ASSESSMENTS OF ZEARALENONE CONTAMINATION IN IN-VITRO MATURATION AND FERTILIZATION OF GAMETES

配偶子の体外成熟および体外受精におけるゼアラレノン汚染の評価

RENTSENKHAND SAMBUU

2013

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph. D)

University of Yamaguchi

2013

THE UNITED GRADUATE SCHOOL OF VETERINARY SCIENCE
YAMAGUCHI UNIVERSITY

We hereby recommend that the thesis prepared by RENTSENKHAND SAMBUU under our supervision, entitled "**Assessments of zearalenone contamination in in-vitro maturation and fertilization of gametes**", be accepted as fulfilling in part for the degree of Doctor of Philosophy

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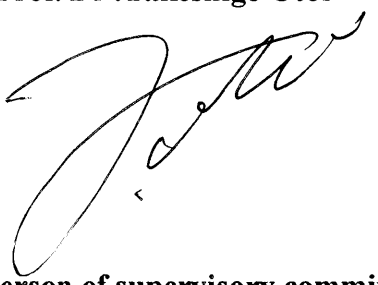

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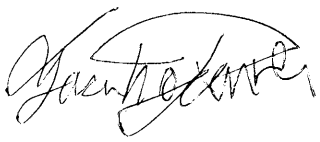

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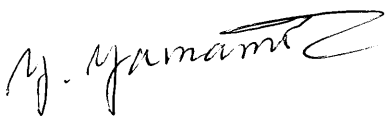

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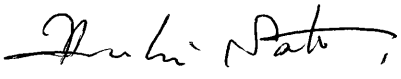

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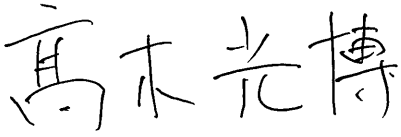

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ACKNOWLEDGMENTS

I would like to take this opportunity to sincerely acknowledge the JAPAN SOCIETY for the PROMOTION of SCIENCE (JSPS), Government of JAPAN for the financial support provided with my scholarship.

I would like to express my deepest respect and most sincere gratitude to my supervisor, Prof. Takeshige Otoi, for his guidance and encouragement at all stages of my work. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist wanted to be. I am indebted to him more than he knows.

Special thanks are due to Prof. Mitsuhiro Takagi, Faculty of Agriculture, Kagoshima University for taking intense academic interest in this study as well as providing valuable suggestions that improved the quality of this study.

I am extremely grateful my home adviser, Dr. Mandakh Begzjav, at Research Institute of Animal Husbandry of Mongolia who sparked my interest in the fascinating field of advanced animal reproduction biotechnology .

I warmly thank to Dr. Zhao Namula, for sharing research papers and guiding me in completion of my research work.

I also extend my thanks to my project leader academician Chadraabal Genden, of Research Institute of Animal Husbandry for his support to carry out some research of the study in the home country.

Foremost, I am incessantly thankful and indebted to my family, especially my mother and father for their always believing in me, for their continuous love and their supports in my decisions. They have been always supporting and encouraging me to do my best in all matters of life.

ABSTRACT

Zearalenone (ZEN), a nonsteroidal estrogen-like mycotoxin produced by *Fusarium* species on several grains, is an estrogen receptor agonist; its distinct estrogenic and anabolic properties in several animal species exert detrimental effects on the reproductive system, resulting in reproductive disorders in domestic animals, particularly in swine. However, the effect of ZEN on the fertilization of sperm and early embryonic development has not been fully reported in the literature.

The first series of experiments were undertaken using liquid chromatography-tandem mass spectrometry to detect ZEN and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) simultaneously in porcine follicular fluid (FF). ZEN and α-ZOL, but not β-ZOL, were detected in all pooled FF samples collected from coexisting follicles (diameter $\geq$ 6 mm) with in 10 ovaries. Furthermore, ZEN and α-ZOL were detected in samples pretreated with β-glucuronidase/arylsulfatase, but not in those left untreated, suggesting that the FF samples contained glucuronide-conjugated forms of the mycotoxins, which might be less harmful to porcine oocytes because of glucuronidation affecting the receptor binding. Nonetheless, the effects of the glucuronide-conjugated forms should be studied, both *in vitro* and *in vivo*.

In the second series of experiments, the influences of acute exposure to zearalenone (ZEN) on porcine oocyte maturation, fertilization, or sperm penetration ability during both *in vitro* maturation and fertilization were evaluated. First, oocytes were cultured in ZEN-containing (0–1000 μg/l) maturation medium and were then fertilized. Second, oocytes maturing *in vitro* without ZEN were fertilized in ZEN-containing fertilization medium. The maturation rates of oocytes and the penetration ability of sperm decreased significantly in the presence of 1000 μg/l of ZEN. However, neither increases in the rates of degeneration and DNA fragmentation of oocytes nor reductions in normal and polyspermic fertilization were observed. ZEN had no effects on sperm penetration rates. However, 1000 μg/l ZEN had positive effects on the rates of normal and polyspermic fertilization. Therefore, from a perspective of normal fertilization, results suggest that acute exposure to ZEN during maturation and fertilization of porcine oocytes, even at a higher concentration, might not affect porcine oocyte fertility.

In the third series of experiments, the effects of *in vitro* exposure of porcine spermatozoa to zearalenone (ZEN) and α-zearalenol (α-ZOL) were studied by evaluating

several parameters of in-vitro fertilization (IVF) systems. For this purpose, boar spermatozoa cultured with semen storage medium containing 0 (control), 10 and 1000 μg/l of ZEN and α-ZOL for 1 week at 5°C were used for IVF of *in vitro* matured oocytes. Overall, no significant differences were found in the rates of total penetration, monospermic fertilization, and polyspermic fertilization of oocytes inseminated with spermatozoa from the different groups. Similarly, ZEN and α-ZOL at 10 and 1000 μg/l showed no detrimental effects on the cleavage and development to blastocysts of oocytes after *in vitro* fertilization. Although the motility, viability, and plasma membrane integrity of spermatozoa decreased significantly after 3 weeks of storage compared to non-stored spermatozoa ($P < 0.05$), ZEN and α-ZOL at the evaluated concentrations exerted detrimental effects on the above parameters, even after 3 weeks of storage. These results indicate that prolonged exposure of boar spermatozoa to ZEN and α-ZOL up to 1000 μg/l under reduced metabolic conditions does not affect their *in vitro* function.

In conclusion, results of this study indicate that ZEN and α-ZOL, but not β-ZOL, are detectable in porcine FF. Moreover, exposure to ZEN during storage of semen and in *in vitro* maturation and fertilization of porcine oocytes, even at a higher concentrations of ZEN, might not affect the meiotic competence or fertility of oocytes.

GENERAL INTRODUCTION

Mycotoxins, which are secondary metabolites synthesized by various fungal species, are toxic compounds that are known to produce widely diverse injurious effects on human and animal health after acute or chronic exposure (Pier, 1981, Marquardt, 1996; Peraica et al.,1999;). Mycotoxins can increase in the field during the growing season and increase during harvesting, drying, storage, or during transport and processing (Mirocha and Christensen, 1974; Scudamore, 1993).

Many different mycotoxins have been recognized and isolated from various *Fusarium* molds and some disease states caused by consumption of cereals containing these toxins by domestic animals as well as by humans. Zearalenone (ZEN), and related compounds α and *β* zearalenol (α and *β*-ZOL) and α and *β* zearalanol (α and *β*-ZAL) are synthesized by several species of *Fusarium* such as *F. graminearum, F. tricinctum, F. moniliforme* and *F. oxysporum* (Placinta et al., 1999). Zearalenone (ZEN) is characterized by its estrogenic properties, which result from competitive binding of ZEN to estrogenic receptors located in the uterus, liver, mammary glands, and hypothalamus. It stimulates the target tissues, producing an estrogenic response, which can adversely affect normal reproductive function (Biehl et al., 1993; Malekinejad et al., 2006b). The natural occurrences of ZEN in agricultural commodities have been reviewed by Yoshizawa (1991) and Zinedine et al., (2007).

Because of the globalization of food and feed trade, the occurrence of mycotoxin contamination is likely to be observed in every region of the world (Binder et al., 2007). ZEN has been reported in maize and poultry feed in countries of the Western Hemisphere: Argentina, Brazil, and the USA (Yoshizawa, 1991; Resnik et al., 1996; Dalcero et al., 1998; Silva and Vargas, 2001). Among agricultural crops, maize has often been contaminated with ZEN, implicating it in cases of hyperestrogenism in farm animals (Shotwell, 1991). In Japan, contamination of cereals (barley and wheat) with ZEN has been reported by Yoshizawa and Jin (1995). The influences of ZEN on reproductive structural and functional parameters are well recognized. Of all domestic species, swine is the most sensitive species, followed by ruminants (Olsen, 1989). Of all stages of maturity, the prepubertal gilt is the most sensitive to ZEN (Haschek and Haliburton, 1986). Vulvovaginitis syndrome in young female swine and anestrous induction in the mature sow have been reported (Osweiler, 1986). A reduction of luteinizing hormone was also

observed in gilts fed ZEN-contaminated feed. In another report, Green et al. (1990) described a decrease of luteinizing hormone, but in boars, ZEN does not adversely affect subsequent reproduction if it is withdrawn from the diet two weeks before exposure. ZEN is also known to induce anestrus in cycling females or delay return into estrus after weaning of the sows. However, at concentration levels of either 5 or 10 ppm diet, ZEN did not affect the proportion of sows returning to estrus, but the interval from weaning to estrus increased (Young et al., 1990). Results of rodent studies suggest that ZEN interferes in female fertility even when observed by reduced pregnancy (Morrissey and Vesonder, 1985).

