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INFLUENCE OF GENISTEIN AND ZEARALENONE ON BOAR SPERMATOZOA MOTILITY *IN VITRO*

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Abstract

Genistein (GEN) belongs among soya phytoestrogens, while zearalenone (ZEA) is a metabolite of the fungi *Fusarium spp.* Both compounds can bind to estrogen receptors, so they can mimic the functions of endogenous estrogens. Pigs very often come into contact with these substances through feeding. The aim of this study was to evaluate the influence of GEN and ZEA on the motility of porcine spermatozoa by the CASA system (computer assisted sperm analysis). The effects of GEN and ZEA, concentration range 0,5 – 20 μM, on the model of boar diluted semen were tested during 2- and 4-hour incubation at 38°C. The data obtained show that GEN and ZEA negatively affect sperm velocity parameters (VCL, VAP, VSL) in a dose and incubation period dependent manner. A significant decrease in sperm velocity parameters was recorded after addition of all the GEN or ZEA concentrations tested. A temporary increase in value was observed in the case of VSL only - after 2 hours of incubation with 0,5 – 10 μM ZEA. Also, a dose dependent increase of immotile spermatozoa corresponded to changes of the sperm velocity. In conclusion, even relatively low doses of the substances tested can negatively affect pig fertility.

Keywords: Pig, sperm, genistein, zearalenone, CASA

Introduction

The quality of boar semen is influenced by various endogenous and exogenous factors such as hormonal background or nutrition, and nutrition can already serve as a pathway for the admission of hormonal active compounds which can affect both male and female.

In this way spermatozoa can be exposed to environmental estrogens which are structurally similar to 17β – estradiol and show estrogenic activity. Among these substances soy phytoestrogens and zearalenone belong. The richest source of soy phytoestrogens are soybeans (*Glycine max*) (Moravcová et Kleinová, 2002). Feedstuff for pigs contains up to 18% soybeans (Zeman et al., 2006). Soy phytoestrogens taxonomically belong among isoflavones, the most important of which is genistein**,** which is, among other things, a known inhibitor of tyrosinprotein kinases (Bajpal et al., 2003). Zearalenone (ZEA) is a mycotoxin produced by Fusarium moulds (Minervini et Dell´Aquilla, 2008). Livestock are exposed to its effects in feed, and pigs are considered the most sensitive species (Benzoni et al., 2008; Fink – Gremmels et Malekinejad, 2007; Agag, 2004; Ravishankar et Karim, 2010). The mechanism of action of both genistein and zearalenone involves their binding to estrogen receptors. In an organism they can demonstrate both estrogenic and antiestrogenic activity (Tapiero et al., 2001; Whitten et Patisaul, 2011). Whether estrogenic and antiestrogenic effects occur in the organism depend not only on the concentration of environmental estrogens, but also on the momentary level of endogenous estrogens (Tapiero et al., 2002) and the type of ER (Minervini et Dell´Aquilla, 2008; Tapiero et al., 2002).

Assessment of mature spermatozoa *in vitro* provides an appropriate model for studying the influence of the environmentals estrogens mentioned above, which can occur in

the female genital tract as a result of the consumption of soy grits or feed with zearalenone contamination. A comparison of genistein and zearalenone effects on boar spermatozoa has not been published yet.

CASA – computer assisted sperm analysis is a practical tool for objective analysis of sperm motility and for classification of various motion categories (Kathiravan et al., 2011). This system is based on capturing consecutive images from microscope by simple chip camera (Quintero – Moreno et al., 2003), and the image obtained is consequently exported to a computer. Image assessment is provided by specific software with the CASA module, which is able to analyse them (Allahbadia, 2005). The data obtained are mathematically processed and individual trajectories are defined in numerical form. The results are expressed by a set of parameters, which accurately define the motion of sperm cells (Quintero – Moreno et al., 2003).

The aim of this study was to assess the motion parameters of boar spermatozoa *in vitro* by CASA.

