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

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Abstract. 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. First, oocytes were cultured in ZEN-containing (0−1000 μg/l) maturation medium and then fertilized. The oocytes maturing in vitro without ZEN were then 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.

Key words: Oocytes, Porcine, Sperm, Zearalenone

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 [1−4]. 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 [5]. 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 [6, 7], as well as viability, motility and acrosome reactions in sperm [8, 9]. Nevertheless, the acute effects of exposure to ZEN during in vitro fertilization remain unknown. This study aimed to examine the effects of exposure to ZEN on porcine oocytes and sperm by using an in vitro maturation (IVM) and in vitro fertilization (IVF) systems to assess the state of nuclear DNA damage and fertilization.

As shown in Table 1, 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 to 100 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 2, exposure to ZEN during IVF did not affect total rates of sperm penetration irrespective of the 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.

Cell and tissue culture systems are useful for evaluating risks posed by toxic compounds such as mycotoxins [5, 10]. Malekinejad et al. [7] 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 (1000 μg/l) of ZEN during maturation can have detrimental effects on the nuclear maturation of porcine oocytes and their subsequent sperm penetration rates after IVF without affecting either DNA fragmentation after IVM or rates of normal or polyspermic fertilization. Moreover, the rates of GVBD in oocytes exposed to 1000 μg/l of ZEN decreased when compared with those of the control oocytes. Therefore, we speculate that the reduction in the maturation rate of oocytes exposed to ZEN may have caused the inhibition of meiotic resumption by ZEN.

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. [7] 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, even the rates of maturation to MII and total fertilization of oocytes in the control group were lower than those reported in other studies [7],
Table 1. Effects of zearalenone exposure of maturing oocytes on meiotic competence, DNA damage and fertilization*

<table>
<thead>
<tr>
<th>ZEN (μg/l)</th>
<th>No. of oocytes examined</th>
<th>GVBD (%)</th>
<th>MII (%)</th>
<th>Degenerated (%)</th>
<th>With DNA fragmentation (%)</th>
<th>No. of oocytes examined</th>
<th>Sperm penetration (%)</th>
<th>Normal fertilization (%)</th>
<th>Polyspermic fertilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>116</td>
<td>108</td>
<td>67</td>
<td>7</td>
<td>8</td>
<td>107</td>
<td>74</td>
<td>16</td>
<td>58</td>
</tr>
<tr>
<td>1</td>
<td>109</td>
<td>(91.1 ± 4.2)</td>
<td>54.7 ± 4.3</td>
<td>(7.3 ± 3.0)</td>
<td>(12.5 ± 5.5)</td>
<td>102</td>
<td>(69.0 ± 2.4)</td>
<td>(22.8 ± 6.4)</td>
<td>(77.2 ± 6.4)</td>
</tr>
<tr>
<td>10</td>
<td>107</td>
<td>(86.5 ± 2.8)</td>
<td>(51.5 ± 6.5)</td>
<td>(12.2 ± 2.9)</td>
<td>(7.4 ± 6.0)</td>
<td>114</td>
<td>(57.1 ± 2.6)</td>
<td>(16.7 ± 7.9)</td>
<td>(83.3 ± 7.9)</td>
</tr>
<tr>
<td>100</td>
<td>108</td>
<td>(88.9 ± 2.7)</td>
<td>(48.0 ± 8.4)</td>
<td>(8.2 ± 2.5)</td>
<td>(8.1 ± 4.1)</td>
<td>103</td>
<td>(63.4 ± 1.8)</td>
<td>(22.5 ± 5.3)</td>
<td>(77.5 ± 5.3)</td>
</tr>
<tr>
<td>1000</td>
<td>109</td>
<td>(83.2 ± 5.9)</td>
<td>(40.1 ± 6.1)</td>
<td>(8.7 ± 4.6)</td>
<td>(4.8 ± 2.5)</td>
<td>110</td>
<td>(49.2 ± 8.0)</td>
<td>(35.6 ± 7.2)</td>
<td>(64.4 ± 7.2)</td>
</tr>
<tr>
<td></td>
<td>(69.4 ± 6.0)</td>
<td>(27.4 ± 5.7)</td>
<td>(14.2 ± 7.6)</td>
<td>(7.3 ± 3.8)</td>
<td></td>
<td></td>
<td>(45.6 ± 3.3)</td>
<td>(29.3 ± 3.2)</td>
<td>(70.7 ± 3.2)</td>
</tr>
</tbody>
</table>

* Percentages are expressed as the mean ± SEM. Five replicated trials were performed. As a control, oocytes were cultured in maturation medium without ZEN. ** Percentage of the total penetrated oocytes. *** Values with different superscripts in the same column differ significantly (P<0.05). ZEN, zearalenone; GVBD, germinal vesicle breakdown; M II, metaphase II.

Table 2. Effects of zearalenone exposure of porcine oocytes during insemination on fertilization*

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

* Percentages are expressed as the mean ± SEM. Eight replicated trials were performed. As a control, oocytes were fertilized with spermatozoa in fertilization medium without ZEN. **Percentage of the total penetrated oocytes. *** Values with different superscripts in the same column differ significantly (P<0.05). ZEN, zearalenone.

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 IVF of porcine oocytes [13]. Li et al. [14] 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 [13]. 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 so 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. [15], 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 por-
cine 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 μg/ml) [16]. Based on the results of our previous study [16], we determined the effect of different ZEN concentrations (1, 10, 100 and 1000 μg/l) in the present study. 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 [7, 16], but also to establish culture systems for risk assessments and to define the mechanism of action of germ cells.

**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, 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, 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% CO2 in air.

**IVF**

IVF was carried out according to the method described by Kikuchi et al. [17] with minor modifications. The sperm-rich fractions of ejaculates were obtained from a large white boar and frozen as described previously [18]. Spermatozoa were thawed and preincubated for 15 min at 38.5 C in TCM 199 medium adjusted to pH 7.8. The preincubated spermatozoa were introduced into fertilization medium containing approximately 10 matured oocytes. The fertilization medium consisted of 90 mM NaCl, 12 mM KCl, 25 mM NaHCO3, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 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 × 106 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) [19] covered with paraffin oil in an atmosphere of 5% O2, 5% CO2 and 90% N2 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 modified version of procedures described previously [20]. 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. Then, they were incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1 h at 38.5 C under 5% CO2 in air. After the TUNEL procedure, the oocytes were counterstained with 25 μg/ml bis-benzimidide (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: (1) 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; (2) polyspermic penetration rate, calculated from the proportion of oocytes forming a single female pronucleus and multiple penetrating sperm nuclei or male pronuclei; and (3) 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 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, 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.

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References