More recent *in vitro* experiments demonstrated that ZEN might affect porcine fertility by inhibiting oocyte maturation (Alm et al., 2002; Malekinejad et al., 2007). Reduction of embryonic survival and sometimes decreased fetal weight occurred when sows were fed a diet containing a high level of ZEN (Etienne and Dourmad., 1994). In prepubertal heifers receiving diet contaminated with zearalenone over three estrus cycles (about 65 days), the conception rate was reduced up to 62–87% (Weawer et al., 1986). Oocyte degeneration and reduced meiotic competence of oocytes were reported by *in vitro* maturation (Alm et al., 2002; Alm et al., 2006). Other studies have demonstrated that zearalenone and its derivatives (α-ZOL and ZEN) inhibit *in vitro* maturation of oocytes to the metaphase II (MII) stage and increase the rates of oocytes with abnormal chromatin (Minervini et al., 2001, Rajkovic et al., 2007). When zearalenone was added at levels of 0.94 or 9.3 μM, most oocytes were unable to complete maturation and were blocked at stages between metaphase I (MI) and telophase I (TI). In young male pigs, moreover, intake of dietary 9 mg/g ZEN from 32 days of age to 1 year decreased sperm concentration and engendered a slight reduction in testicular and epididymal weights (Haschek and Haliburton, 1986).

It has been demonstrated that ZEN levels in follicular fluid were often low, but they still reduced the conception rate (Minervini and Dell'Aquila, 2008). Alm et al. (2002) reported that the development of *in vivo*-produced porcine zygotes to blastocysts was influenced by α-ZOL. At 7.5 μM concentration, the percentage of zygotes that developed to blastocysts tended to decrease. Increasing concentrations of mycotoxins in the culture medium also increased the embryo degeneration. However, mycotoxin concentration in follicular fluid has been shown to vary according to the duration of follicular exposition to zearalenone. Follicular cysts have a higher concentration of zearalenone than normal follicles do (detection rates 35% and 18.8%) (Takagi et al., 2008). Under controlled feeding experiments, interestingly, ewes were more sensitive to zearalenone than sows

were (Smith et al., 1990). The ovulation rate and cycle length decreased, and the estrous duration increased, but the pregnancy rate was unaffected (Smith et al., 1992).
Some studies have demonstrated toxic effects of ZEN on the reproductive performance of animals as described above, but little information is available in the literature in relation to the effects of ZEN and its derivatives on the viability and development of sperm and oocytes.

Further research must be undetaken, not only to establish databases for ZEN and its metabolites in follicular fluid samples derived from donor animals for which the reproductive stage and feeding status are defined, but also to clarify the individual effects of mycotoxins on the maturation of porcine oocytes *in vivo*.

CHAPTER 1

Detection of Zearalenone and Its Metabolites in Naturally Contaminated Porcine Follicular Fluid by Using Liquid Chromatography-Tandem Mass Spectrometry

ABSTRACT

Zearalenone (ZEN) and its metabolites are important nonsteroidal estrogenic mycotoxins that cause reproductive disorders in domestic animals, especially pigs. We aimed to simultaneously detect ZEN and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) in porcine follicular fluid (FF) by liquid chromatography-tandem mass spectrometry. ZEN and α-ZOL, but not β-ZOL, were detected in all pooled FF samples collected from coexisting follicles (diameter ≥ 6 mm) within 10 ovaries. Furthermore, ZEN and α-ZOL were detected in samples pretreated with β-glucuronidase/arylsulfatase, but not in those left untreated, suggesting that the FF samples contained glucuronide-conjugated forms of the mycotoxins that may be less harmful to porcine oocytes due to glucuronidation affecting the receptor binding. Nonetheless, the effects of the glucuronide-conjugated forms should be studied, both in vitro and in vivo.

INTRODUCTION

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin that is produced by *Fusarium* species on several grains. Despite its low acute toxicity and carcinogenicity, ZEN and its metabolites exhibit distinct estrogenic and anabolic properties in several animal species because of their agonistic effect on the estrogenic receptor. Thus, they affect the reproductive system and play important roles in reproductive disorders in domestic animals, particularly swine (Diekman and Green, 1992; Coulombe, 1993; Fink-Gremmels and Malekinejad, 2007; Zinedine et al., 2007).

The presence of environmental pollutants with potential reproductive toxicity in the follicular fluid (FF) of livestock may be of particular importance because the oocyte completes maturation before ovulation within the FF (Kamarianos et al., 2003). Recently, Takagi et al, (2008) reported that the FF was naturally contaminated with ZEN and its metabolites and showed the *in vitro* effects of ZEN on oocyte maturation in cattle. However, no reports are available on the contamination of porcine FF by ZEN and its metabolites.

MATERIALS AND METHODS

Chemicals and solvents

ZEN was purchased from MP Biomedicals (Zen, 10 mg; Eschwege, Germany), α-Zol and β-Zol were purchased from Sigma (Z2000 and Z01665, respectively; 5 mg each; St. Louis MO, USA). Methanolic stock solutions with 1000 μg/L ZEN were stored at 4° C away from the light. Ammonium acetate, high-performance liquid chromatography (HPLC)-grade methanol, and HPLC-grade acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Sample preparation

Ovaries from cross-bred gilts (unknown age) were collected at a local slaughterhouse and transported to the laboratory in 0.9 % physiological saline at 35° C within 3 h, and the ovaries were stored at -30° C until sample preparation for (LC/MS/MS) analysis. Once the ovaries were thawed, FF was aspirated from follicles with a diameter >6 mm by using a 20-ml syringe fitted with an 18-G needle and pooled for each individual ovary. The volume of fluid collected from each ovary was recorded, and the FF was centrifuged at 3000 rpm for 15 min and subjected to LC/MS/MS. A total of 10 pooled FF samples from 10 porcine ovaries were examined.

LC/MS/MS analysis

The concentrations of ZEN and its metabolites, α-ZOL and β–ZOL, in FF were determined by LC/ MS/ MS as described previously with some modifications (Takagi et al., 2008). Briefly, FF (0.25 ml) was mixed with 4 ml of 50 mM sodium acetate butter (pH 4.8), and the mixture was incubated for 15 h at 37° C with 8μl of β glucuronidase/arylsulfatase solution. The sample was loaded onto a C18 solid-phase extraction (SPE) column (Strata, Phenomenex, Torrance, CA, USA) preconditioned with 3ml of methanol followed by 2 ml of 20mM Tris buffer (pH 8.5)/methanol (80/20). The column was washed with 2 ml of 20 mM Tris buffer (pH 8.5)/ methanol (80/20) and 3 ml of methanol (40%) and then centrifuged for 10 min at 2000 rpm to dry it out. The analytes were slowly eluted with 1 ml of 80% methanol (flow rate: 15 drops per min). The eluate was evaporated to dryness under a nitrogen stream.

The dried residue was redissolved with 1 ml of 75% acetonitrile and mixed thoroughly. It was then loaded onto an immunoaffinity column (IAC; Easi-Extract Zearalenone; R-Biopharm, Darmstadt, Germany) and treated on the basis of the manufacturer's instructions with minor modifications. Briefly, 1 ml of acetonitrile and 20 ml of PBS were mixed well, and the diluted medium was then loaded onto the IAC. The IAC was washed with 10 ml of PBS, and the analytes were slowly eluted with 1.5 ml of 100% acetonitrile (flow rate: 1 drop per second). The eluate was again dried under a nitrogen stream, and the residue was redissolved in exactly 100 μl of 80% methanol. Then, 20μl of the reconstituted solution was injected into the LC/MS/MS system.

LC/MS/MS was performed with an API 2000 system (Applied Biosystems, Foster City, CA,USA) equipped with an electrospray ionization interface and HPLC system (1200 Series; Agilent Technologies, Palo Alto, CA, USA). Briefly, chromatographic separation was achieved on an Inertsil ODS-3 column (4.6 (id) **x** 150 mm **x** 5μm: GL Science, Tokyo, Japan) at 40°C. The mobile phase comprised methanol (A) and water (B), and the following gradient conditions were used to separate the analytes: a linear increase in solvent A from 50 to 100% over 5 min and isocratic elution for 10 min at a flow rate of 200 ml/min. The column was isocratically re-equilibrated with 50% of solvent B applied for 7 min at a flow rate of 1000 ml/min. For LC/MS/MS analysis, a multiple reaction monitoring (MRM) system was used in the negative mode for the transition of ZEN *(m/z:* 317.0-130.5) and α/β-ZOL *(m/z:* 319.0-129.9). For each analyte, the instrument parameters were optimized by analysis of the corresponding standard solution (1.0 mg/I in methanol) at a flow rate of 10 ml/min, injected using a syringe pump integrated in the API-2000 mass spectrometer. The electrospray conditions for ZEN and α/β-ZOL were as follows: curtain gas, 20 psi; ion-spray voltage, -4500 V; turbo temperature, 500 C: collision energy, -48 eV for ZEN and -44 eV for α/β-ZOL; declustering potential, -36 V for ZEN and -51 Vfor α/β -ZOL: focusing potential, -260 V for ZEN and -280 V for α/β -ZOL; and entrance potential, -9.0 V for ZEN and -8.5 V for a/β-ZOL. Nitrogen was used as the nebulizer, curtain and collision gas. The mean recovery rates for ZEN, α -ZOL and β-ZOL were 116, 121 and 56%, respectively.

RESULTS

We aimed to simultaneously detect ZEN and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) in porcine follicular fluid (FF) by liquid chromatography-tandem mass spectrometry.

As shown in Table 1, ZEN and α-ZOL were detected in all 10 FF samples supplemented with β- glucuronidase/arylsulfatase solution during preincubation, while the 10 FF samples that were not treated with β-glucuronidase/arylsulfatase solution did not contain ZEN or α –ZOL, even at trace levels. Additionally, β-ZOL was not detected even at trace levels, irrespective of β- glucuronidase/arylsulfatase treatment.

Figure 1 shows representative LC/MS/MS chromatograms of the porcine FF samples contaminated with ZEN and α-ZOL. The mean (± SEM) concentrations of ZEN and α-ZOL were 38.9 ± 4.0 pg/ml (max and min: 54.8 and 15.2 pg/ml, respectively) and I7.6 ± 1.7 pg/ml (max and min: 26.4 and 10.0 pg/ ml, respectively), respectively.