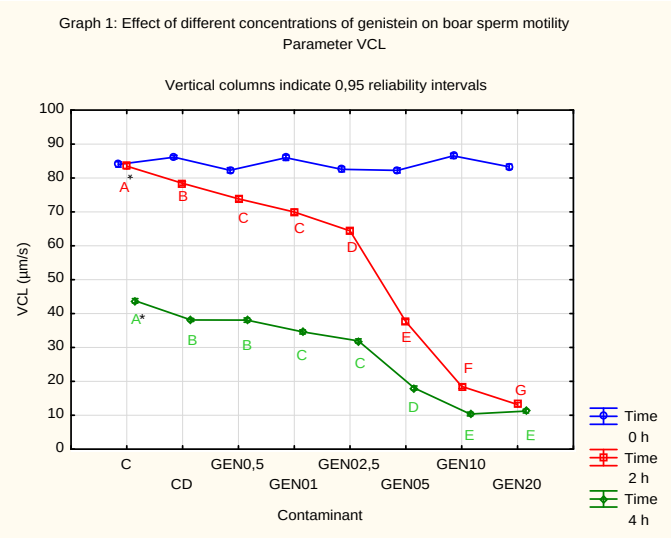
Materials and methods

Spermatozoa were obtained from France hybrids boars as commercial short – term preserved insemination doses. The doses were kept during transportation and storage at 17°C and were processed on the day of collection or on the following day. Genistein (GEN) and zearalenone (ZEA) were dissolved in DMSO (dimethyl sulfoxide) to a final concentration of 2µl DMSO/ 998 µl ejaculate and 0,5; 1; 2,5; 5; 10; 20 µM GEN or ZEA. The acquired results were confronted with pure control and control with DMSO. All of the instruments and laboratory equipment were heated to 38°C. For control, all samples were examined after 4 min incubation before the addition of GEN or ZEA. Spermatozoa were incubated for 2 and 4 hours at 38°C in a waterbath. The samples were then injected into the chamber of calibrated Leja® microscopic slide and assessed by a Nikon E 600 microscope with a heating plate, objective lens PH 10x with negative phase contrast and a Jenoptik Prog Res CT1 camera. Capturing and image analyses were performed using the software Nis Elements 3.2 (Laboratory Imaging, Prague). The parameters assessed were VCL (curvilinear velocity), VSL (straight line velocity) and VAP (average path velocity). Based on the parameters obtained, spermatozoa were divided into the categories of motile and immotile. Distinct differences at the level of velocity parameters can be related to the high sensitivity of the CASA system. Choosing a significance level of $p= 1 \times 10^{-6}$ should be sufficient for the separation of rezidual variability and the experimental scheme of variability. For the statistical analysis program Statistica.cz version 10 was used. All data were subjected to multifactorial ANOVA system.

Results and discussion

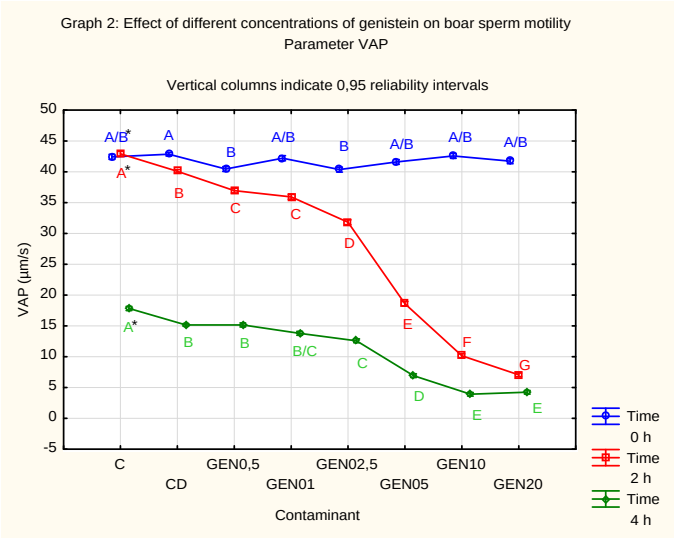
Our data show that both of the tested contaminants considerably affected the values of motion parameters of boar spermatozoa. In our experiment a statistically significant decrease of sperm motility was observed after addition of various concentrations of GEN already 2 hours after incubation. This was confirmed by a rapid decrease of motion parameters marked mainly from the concentration of 5 µM (graphs 1 – 3). A significant increase in immotile spermatozoa, especially in the samples with 10 and 20 µM supplementation (graph 4) correlates with this fact. After 4 hours incubation another decrease in velocity motion parameters in a dose dependent manner was recorded in all samples (graphs 1 – 3). Related increase in the immotile sperm ratio was also observed (graph 4).

