confirmed that the operation of the mammary gland tumors in advanced pregnancy sows and it is not possible in this case performed an abortion or fetal death in the last third of pregnancy. Healing the wounds of the udder was completed within 24 days. Implemented procedures that were used during the surgical treatment and postoperative therapy proved to be good practice in the applicable in these tumorous formation in the mammary glands of pigs. After 6 weeks the sow was farrowed six live pigs, and then castrated. On the udder or anywhere on the body there were observed no changes that would indicate metastatic formations.

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## AKTINOMIKOTIČNI GRANULOM KOD VISOKO GRAVIDNE KRMAČE (PRIKAZ SLUČAJA)

TIHOMIR PETRUJKIĆ, VLADIMIR KUKOLJ, BRANKO PETRUJKIĆ, BRANISLAV STANKOVIĆ

**Izvod**

O ovom radu je opisan slučaj aktinomikotičnog granuloma kod visoko gravidne krmače, koja je hirurški tretirana. Krmača rase Landras, stara oko 3 godine i telesne mase oko 200 kg, je vlasništvo jedne privatne farme u istočnoj Srbiji. Krmača je normalno jela i manifestovala normalno ponašanje. Kod prvog pregleda, tumor je bio veličine pesnice, a kod drugog, 81 dan posle parenja, tumor je bio veličine 5 litara. Pregledom je dijagnostikovana purulentna granulomatozno-aktinomikotična inflamacija mlečne žlezde. Ova dijagnoza je potvrđena histopatološkim pregledom. Ovaj slučaj pokazuje da je moguće uspešno odstraniti mamarni tumor krmače u kasnoj gravidnosti. Primenjena hirurška procedura i kasniji trtetman pokazuju da mogu biti dobra praktična metoda u saniranju mamarnog tumora krmače. Posle 6 nedelja, krmača je oprasila 6 prasadi, a zatim je bila kastrirana. Uočenu su promene koje ukazuju na pojavu metastaza.

**Ključne reči:** visoko gravidna krmača, actinomycosis, vime, hirurški tretman.

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