CHAPTER 2

Effects of Exposure to Zearalenone on Porcine Oocytes and Sperm During Maturation and Fertilization in Vitro

ABSTRACT

This study reports about the *in vitro* effects of ZEN exposure on both porcine oocytes and sperm during in vitro maturation (IVM) and in vitro fertilization (IVF), respectively, with microscopic observations such as meiotic competence, the ability of sperm to penetrate the ooplasm, pronucleus formation, and development to the blastocyst stage. First, oocytes were cultured in ZEN-containing (0–1000 μg/l) maturation medium and then fertilized. The oocytes maturing *in vitro* without ZEN were fertilized in ZEN-containing fertilization medium. The maturation rates of oocytes and penetration ability of sperm decreased significantly in the presence of 1000 μg/l of ZEN. However, neither increases in the rates of degeneration and DNA fragmentation of oocytes nor reductions in normal and polyspermic fertilization were observed. ZEN did not affect the sperm penetration rates; however, 1000 μg/l ZEN had positive effects on normal and polyspermic fertilization rates. Therefore, it can be suggested that an acute exposure of porcine oocytes during maturation and of oocytes and sperm during fertilization to ZEN up to 1000 μg/l may not affect the fertility of the oocytes.

INTRODUCTION

Zearalenone (ZEN) is a nonsteroidal estrogen-like mycotoxin produced by *Fusarium* species on several grains. It is an estrogen receptor agonist: its distinct estrogenic and anabolic properties in several animal species exert detrimental effects on the reproductive system resulting in reproductive disorders in domestic animals, particularly in swine (Diekman and Green, 1992; Coulombe, 1993; Fink-Gremmels, Malekinejad, 2007; Zinedine et al., 2007). Although in vitro culture systems do not always provide accurate predictions of toxicity in animals, they can be used to assess risks and can help to define the mechanisms by which mycotoxins act on germ cells (Minervini and and Dell'Aquila, 2008).

Several *in vitro* culture assays have been employed to determine the effect of ZEN and its metabolites on the reproductive organs of swine. Previous *in vitro* experiments revealed that exposure to these mycotoxins affects oocyte maturation, pronucleus formation and embryonic development (Alm et al., 2002; Malekinejad et al., 2007), as well as viability, motility and acrosome reactions in sperm (Tsakmakidis et al., 2006; Benzoni et al.,2008). Nevertheless, the acute effects of exposure to ZEN during *in vitro* fertilization remain unknown.

MATERIALS AND METHODS

Collection and maturation of oocytes

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% physiological saline at 35° C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles 3 to 6 mm in diameter by using an 18-gauge needle connected to a 5-ml disposable syringe. They were collected in modified phosphate-buffered saline (m-PBS; Nippon Zenyaku Kogyo CO. Ltd., Fukushima, Japan) supplemented with 100 IU/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. Only COCs with a uniform ooplasm and compact cumulus cell mass were used in this experiment.

The COCs were cultured for 22 h in a maturation medium, tissue culture medium (TCM) 199 medium (Earle's salts) with 25 mM HEPES buffer (Invitrogen Corp., Carlsbad, CA, USA), 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma, St. Louis, MO, USA), 50 μM sodium pyruvate (Sigma), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries, Osaka, Japan), 1 μg/ml 17β-estradiol (Sigma), 50 μM β-mercaptoethanol (Wako), 10 IU/ml equine chorionic gonadotropin (Kawasaki-Mitaka, Kawasaki, Japan), 10 IU/ml human chorionic gonadotropin (Kawasaki-Mitaka), and 50 μg/ml gentamicin (Sigma). Approximately 15 to 25 COCs were cultured in 500 μl of the maturation medium in a 35 × 10-mm Petri dish (Falcon, Franklin Lakes, NJ, USA) for 22 h. They were then transferred to the maturation medium without hormones and cultured for an additional 22 h. All cultures were performed in a humidified incubator at 38.5° C containing 5% CO_2 in air.

In vitro fertilization

In vitro fertilization was carried out according to the method described by Kikuchi et al (2002) with minor modifications. The sperm-rich fractions of ejaculates were obtained from a large white boar and frozen as described previously (Yuge et al., 2003). Spermatozoa were thawed and preincubated for 15 min at 38.5°C in TCM 199 medium adjusted to pH 7.8. The preincubate spermatozoa were introduced into fertilization medium containing approximately 10 matured oocytes. The fertilization medium consisted of 90 mM NaCl, 12 mM KCl, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate (Sigma), 3 mg/ml bovine serum albumin (BSA; fatty acid-free, Sigma), 5

mM caffeine (Sigma), and 50 μg/ml gentamicin. The final sperm concentration was adjusted to 1×10^6 cells/ml. The oocytes were coincubated with spermatozoa for 5 h. The cumulus cells and spermatozoa were removed from the inseminated oocytes by mechanical pipetting, and the denuded oocytes were subsequently transferred to a culture medium. Putative zygotes were cultured in Porcine Zygote Medium (PZM-5; IFP, Yamagata, Japan) (Yoshioka et al., 2002) covered with paraffin oil in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 38.5° C.

Assessment of oocyte nuclear status, DNA damage, and fertilization

At the end of the IVM culture, oocytes were mechanically denuded from cumulus cells in Dulbecco's PBS (Invitrogen) supplemented with 1 mg/ml hyaluronidase (Sigma). The denuded oocytes in each group were analyzed in terms of DNA damage and nuclear status by using a combination of nuclear staining and TUNEL by a modification of procedures described previously (Otoi et al., 1999). Briefly, the oocytes were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. They were then permeabilized in PBS containing 0.5% (v/v) Triton-X100 for 1 h and incubated in PBS containing 10 mg/ml BSA (blocking solution) overnight at 4°C. Next, they were incubated in fluorescein -conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1h at 38.5° C under 5% CO_2 in air. After the TUNEL procedure, the oocytes were counterstained with 25 μg/ml bisbenzimide (Hoechst 33342, Sigma) for 30 min. They were then washed in blocking solution, treated with an antibleaching solution (SlowFade; Molecular Probes, Eugene, OR, USA), mounted on a glass slide and sealed with clear nail polish. Labeled oocytes were examined using a Nikon Diaphot microscope fitted with epifluorescent illumination. To assess the relationship between nuclear status and DNA damage, they were classified according to their chromatin configuration as germinal vesicles, condensed chromatin, metaphase I, anaphase I and telophase I, or MII. Those with a diffusely stained cytoplasm characteristic of nonviable cells, and those in which the chromatin was unidentifiable or not visible, were excluded from the analysis of DNA damage. At 10 h after IVF, the presumptive zygotes were mounted on a glass slide and fixed with acetic acid/ethanol (1:3 v/v) for 48 to 72 h. The fixed oocytes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope.

The following parameters of fertilization were assessed: (Diekman and Green 1992) total sperm penetration rate, calculated from the proportion of oocytes forming a single female pronucleus and single or multiple penetrating sperm nuclei or male pronuclei; (Coulombe,

1993) polyspermic penetration rate, calculated from the proportion of oocytes forming a single female pronucleus and multiple penetrating sperm nuclei or male pronuclei; and (Fink-Gremmels and Malekinejad, 2007) male pronucleus formation rate, calculated from the proportion of oocytes with a male pronucleus.

Experimental design

In the first experiment, the toxic effects of ZEN in the maturation medium on the meiotic maturation and fertilization of porcine oocytes were assessed. The oocytes were randomly allotted for experimental treatment. COCs were cultured in maturation medium supplemented with 1, 10, 100, or 1000 μg/l of ZEN during the complete IVM culture period. In the control sample, oocytes were cultured in the maturation medium without ZEN (control group). Paraffin oil was not used to cover the culture dishes to prevent the absorption of ZEN from the culture medium. At the end of IVM, the meiotic status of the oocytes was examined as described above. At the end of the maturation culture, some of the oocytes were fixed to examine nuclear status and DNA damage, and others were subjected to IVF for evaluations of sperm penetration and pronuclear formation at 10 h after IVF.

In the second experiment, the effects of adding ZEN to the IVF medium on the fertilization of porcine oocytes were evaluated. All COCs were cultured in maturation medium without ZEN. After the maturation culture, the COCs were fertilized with spermatozoa in fertilization medium (500 μl) supplemented with 0, 1, 10, 100, or 1000 μg/l of ZEN. Paraffin oil was not used to cover the fertilization medium. At the end of IVF, the fertilization status of the oocytes was examined as described above.

Statistical analysis

Statistical analyses were carried out with an analysis of variance (ANOVA) and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts Inc., Berkeley, CA, USA) program. All percentage values were subjected to arcsine transformation before the statistical analysis. Data were expressed as the mean ± SEM. Differences of $P < 0.05$ were considered significant.

RESULTS

The influence of acute exposure to zearalenone (ZEN) on porcine oocyte maturation, fertilization, or sperm penetration ability during both *in vitro* maturation and fertilization was evaluated.

As shown in Table 2, exposure to 1000 μg/l of ZEN had a negative effect on the meiotic competence of porcine oocytes ($P < 0.05$). However, there were no significant differences among the groups with respect to the percentages of oocytes showing degeneration and DNA damage. Exposure to100 and 1000 μg/l of ZEN during maturation culture decreased the total rate of sperm penetration ($P < 0.05$) compared with the control group, but did not influence the rates of normal or polyspermic fertilization of oocytes.

As shown in Table 3, exposure to ZEN during IVF did not affect total rates of sperm penetration irrespective of ZEN concentration. However, exposure to 1000 μg/l of ZEN had a positive effect on the rates of normal and polyspermic fertilization of oocytes ($P < 0.05$) compared with the rates in the control group.