Graph 1



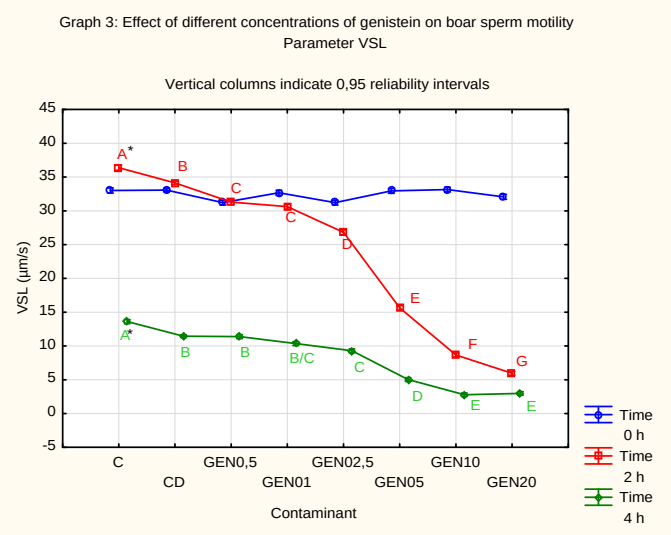
*values marked with different indices of the same color are statistically significantly different at a significance level $p < 1 \times 10^{-6}$

Graph 2



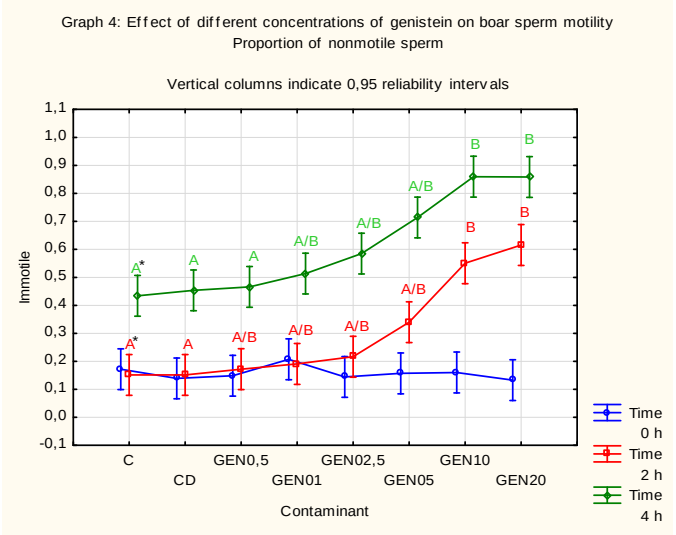
*values marked with different indices of the same color are statistically significantly different at a significance level $p < 1 \times 10^{-6}$

Graph 3



*values marked with different indices of the same color are statistically significantly different at a significance level $p < 1 \times 10^{-6}$

Graph 4



*values marked with different indices of the same color are statistically significantly different at a significance level $p < 0,05$

Several studies have been concerned with the influence of genistein on sperm motility. However, they were conducted on different animal models with various experimental protocols, so the results obtained are ambiguous. While Adeoya – Osiguwa et al. (2003), Eustache et al. (2009) and Martinez – Soto et al. (2010) demonstrated that genistein has an influence on sperm motility, Pukazhenti et al. (1998), Hinsch et al. (2000) and Menzel et al. (2007) obtained different results. None of these author collectives worked with boar sperm. Our work is in methodology most relevant to the study of Martinez – Soto et al. (2010), which described the influence of freezing and thawing media with the addition of GEN on human sperm. The tested concentrations were 0,1 µM, 1 µM and 10µM GEN in the freezing medium