CHAPTER 3

Effects of Long – Term In Vitro Exposure of Ejaculated Boar Sperm to Zearalenone and α- Zearalenol in Sperm Liquid Storage Medium

ABSTRACT

The effects of *in vitro* exposure of porcine spermatozoa to zearalenone (ZEN) and α-zearalenol (α-ZOL) were studied by evaluating several parameters of an *in vitro* fertilization (IVF) system. For this purpose, boar spermatozoa cultured with semen storage medium containing 0 (control), 10 and 1000 μg/l of ZEN and α-ZOL for 1 week at 5°C were used for IVF of *in vitro* matured oocytes. Overall, there were no significant differences in the rates of total penetration, monospermic fertilization, and polyspermic fertilization of oocytes inseminated with spermatozoa from the different groups. Similarly, ZEN and α-ZOL at 10 and 1000 μg/l did not have detrimental effects on the cleavage and development to blastocysts of oocytes after *in vitro* fertilization. Although the motility, viability, and plasma membrane integrity of spermatozoa significantly decreased after 3 weeks of storage compared to non-stored spermatozoa ($P < 0.05$), ZEN and α-ZOL at the evaluated concentrations did not exert detrimental effects on the above parameters, even after 3 weeks of storage. These results indicate that prolonged exposure of boar spermatozoa to ZEN and α-ZOL up to 1000 μg/l under reduced metabolic conditions does not affect their *in vitro* function.

INTRODUCTION

Zearalenone (ZEN) is a nonsteroidal estrogen-like mycotoxin produced by *Fusarium* species on several grains. Zearalenone and one of its metabolites α-zearalenol (α-ZOL), present in the blood in swine, are estrogen receptor agonists; their distinct estrogenic and anabolic properties in several animal species cause it to exert detrimental effects on the reproductive system, resulting in reproductive disorders in domestic animals, particularly in swine (Diekmann and Green, 1992; Coulombe, 1993; Fink-Gremmel and Malekinajad, 2007; Zinedine et al., 2007). To date, the toxic effects of ZEN and α-ZOL on female reproductive performance have been widely investigated, including oocyte maturation, pronucleus formation, and embryonic development (Alm et al., 2002; Malekinajad et al., 2007).

However, few studies have reported on the influences of ZEN and α-ZOL in the male, possibly due to the lack of adverse effects on the reproductive potential of mature boars *in vivo*, with the exception of a previously reported reduction in plasma testosterone levels (D'Mello et al., 1999; Minervini and Dell'Aquila, 2008). Although *in vitro* culture systems do not always provide accurate predictions of toxicity in animals, they can be used for risk identification and can help to define the mechanisms by which mycotoxins act on germ cells (Minervini and Dell'Aquila, 2008). Several *in vitro* culture assays have been employed to determine the effect of ZEN and its metabolites on viability, motility, and acrosome reactions in sperm cells (Tsakmakidis et al., 2006; Benzoni et al., 2008).

We also recently reported the *in vitro* effects of ZEN exposure on both porcine oocytes and sperm during in vitro maturation (IVM) and in vitro fertilization (IVF), respectively, with microscopic observations such as meiotic competence, the ability of sperm to penetrate the ooplasm, pronucleus formation, and development to the blastocyst stage (Sambuu et al., 2011b). The results of these experiments suggested that acute exposure of porcine oocytes and sperm to ZEN during the periods of IVM (44 h) and IVF (5 h), even at high ZEN concentrations (up to 1 ppm, equivalent to 1000 μg/l), might not affect their meiotic competence or fertility. On the other hand, it was recently suggested that although ZEN chemicals are ubiquitous, they are generally considered a potential hazard for both human and animal health, particularly when they are absorbed in high amounts or over a long exposure time (Massart and Saggese, 2010). Indeed, previous reports have demonstrated lower testicular weight and decreased motility of spermatozoa in boars after

continuous ingestion of low ZEN concentrations, indicating that chronic exposure to ZEN may affect testicular epididymal function (Young and King, 1986; Tsakmakidis et al., 2006).

However, to our knowledge, no report on long term *in vitro* exposure (>48 h) of boar spermatozoa to ZEN and α-ZOL are available, possibly due to the concentration-independent toxicity of all mycotoxins that is induced by long-term *in vitro* incubation/storage of boar sperm (Minervini and Dell'Aquila, 2008). Thus, the use of another *in vitro* storage method to confirm the effects of long-term ZEN exposure of boar sperm is critical.

Recently, Namula et al. (2011) confirmed the feasibility of storing boar sperm at 5°C for a 2-week period using a newly modified storage extender of boar sperm, by evaluating both sperm morphology and *in vitro* embryonic development following the fertilization of IVM oocytes by these spermatozoa.

This study aimed to examine the effects of *in vitro* exposure of porcine sperm to both ZEN and α-ZOL by evaluating several parameters of sperm in the process of *in vitro* fertilization.

MATERIALS AND METHODS

Semen collection and preparation

Sperm-rich fractions of ejaculates were collected weekly from three Large White boars (age, 1.5–2 years) maintained at the Tokushima Prefectural Livestock Research Institute using the gloved-hand technique. The sperm-rich fraction from each boar was diluted tree times with a modified Modena solution (Funahashi and Sano, 2005) without glucose but supplemented with D(-) fructose and skim milk, which consisted of 27.5 mg/ml D(-) fructose (Wako Pure Chemical Industries, Osaka, Japan), 6.9 mg/ml trisodium citrate dihydrate (Wako Pure Chemical Industries), 2.35 mg/ml ethylenediaminetetraacetic acid, disodium salt (EDTA-2Na, Wako Pure Chemical Industries), 1.0 mg/ml sodium hydrogen carbonate (Wako Pure Chemical Industries), 2.9 mg/ml citric acid monohydrate (Wako Pure Chemical Industries), 5.65 mg/ml Tris [hydroxymethy] aminomethane (Sigma-Aldrich, St. Louis, MO, USA), 7.5 mg/ml skim milk (Snow Brand Milk Products, Co., Ltd., Hokkaido, Japan), and 0.2 mg/ml amikamycin sulfate (Meiji, Tokyo, Japan) in distilled water.

The diluted semen in a 50-ml glass tube was placed in a refrigerator at 15°C for 3 h and then transported to the laboratory at 15° C within 24 h after collection. The semen from each boar in Modena solution was centrifuged at 550 × *g* for 2 min. After discarding the supernatant, the pellet was mixed and then diluted with Modena solution supplemented with 10 and 1000 μg/l of ZEN or α-ZOL to give a final sperm concentration of 1×10^8 cells/ml. The semen samples (about 5 ml) were then transferred into 15-ml polystyrene conical tubes. To avoid cooling too rapidly and to reduce the effects of cold temperature shock to the spermatozoa during the chilling process, the tubes were placed in a 500-ml glass beaker containing 350 ml of water at 15° C, then cooled in the refrigerator at 5°C and stored for 3 weeks. An aliquot (100 μl) of each sample was used for the analyses of sperm characteristics before storage, and at 1 and 3 weeks after storage, as described below. The concentrations of both ZEN and α-ZOL supplemented into the Modena solution were measured using liquid chromatography-tandem mass spectrometry (LC/MS/MS) for the accurate confirmation of each concentration as described previously (Takagi et al., 2011) with minor modifications. Briefly, 0.5 ml each of storage medium samples collected at day 0 (before culture) and 3 weeks after storing were diluted with 20 ml phosphate-buffered saline (PBS; pH 7.0 to 7.2), and the solution was loaded onto an immunoaffinity column

(IAC; DZT MS Prep, R-Biopharm Rhone Ltd, Glasgow, Scotland, UK), followed by the addition of 20 ml distilled water to wash the IAC. The IAC was then washed with 1 ml 100% methanol and dried, and the volume of the eluted solution was adjusted to exactly 1 ml with acetonitrile. Then, 20 µl of the reconstituted solution was injected into the LC/MS/MS. Overall, the concentrations of ZEN and α-ZOL supplements, at a final expected concentration of 1000 µg/l, were 953 and 825 µg/l, and 1155 and 1029 µg/l, at day 0 and 3 weeks, respectively. These results clearly indicate that both ZEN and α-ZOL were present at the required concentrations in the storage medium during the 3-week culture period.

Sperm quality assessment

Sperm motility was assessed using a computer-assisted sperm motility analysis (CASA) system (Sperm Class Analyze; Microptic, Barcelona, Spain). Briefly, for each sample, a 5-µl aliquot of semen was placed on the analyzer chamber (Leja Products B.V., Nieuw-Vennep, The Netherlands) which was maintained at 37°C during analysis. The CASA system was based upon the analysis of 16 consecutive digitalized photographic images obtained from a single field at magnification of 200 × on a dark field. These 16 consecutive photographs were taken at a time lapse of 0.64 s, which indicates an image capture velocity of one photograph every 40 ms. Five separate fields were taken for each sample. Total sperm motility was defined as the percentage of spermatozoa that had any form of motility.Sperm viability was assessed by a live/dead stain combination (SYBR-14/propidium iodide [PI], Fertilight Kit; Molecular Probes, Eugene, OR, USA) with minor modifications (Blanco et al., 2000). Briefly, a seminal aliquot (5 µl) was mixed with a 50-µl solution containing 5 µl PI (diluted 1:100 in distilled water) and 1 µl SYBR-14 (diluted 1:100 in dimethyl sulfoxide (DMSO)). Assessments of 100 spermatozoa were made in duplicate aliquots for every sample and evaluated under a fluorescent microscope (400×, Optiphot-2; Nikon, Tokyo, Japan) with 480-nm wavelength excitation filters. Plasma membrane integrity of sperm was assessed by the hypoosmotic swelling test (Ahmad et al., 2003).

Briefly, a seminal aliquot (10 µl) was mixed with 100 µl of 100 mmol/L hypoosmotic sucrose solution (150 mOsm/L), which consisted of 13.5 mg/ml D(-) fructose, and 7.35mg/mL trisodium citrate dihydrate in pure water. After incubation of the samples for 10 min at 37°C, 10 µl of each sample was placed on a slide and overlaid with a cover glass.

The plasma membrane integrity of sperm was expressed as the percentage of sperm with curled tails (intact plasma membrane) in total spermatozoa. Assessments of 100 spermatozoa were made in 3 fields from 1 aliquot for every sample using a phase-contrast microscope (400×, Nikon TE300, Tokyo, Japan). Acrosomal integrity was measured by fluorescein isothiocyanate FITC-labeled peanut agglutinin (FITC-PNA; Vector Laboratories Inc., Burlingame, CA, USA) and PI staining. The samples were spread over the slides, air dried at room temperature, and fixed with absolute ethanol for 10 min at room temperature. After drying, FITC-PNA solution (100μg/ml) in PBS was spread over each slide, and the slides were then incubated in a dark, moist chamber for 30 min at 37°C, followed by the addition of PI stain solution and incubation for an additional 10 min. They were then sufficiently rinsed with PBS, air-dried, and then mounted with 10 ml of antifade solution (glycerol: PBS, 1:1) to preserve fluorescence. A coverslip was then applied, and the edges were sealed with colorless nail polish. Acrosome status (intact or reacted) was recorded according to the FITC-PNA staining pattern (Okazaki et al., 2009). A total of 100 spermatozoa were counted in at least three different fields. The acrosomal integrity was expressed as the mean percentage of acrosome-intact spermatozoa.

Oocyte collection and in vitro maturation

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% physiological saline at 35°C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles 3-6 mm in diameter using an 18-gauge needle connected to a 5-ml disposable syringe. The COCs were collected in modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/ml penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). Only COCs with a uniform ooplasm and compact cumulus cell mass were used in this experiment.

The COCs were cultured for 22 h in a maturation medium, tissue culture medium (TCM) 199 (Earle's salts) with 25 mmol/L HEPES buffer (Invitrogen Corp., Carlsbad, CA), 10% (v/v) porcine follicular fluid, 0.6 mmol/L cysteine (Sigma-Aldrich), 50 μmol/L sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries), 1 μg/ml 17β-estradiol (Sigma-Aldrich), 50 μmol/L β-mercaptoethanol (Wako Pure Chemical Industries), 10 IU/ml equine chorionic gonadotropin (Kawasaki-Mitaka, Kawasaki, Japan), 10 IU/ml human chorionic gonadotropin (Kawasaki-Mitaka), and 50 μg/ml gentamicin (Sigma-Aldrich). They were then transferred to the maturation medium without hormone

and cultured for an a additional 22h. All culture were grown in a 35°C humidified incubator containing 5% CO_2, in air

In vitro fertilization (IVF) and the assessment

In vitro fertilization was carried out according to the method described by Yoshioka et al. (2008) with minor modifications. Spermatozoa stored in each Modena solution supplemented with ZEN or α-ZOL were transferred into 5 ml of fertilization medium (PFM; Research Institute for the Functional Peptides Co.,Yamagata, Japan) in a 15-ml test tube and then were washed by centrifugation at 500 × *g* for 5 min. The pellet of spermatozoa was resuspended in the fertilization medium to give a final sperm concentration of 1×10^7 cells/ml. A portion (50 μl) of spermatozoa was introduced into 50 μl of fertilization medium containing 10–20 mature oocytes. The final sperm concentration was adjusted to 5×10^6 cells/ml.

The oocytes were co-incubated with spermatozoa for 20 h in a 38.5°C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. After co-incubation with spermatozoa for 20 h, the inseminated oocytes were denuded from the cumulus cell and the attached spermatozoa by mechanical pipetting. To assess the fertility of spermatozoa, some presumptive zygotes were mounted on a glass slide and fixed with acetic acid: ethanol (1:3 v/v) for 48-72 h. The fixed zygotes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined under a phase contrast microscope. Oocytes containing both female and male pronuclei were considered as fertilized and categorized as normal or polyspermic according to the number of swollen sperm heads and pronuclei in the cytoplasm.

Embryo culture (IVC) and the assessment

To evaluate their ability to develop to the cleavage and blastocyst stages, the inseminated oocytes were subsequently transferred to 100 μl droplets of PZM-5(Research Institute for the Functional Peptides Co., Yamagata, Japan) in a 38.5°C humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. Each droplet contained approximately 10 presumptive zygotes. At 72 h after insemination, all cleaved embryos were transferred into fresh PZM-5 supplemented with 5% (v/v) fetal bovine serum (Invitrogen Corp.), and then cultured for an additional 5 days to evaluate their ability to develop to the blastocyst

stage. On day 8 (day 0 = insemination), all embryos were fixed and permeabilized for 15 min at room temperature in PBS containing 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) and then placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. Embryos were then placed in a drop of mounting medium on a slide. The mounting medium consisted of 90% (v/v) glycerol containing 1.9 μM Hoechst 33342 (Sigma-Aldrich). The embryos were subsequently overlaid with a cover slip supported by four droplets of vaseline/paraffin and incubated for 1 night at 4°C. The embryos were examined under a fluorescence microscope with a 355-nm wavelength excitation filter. Embryos clear blastocole and cells were defined as blastocysts. The numbers of cleaved embryos and blastocysts were recorded.

Statistical analysis

Statistical analyses were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) test using the STATVIEW (Abacus Concepts, Inc, Berkeley, CA, USA) program. All percentage data were subjected to arc sin transformation before statistical analysis. Data were expressed in terms of the mean and SEM. Differences at a probability value (P) of 0.05 or less were considered to be significant.

RESULTS

Fertilization and development of oocytes inseminated with spermatozoa stored with ZEN and α ZOL for 1 week

The fertilization and development of oocytes inseminated with spermatozoa stored in Modena solution with ZEN and α-ZOL at 5° C for 1 week are shown in Tables 4 and 5. There were no significant differences in the percentages of total penetration, monospermic fertilization, and polyspermic fertilization of oocytes inseminated with spermatozoa from the different groups, irrespective of the concentrations of ZEN and α-ZOL. Similarly, ZEN and α-ZOL had no detrimental effects on the cleavage and development to blastocysts of oocytes after IVF.

Quality of spermatozoa stored with ZEN and α-ZOL for 3 weeks

The motility, viability, plasma membrane integrity, and acrosomal integrity of spermatozoa stored in Modena solution with ZEN and α-ZOL at 5° C for 3 weeks were analyzed. ZEN and α-ZOL showed no detrimental effects on all parameters of spermatozoa, irrespective of their concentrations, even when the spermatozoa were stored for 3 weeks (Figs.2 and 3). However, the percentages of motility, viability, and plasma membrane integrity of spermatozoa were significantly decreased after 3 weeks of storage when compared to non-stored spermatozoa ($P < 0.05$).

OVERALL DISCUSSION AND CONCLUSIONS

It has been reported that reproductive system is a major target of zearalenone (Minervini and Dell'Aquila, 2008). Zearalenone was identified in 1952 and its chemical structure was determined in 1966 (Urry, et al., 1966). Alpha-ZEA might bind to estrogen receptors 1 and 2 decrease fertility, increase embryolethal absorption, reduce litter size, and change the weight of the adrenal and the pituitary glands (Creppy, 2002).

In the first series of experiment, we detected ZEN and α-ZOL but not β-ZOL in porcine FF only after the FF was treated with β-glucuronidase/arylsulfatase solution. This finding strongly suggests that ZEN and α-ZOL are present within porcine follicles in their glucuronide –conjugated forms within porcine follicles. To our knowledge, this is the first time that ZEN and its metabolites have been detected in naturally contaminated porcine FF. Although the conditions of the sows prior to slaughter are unknown, we can assume that they were given feed naturally contaminated with ZEN, since this mycotoxin and α-ZOL, but not β-ZOL, were detected in all 10 FF samples. Additionally, studies have clearly shown that hepatic biotransformation converts ZEN in porcine FF predominantly into α-ZOL, and ZEN may even be extrahepatically biotransformed into α-ZOL in porcine granulosa cells through the action of 3α-hydroxysteroid dehydrogenase, as reported by Malekinejad et al, (2006a, b). Our results strongly support these previous findings.

Generally, blood estradiol-17β (E2) concentrations during the porcine estrous cycle range from 10 to 50 pg/mL (Cox and Tubbs, 1977), and this hormone acts on E2 receptor-positive reproductive organs such as the oviducts, endometrium, and uterine glands. In the present study, the maximum ZEN and α-ZOL concentrations in the FF were 54.8 pg/mL and 26.4 pg/mL, respectively. These concentrations are within the normal range for the E2 concentration in circulating blood during the estrous cycle of sows. Generally, chronic exposure to environmental pollutants is expected to lead to their accumulation in body fat and serum and consequently in FF (Wolff et al., 1983;Kamarianos et al., 2003). Therefore, as we suggested in our previous study with bovine FF samples (Takagi, et al., 2008), the levels of both ZEN and α-ZOL in the FF may greatly depend on the blood ZEN concentration. Thus, the current results suggest that ZEN and α-ZOL bind to E2 receptors and functionally affect the genital tract and accessory genital glands of sows.

Further, in the present study, we examined the glucuronide conjugation of ZEN and its metabolites within porcine follicles by examining FF with and without β-glucuronidase/arylsulfatase treatment during extraction. Interestingly, the ZEN and α-ZOL detected in the porcine FF were entirely in the glucuronide-conjugated forms, which would not bind to E2 receptors within the oocytes, and we could not detect the no glucuronide-conjugated forms even in trace amounts. Glucuronidation of ZEN and its metabolites in the liver is much more frequent in swine than in other animals (Malekinejad , et al., 2006b).

Therefore, the results of the present study indicate that porcine oocytes within the follicular environment in vivo may not be affected by exposure to ZEN and its metabolites during maturation under normal feed conditions. Alm et al, (2006) investigated the effects of feeds naturally contaminated with *Fusarium* toxins [deoxynivalenol (DON) above the critical concentration and ZEN below the critical concentration] for 35 days and found that high concentrations of these mycotoxins were associated with oocyte degeneration and reduced meiotic competence after in vitro maturation in swine. Additionally, Malekinejad et al. (2007) reported that ZEN and DON could cause abnormal spindle formation, leading to less fertile oocytes and embryos with abnormal ploidy, and that the effects of ZEN and DON were not synergistic. Obviously, further research is required, not only to establish databases for ZEN and its metabolites in FF samples, but also to clarify the individual effects of these mycotoxins on the maturation of porcine oocytes in vivo. Additionally, it may be worth elucidating the effects of glucuronide-conjugated ZEN and α-ZOL on porcine oocytes in vitro.