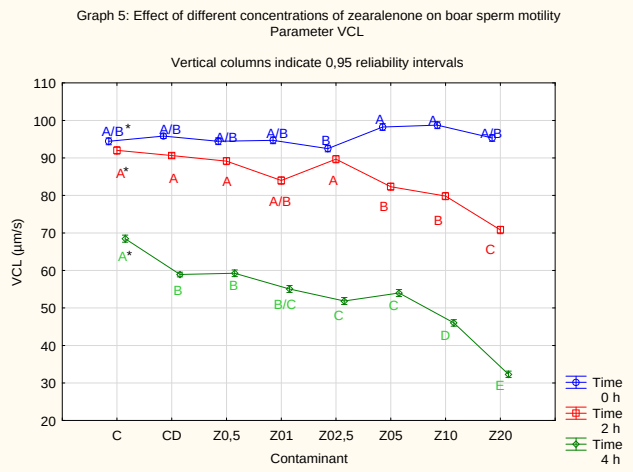
and 1 µM and 10 µM in the thawing medium. Sperm were thawed at a constant temperature of 37°C, incubation received 60 min. In agreement to our results Martinez – Soto et al. (2010) recorded a decrease in motile spermatozoa and progressive motile spermatozoa in a dose dependent manner both in the freezing and thawing medium. In the case of the thawing medium decrease of VCL, VSL and VAP was also observed. Also, publications by a Hinsch et al. (2000) and Menzel et al. (2000) were similar to our experiment concerning the temperature and concentration of genistein. They worked with cryopreserved bovine spermatozoa. Hinsch et al. (2000) worked with concentrations of GEN of 0,74 µM and 7,4 µM, and samples after thawing were kept at 38°C. Menzel et al. (2007) worked with concentrations of GEN of 0,074 µM, 0,74 µM and 7,4 µM, and samples after thawing were kept at 38,5°C. In both studies GEN did not affect sperm motility. However, bovine spermatozoa can be less sensitive to this phytoestrogen than porcine spermatozoa.

In our experiment zearalenone as well as genistein inhibited sperm motility in a concentration dependent manner.

After 2 hours of incubation ZEA induced a decrease in the velocity motion parameters statistically significant at 5 – 20 µM concentrations. However, in the case of VSL except 20 µM ZEA an increase in this parameter was recorded. Differences between pure control, control with DMSO and samples with 0,5 – 2,5 µM of ZEA were minimal. Significant deviations were observed after the addition of 5 – 20 µM ZEA (graphs 5 – 7). The ratio of immotile spermatozoa continuously increased in all tested groups with the highest manifestation in the sample with 20 µM ZEA (graph 8),

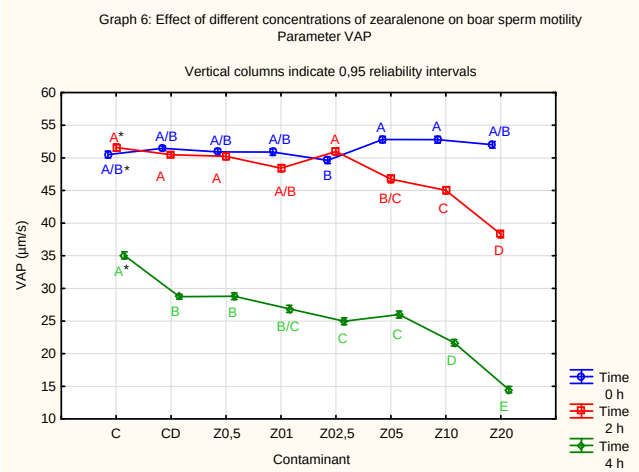
After 4 hours of incubation a rapid velocity decrease at all the concentrations tested occured in a dose dependent manner. Simultaneously the differences between control and experimental samples were more marked, mainly at 10 and 20 µM ZEA (graphs 5 – 7). An increase in immotile spermatozoa in a dose dependent manner was also determined, however, this effect was not statistically significant (graph 8).

Graph 5



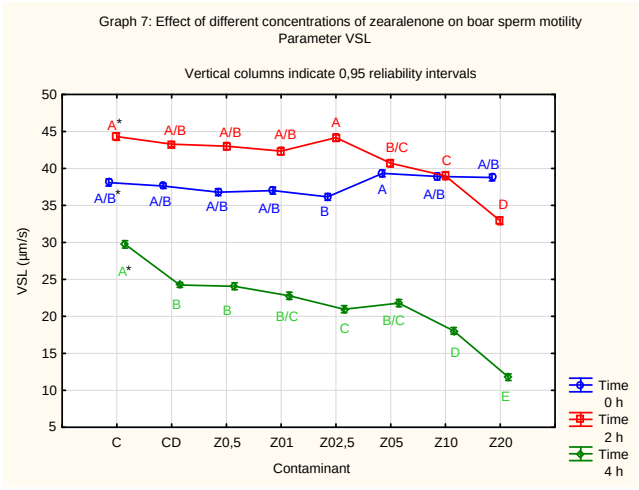
*values marked with different indices of the same color are statistically significantly different at a significance level p < 1×10^{-6}