In conclusion, our results indicated that ZEN and α-ZOL, but not β-ZOL, can be detected in porcine FF by LC/MS/MS analysis, even though their concentrations were rather low. The detected ZEN and α-ZOL were entirely in the glucuronide-conjugated forms. It may be necessary to consider the use of in vitro culture systems for evaluating the effects of ZEN and its metabolites on reproductive function in farm animals, especially with regard to porcine oocytes.

In the second series of experiments, cell and tissue culture systems seems to be are useful for evaluating risks posed by toxic compounds such as mycotoxins (Minervini and Dell'Aquila, 2008; Tiemann and Danicke, 2007). Malekinejad et al. (2007) reported that ZEN and its metabolites reduce fertility *in vitro* by altering spindles during meiosis, leading to less fertile oocytes and mixoploid embryos. In our IVM/IVF system, we confirmed that exposure to a high concentration (1000μg/l) of ZEN during maturation can

have detrimental effects on the meiotic competence and fertility of porcine oocytes without affecting either DNA fragmentation after IVM or rates of normal or polyspermic fertilization after IVF.

A significant decrease in the metaphase II (MII) rate was evident in the group exposed to 1000 μg/l of ZEN when compared with that in the control group. However, such a difference was not due to DNA fragmentation. Malekinejad et al. (2007) reported that ZEN (0.312 μM; approximately equivalent to 100 μg/l) inhibits maturation of porcine oocytes *in vitro* and increases the percentage of oocytes that contain aberrant nuclei. In the present study, the rates of maturation to MII and total fertilization of oocytes in the control group were lower than those reported in other studies (Kikuchi et al., 2002 ; Suzuki et al., 2000). Moreover, porcine oocytes were cultured in maturation and fertilization media that were not covered by paraffin oil to prevent the absorption of ZEN by oil from the culture medium. Therefore, the differences in the rates of maturation, DNA fragmentation, and fertilization of oocytes observed between the previous studies and the present study might be attributable in part to the culture conditions.

Despite a significant reduction in the rate of penetration by sperm, there were no significant effects of ZEN on normal or polyspermic fertilization in the present study. A high frequency of polyspermy is a major problem with the IVF of porcine oocytes (Wang et al., 1994; Li et al., 2004) examined the effects of estradiol-17β (E2) on the maturation and subsequent fertilization of porcine oocytes *in vitro*, and found that E2 inhibited both the nuclear and cytoplasmic maturation of cumulus-enclosed oocytes, possibly by suppressing production of progesterone by cumulus cells through an estrogen receptor - mediated pathway. It was suggested that both the exocytosis of cortical granules and the events that precede it are important in establishing a functional block to polyspermy (Wang et al., 1994). Therefore, on the basis of our results, the adverse effects of ZEN at a concentration of up to 1000 μg/l on the cytoplasmic maturation of porcine oocytes *in vitro* seemed not to be severe, although hardening of the zona pellucida may have occurred owing to the release of cortical granules.

In the present study, no significant change in the sperm penetration rate was observed when oocytes were fertilized with spermatozoa in IVF medium supplemented with ZEN. Moreover, exposure to 1000 μg/l of ZEN had a positive effect on the rates of normal and polyspermic fertilization of oocytes. Our results contradict a previous study by

Tsakmakidis et al. (2007), who reported a significant decrease in the number of tightly attached spermatozoa in an *in vitro* hemizona assay with ZEN-supplemented medium. They suggested that ZEN affects the sperm-zona interaction by reducing the ability of boar spermatozoa to bind to the zona pellucida. In their study, boar semen was exposed to 40 to 80 μg/ml of ZEN and α-ZOL *in vitro*, in which the ZEN concentration was 40 to 80 times higher than the maximum concentration of ZEN (1000 μg/l) used in the present study. Therefore, this difference may reflect the difference in the concentration of ZEN to which the sperm were exposed. Our results suggest that exposure of sperm and oocytes to a ZEN concentration of up to 1000 μg/l during the IVF period does not have a detrimental effect on the ability of boar sperm to penetrate the zona pellucida and form a pronucleus. In a previous study, we found that ZEN could be detected in porcine follicular fluids collected from follicles with diameters of ≥ 6 mm, but its concentrations, which might have reflected the physiological level in porcine follicular fluids, was rather low (<55 pg/ml) (Sambuu et al., 2011a). Our results showed that exposure to ZEN during IVM of porcine oocytes at a concentration of up to 100 μg/l did not affect the meiotic competence or fertility after IVF even though the exposure concentration of ZEN was much higher. In addition, *in vitro* ZEN exposure during IVF did not influence the ability of sperm to penetrate the zona pellucida or form a pronucleus. Therefore, acute exposure to ZEN during IVM and fertilization of porcine oocytes, even at a higher concentration of ZEN, may not affect the meiotic competence or fertility of oocytes. However, further study is required not only to create databases of the concentrations of both ZEN and its metabolites in follicular fluid samples as suggested previously (Malekinejad et al., 2007; Sambuu et al., 2011), but also to establish culture systems for risk assessments and to define the mechanism of action of germ cells.

In the third series of experiments, we aimed to clarify the long-term exposure effects of ZEN and α-ZOL on boar spermatozoa using our modified storage medium and *in vitro* culture system for boar spermatozoa in order to simulate ZEN and α-ZOL contamination of ejaculated semen. It has been suggested that the negative effects of ZEN and α-ZOL on boar fertility could be the consequence of the interaction between ZEN or α-ZOL and estrogen receptors found in boar spermatozoa (Rago et al., 2007; Benzoni et al., 2008). Although previous studies of the effects of *in vivo* exposure (ingestion) to ZEN on the reproductive potential of immature boars (Young and King, 1986) have revealed

detrimental effect of reduced total motile spermatozoa count, possibly due to the occurrence of multiple interactions in the course of spermatogenesis (Minervini and Dell'Aquila, 2008), the results of our study suggest that direct exposure of boar spermatozoa to ZEN or α-ZOL at concentrations up to 1000 μg/l under reduced metabolic conditions (5° C) might not affect sperm cell quality.

Several previous reports are available regarding the effects of *in vitro* exposure of porcine spermatozoa to ZEN and its metabolites, evaluated by several parameters. Tsakmakidis et al. (2006, 2007) reported the *in vitro* effects of ZEN and α-ZOL exposure on (1) boar sperm characteristics (motility, viability, and acrosome reaction) using several doses of ZEN and α-ZOL (range, 125-250 μM) and a 4-h exposure time, and (2) sperm-zona pellucida interaction by the hemizona assay using 40, 60, and 80 μg/ml each of ZEN and α-ZOL, with a 1-h exposure time. This study confirmed the time- and dose-dependent direct toxic effects of ZEN and α-ZOL on boar spermatozoa as well as on the binding ability of boar sperm to the zona pellucida. Tsakmakidis et al. (2008) additionally studied the *in vitro* effects of ZEN and α-ZOL on the motility and nuclear chromatin integrity (NCI) of boar spermatozoa at levels ranging from 10-30 μg/ml after 4 h of exposure, and observed no effect on sperm motility, whereas the effect on sperm NCI was dependent on the individual. Additionally, Benzoni et al. (2008) used computer-aided sperm analysis to examine the *in vitro* effects of direct exposure for 24 and 48 h to ZEN and α-ZOL at several concentrations ranging from 1×10^{-8} to 1 μmol/L, and reported that α-ZOL proved more active in denaturing the chromatin structure, while ZEN had a significant effect on viability at concentrations much lower than the blood and urine levels found in sows fed with contaminated feed. Although we did not focus on the physiological levels of ZEN and α-ZOL in boar seminal plasma in the present study, we selected 2 concentrations (10 μg/l, or approximately 3.12×10^{-2} μmol/L; and 1000 μg/l, or approximately 3.12 μmol/ for evaluating their toxic effects, based on our previous studies of ZEN concentrations in porcine follicular fluid (Sambuu et al., 2011a, b) and in the blood and urine of pigs fed with or without ZEN-contaminated feeds (Olsen et al., 1985). Our results indicate that although a significant time-dependent reduction of motility and viability of spermatozoa was observed after 3 weeks of storage compared with spermatozoa that had not been presented, no harmful effects of ZEN and α-ZOL exposure were seen in any of the parameters evaluated by sperm morphological observation (3 weeks after), and in developmental rates to the blastocyst stage after IVF with spermatozoa stored for 1 week.

Thus, we conclude that either ZEN or α-ZOL contamination in the ejaculated semen may be critical for the purpose of artificial insemination after liquid preservation at 5° C, under the normal/physiological feeding conditions of boar management. In the present study, we also examined the fertilization of oocytes inseminated with porcine spermatozoa without exposure to ZEN and α-ZOL (control group) during storage at 5°C for 1 week; the rates of sperm penetration and monospermic fertilization were 53.0% and 30.6%, respectively. In our previous study (Sambuu et al., 2011a), we examined the effects of ZEN exposure on both porcine oocytes and spermatozoa during the insemination period for 5 h in a similar *in vitro* fertilization system, and the rates of sperm penetration and monospermic fertilization in the control group of this previous study were 44.2% and 41.2%, respectively. These results are comparable and clearly confirm that the new semen storage method used in the present study is as effective as the commonly used method in the porcine industry involving Modena medium, at least in terms of *in vitro* culture viability. The rate of development to the blastocyst stage (19.3%) after *in vitro* culture in the present study may also support this premise because it has been previously reported that sperm DNA damage does not impair fertilization of the oocyte or the completion of the first 2–3 cleavages, but blocks blastocyst formation by inducing apoptosis (Fatehi et al., 2006).

In conclusion, the results of our present study suggest that direct exposure of boar spermatozoa to ZEN or α-ZOL up to concentrations of 1000 μg/l under reduced metabolic conditions might not affect sperm quality.

In overall conclusion, we found that, in the first series of experiment, the FF samples contained glucuronide-conjugated forms of the mycotoxins which may be less harmful to porcine oocytes due to glucuronidation affecting the receptor binding. In the second series of experiments, we demonstrated that an acute exposure to ZEN during maturation and fertilization of porcine oocytes, even at a higher concentration, may not affect the fertility of porcine oocytes. In the third series of experiment, we found that prolonged exposure of boar spermatozoa to ZEN and α-ZOL up to 1000 μg/l under reduced metabolic conditions does not affect their *in vitro* function. However, further study is required not only to create databases of the physiological concentrations of ZEN and α-ZOL in seminal plasma, but also to establish *in vitro* culture systems for risk characterization and to define the mechanism of action of ZEN and α-ZOL on spermatic cells at each stage of spermatogenesis.

TABLES AND FIGURES

Table 1. Concentrations of ZEN, α-ZOL, and β -ZOL in porcine follicular fluid

No. of FF	Concentration		
	ZEN (pg/mL)	α-ZOL (pg/mL)	β-ZOL (pg/mL)
With β-glucuronidase/arylsulfatase treatment			
1	26.6	23.1	ND
2	28.7	11.8	ND
3	15.2	10.0	ND
4	50.4	26.4	ND
5	39.7	23.7	ND
6	50.0	18.4	ND
7	42.0	14.3	ND
8	47.6	16.6	ND
9	54.8	14.6	ND
10	34.0	16.8	ND
Mean ± SEM	38.9 + 4.0	17.6 ± 1.7	
Without β-glucuronidase/arylsulfatase treatment			
1	ND	ND	ND
2	ND	ND	ND
3	ND	ND	ND
4	ND	ND	ND
5	ND	ND	ND
6	ND	ND	ND
7	ND	ND	ND
8	ND	ND	ND
9	ND	ND	ND
10	ND	ND	ND

ND: Not detected

Table 2. Effects of zearalenone exposure of maturing oocytes on meiotic competence, DNA damage, and fertilization

ZEN (μg/l)	No. of oocytes examined	No. (%) of oocytes				No. of oocytes examined	No. (%) of oocytes with		
		GVBD	MII	Degenerated	With DNA fragmentation		Sperm penetration	Normal fertilization**	Polyspermic fertilization**
Control	116	108 (91.1 ± 4.2)[a]	67 (54.7 ± 4.3)[a]	7 (7.3 ± 3.0)	8 (12.5 ± 5.5)	107	74 (69.0 ± 2.4)[a]	16 (22.8 ± 6.4)[a,b]	58 (77.2 ± 6.4)[a,b]
1	109	95 (86.5 ± 2.8)[a,b]	61 (51.5 ± 6.5)[a]	12 (12.2 ± 2.9)	5 (7.4 ± 6.0)	102	58 (57.1 ± 2.6)[b,c]	11 (16.7 ± 7.9)[a]	47 (83.3 ± 7.9)[a]
10	107	96 (88.9 ± 2.7)[a]	54 (48.0 ± 8.4)[a]	8 (8.2 ± 2.5)	7 (8.1 ± 4.1)	114	72 (63.4 ± 1.8)[a,c]	16 (22.5 ± 5.3)[a,b]	56 (77.5 ± 5.3)[a,b]
100	108	91 (83.2 ± 5.9)[a,b]	45 (40.1 ± 6.1)[a,b]	10 (8.7 ± 4.6)	4 (4.8 ± 2.5)	103	50 (49.2 ± 8.0)[b]	18 (35.6 ± 7.2)[b]	32 (64.4 ± 7.2)[b]
1000	109	77 (69.4 ± 6.0)[b]	31 (27.4 ± 5.7)[b]	14 (14.2 ± 7.6)	5 (7.3 ± 3.8)	110	50 (45.6 ± 3.3)[b]	15 (29.3 ± 3.2)[a,b]	35 (70.7 ± 3.2)[a,b]

*Percentages are expressed as mean ± SEM. Five replicated trials were performed. As a control, oocytes were cultured in maturation medium without ZEN.

**Percentage of number of total penetrated oocytes.

[a-c] Values with different superscripts in the same column differ significantly ($P < 0.05$).

ZEN, zearalenone; GVBD, germinal vesicle breakdown; MII, metaphase II

Table 3. Effects of zearalenone exposure of porcine oocytes during insemination on fertilization*

ZEN (μg/l)	No. of oocytes examined	No. (%) of oocytes with		
		Sperm penetration	Normal fertilization**	Polyspermic fertilization**
Control	123	55 (44.2 ± 2.5)	23 (41.2 ± 3.5)[a]	32 (58.8 ± 3.5)[a]
1	131	50 (36.6 ± 5.0)	23 (44.7 ± 5.5)[a,b]	27 (55.3 ± 5.5)[a,b]
10	126	45 (36.1 ± 6.4)	23 (51.7 ± 5.0)[a,b]	22 (48.3 ± 5.0)[a,b]
100	114	41 (35.9 ± 2.2)	17 (41.9 ± 4.2)[a]	24 (58.1 ± 4.2)[a,b]
1000	103	41 (38.0 ± 5.3)	23 (56.3 ± 5.4)[b]	18 (43.8 ± 5.4)[b]

*Percentages are expressed as mean ± SEM. Eight replicated trials were performed. As a control, oocytes were fertilized with spermatozoa in fertilization medium without ZEN.

**Percentage of number of total penetrated oocytes.

[a-b]Values with different superscripts in the same column differ significantly ($P < 0.05$).

Table 4. Fertilization of oocytes inseminated with porcine spermatozoa exposed to zearalenone (ZEN) and α-zearalenol (α-ZOL) at various concentrations during storage at 5°C for 1 week*

Treatment	Concentration (μg/l)**	No. of oocytes examined	No. (%) of oocytes with	
			Sperm penetration	Monospermic fertilization***
Control	0	120	62 (53.0 ± 4.3)	19 (30.6 ± 1.4)
ZEN	10	91	47 (56.2 ± 6.9)	15 (28.4 ± 7.3)
ZEN	1000	91	47 (59.4 ± 11.2)	14 (28.1 ± 4.1)
α-ZOL	10	97	45 (48.1 ± 5.3)	13 (29.3 ± 1.4)
α-ZOL	1000	100	59 (64.9 ± 8.8)	18 (30.6 ± 2.7)

*Percentages are expressed as the mean ± SEM. Five replicated trials were carried out.

**As a control, oocytes were fertilized with spermatozoa stored without ZEN and α-ZOL for 1 week.

*** The proportions of monospermic fertilization was calculated by dividing the number of monospermic fertilized oocytes by the total number of fertilized oocytes.

Table 5. Development of oocytes inseminated with porcine spermatozoa exposed to zearalenone (ZEN) and α-zearalenol (α-ZOL) at various concentrations during storage at 5°C for 1 week*

Treatment	Concentration (μg/l)**	No. of oocytes examined	No. (%) of embryos	
			cleaved	developed to blastocysts
Control	0	174	117 (68.0 ± 4.2)	33 (19.3 ± 2.0)
ZEN	10	157	98 (62.7 ± 4.5)	25 (16.0 ± 2.8)
ZEN	1000	160	107 (66.7 ± 5.1)	25 (15.8 ± 3.0)
α-ZOL	10	157	99 (62.5 ± 5.6)	23 (14.6 ± 1.6)
α-ZOL	1000	149	101 (66.9 ± 4.1)	28 (19.1 ± 2.4)

*Percentages are expressed as the mean ± SEM. Five replicated trials were carried out.