Graph 6



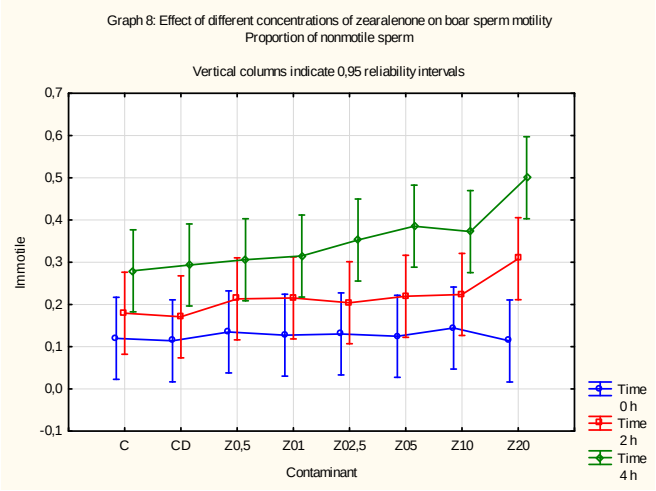
*values marked with different indices of the same color are statistically significantly different at a significance level p < 1×10^{-6}

Graph 7



*values marked with different indices of the same color are statistically significantly different at a significance level $p < 1 \times 10^{-6}$

Graph 8



*values marked with different indices of the same color are statistically significantly different at a significance level $p < 0,05$

ZEA and its influence on the motility of boar spermatozoa has been the focus of attention of several authors. This substance was tested in a wide spectrum of concentrations from picomolar to milimolar amounts. However, most of studies used higher concentrations than in our experiment. The negative effects of ZEA on sperm motility parameters were confirmed by Rajkovic et al. (2007) who incubated boar sperm with a very high amount of ZEA 3,1 – 310 mM, although concentration 3,1 was not effective. The effects on motility were expressed as a decrease of percentage of progressive motile spermatozoa. Tsakmakidis et al. (2006, 2007) assessed the effect of ZEA after the addition of 125 µM, 187,5 µM and 250 resp. 40 µg/l, 60 µg/l and 80 µg/l. The incubation periods were 1, eventually 2, 3 and 4 hours at 38,5°C. A significant decrease in motility was observed after 1 hour of incubation (Tsakmakidis et al. 2006, 2007), and capturing after each following hour led to another depression in sperm progressive motion. After 4 hours of incubation steep decline in motility was observed. In the case of 250 µM, the percentage of progressive motile spermatozoa was almost zero (Tsakmakidis et al., 2006). This effect was dose dependent, which corresponds to our results.

However, in the study of Tsakmakidis et al. (2008) no effect on sperm motility after the addition of 31,4 µM, 62,8 µM or 94,2 µM ZEA was observed. Incubation conditions were similar to previous studies at 38,5°C/ 4 hours. This contradiction of our results could be caused by the different method of sperm motility assessment. While Tsakmakidis et al. (2008) assessed motility by subjective evaluation, we used an objective system of computer assisted sperm analysis (CASA).

Lower concentrations of ZEA (0,2 pM – 20 µM) were tested by Benzoni et al. (2008). In this study the CASA system for motility evaluation was used. Contrary to our results, no effects on VCL and VSL after 5, 16 and 24 h incubation were observed. This contrast could be caused by the different temperature during incubation. Benzoni et al. (2008) incubated spermatozoa at 18°C, which corresponds to the preservation temperature, while our samples

were maintained at 38°C, which corresponds to the body temperature recommended by Verstegen et al. (2002).

Conclusion

In our experimental conditions genistein significantly inhibited the motility of boar spermatozoa in a dose dependent manner from 0,5 μM, and zearalenone had the same negative effect from concentration 5 μM. Contrary to published studies, in our experiments *in vitro* the effects of both substances were confirmed at a relatively low concentration. As GEN is a considerable part of pig nutrition and ZEA mycotoxicosis is a common problem at pig farms, it is possible that these compounds can affect reproduction also *in vivo*.

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