**As a control, oocytes were fertilized with spermatozoa stored without ZEN and α-ZOL for 1 week.

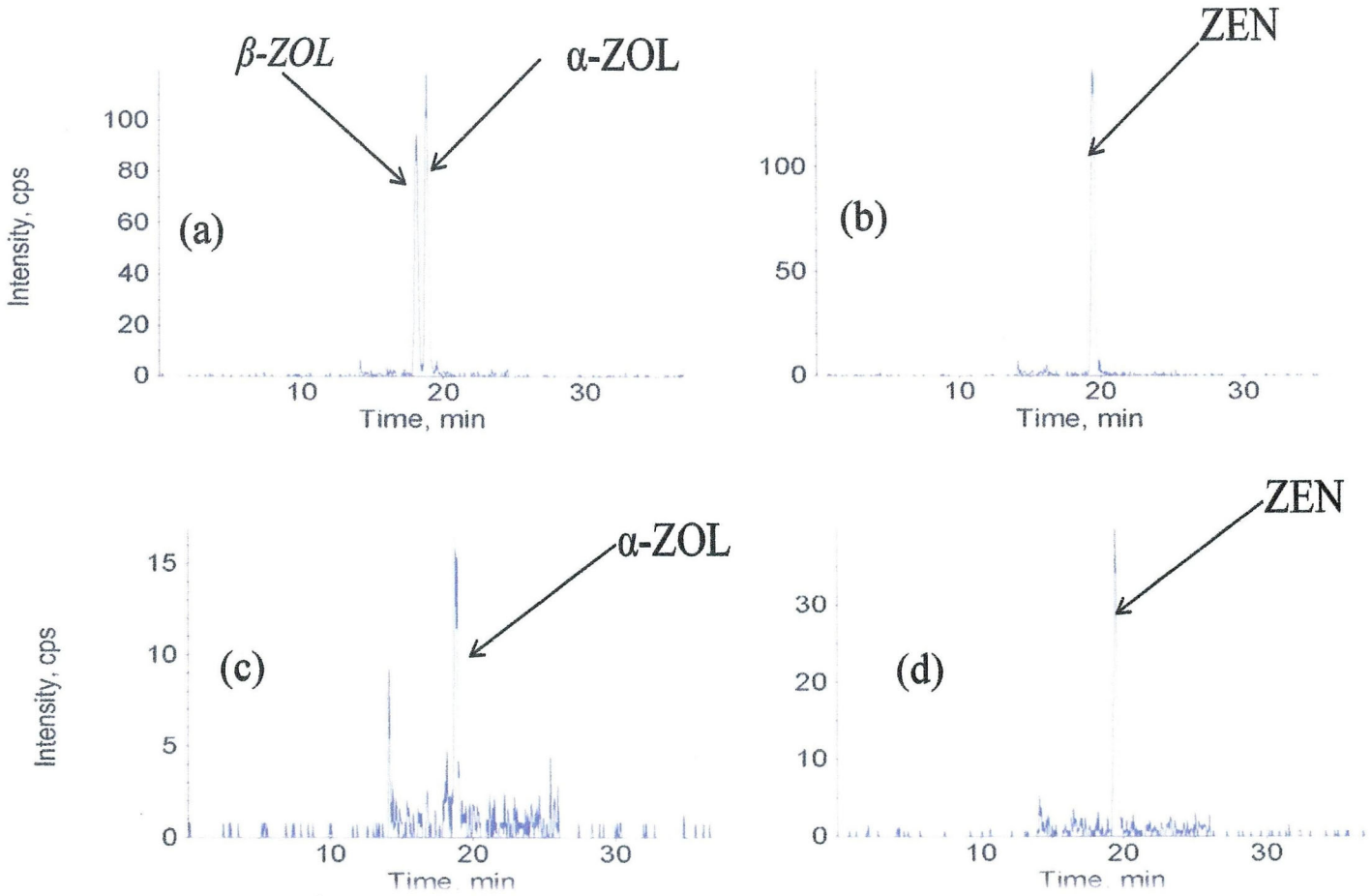

Fig.1. Representative LC/MS/MS chromatograms of (a) standard for both α-ZOL and β-ZOL (0.1μg/l), (b) the standard for ZEN (0.1μg/l), (c)) both α-ZOL and β-ZOL from porcine follicular fluid sample No. 4. and (d)ZEN from porcine follicular fluid sample No. 4.

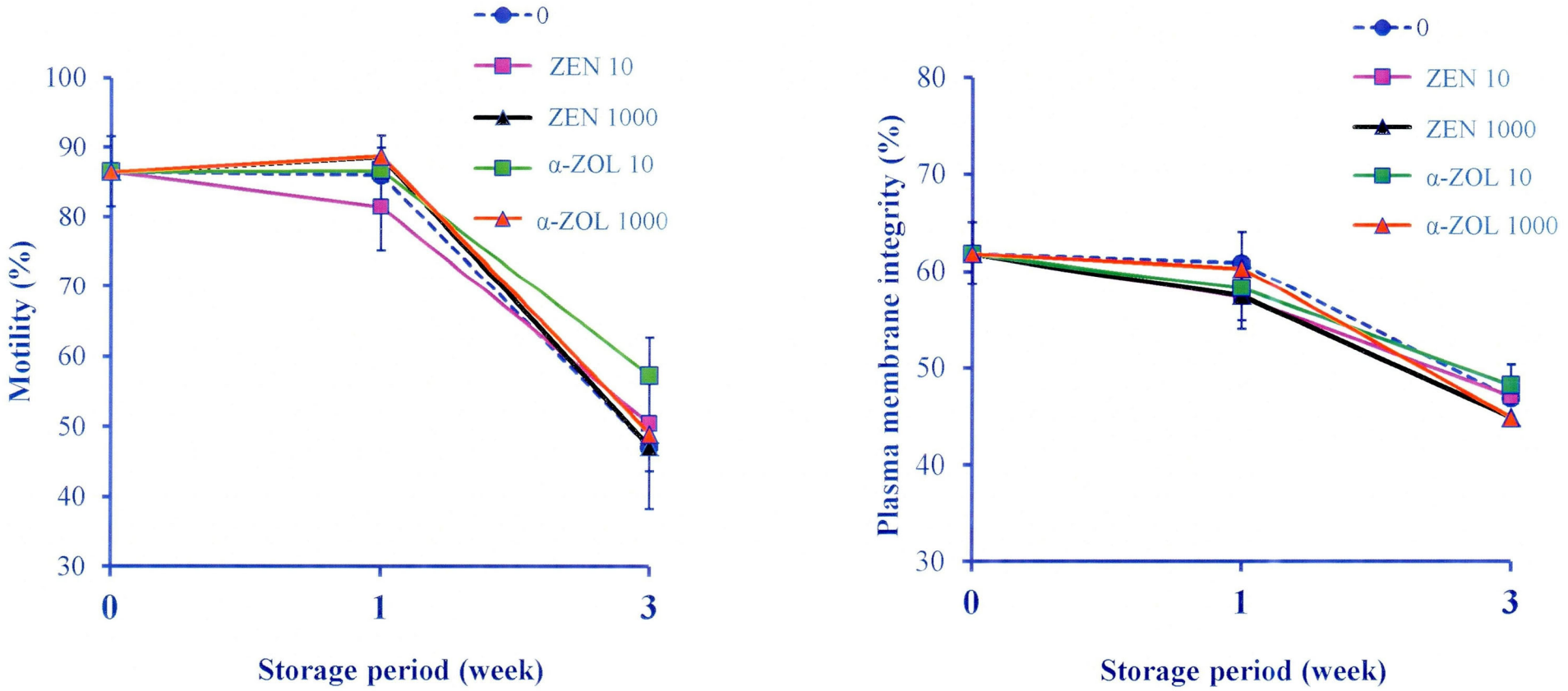

Fig.2. Percentages of sperm motility (Left) and plasma membrane integrity (Right) of boar spermatozoa stored in Modena solution with either 10 μg/l (■) zearalenone (ZEN), 1000 μg/l ZEN (▲), 10 μg/l (■) α-zearalenol (α-ZOL), 1000 μg/l (▲) α-ZOL, or without ZEN and α-ZOL (control, ●) at 5°C for 3 weeks. Four replicated trials were carried out. *The mean values of these parameters for sperm stored with 10 μg/l α-ZOL differs significantly from those of control sperm as well as those stored with 10 μg/l ZEN ($P < 0.05$).

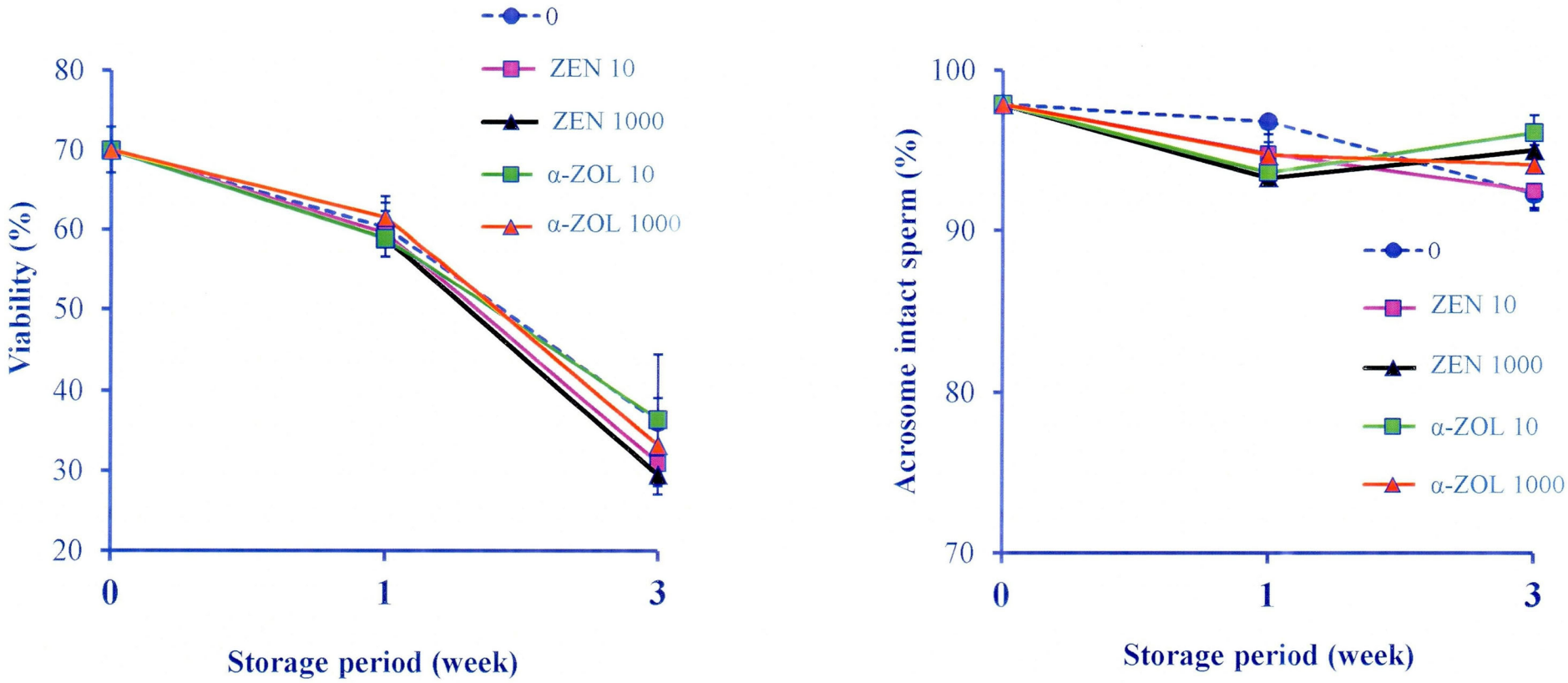

Fig.3. Percentages of sperm viability (Left) and acrosome intact sperm (Right) of boar spermatozoa stored in Modena solution with either 10 μg/l (■) zearalenone (ZEN), 1000 μg/l ZEN (▲), 10 μg/l (■) α-zearalenol (α-ZOL), 1000 μg/l (▲) α-ZOL, or without ZEN and α-ZOL (control, ●) at 5°C for 3 weeks. Four replicated trials were carried out. *The mean values of these parameters for sperm stored with 10 μg/l α-ZOL differs significantly from those of control sperm as well as those stored with 10 μg/l ZEN ($P < 0.05$).

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