



Zearalenone (ZEN) disrupts the anti-inflammatory response of bovine oviductal epithelial cells to sperm *in vitro*

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ABSTRACT

Dietary contamination by Zearalenone (ZEN) has a detrimental effect on bovine fertility. Recently, we showed a novel anti-inflammatory response of bovine oviductal epithelial cells (BOEC) to active sperm cells *in vitro*. The aim of the present study was to investigate the effect of ZEN exposure of BOEC on the immune-related cytokine expression in response to bovine sperm. At concentrations of 100 and 1000 ng/mL, ZEN induced the expression of *TNF* and *IL1B* (pro-inflammatory cytokines) as well as *IL8* (chemokine) in BOEC in a dose-dependent manner. Furthermore, ZEN induced *PTGES* expression and PGE_2 secretion in BOEC. Sperm co-culture induced an anti-inflammatory response in BOEC with upregulation of *TGFB*, secretion of PGE_2 and downregulation of *TNF*. Most importantly, ZEN at 1–1000 ng/mL eliminated the response of BOEC to sperm. Estradiol-17 β (5 ng/mL) treatment did not produce the same effects as ZEN, suggesting that the response of BOEC to ZEN is, at least in part, not mediated by estrogen receptors. Taken together, ZEN can produce inflammatory effects on BOEC by stimulating the expressions of pro-inflammatory cytokines and disrupt the normal interaction between sperm and BOEC at the level of cytokine expressions and PGE_2 production. Thus, exposure of the bovine oviduct to ZEN may negatively affect sperm survival and reduce fertility.

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1. Introduction

Fusarium fungi have traditionally been associated with temperate climatic conditions, since they require somewhat lower temperatures for growth and mycotoxin production [1]. The most toxicologically important *Fusarium* mycotoxins are zearalenone (ZEN), fumonisin B1 (FB1) and trichothecenes (including deoxynivalenol (DON) and T-2 toxin (T-2)). ZEN is a non-steroidal estrogenic mycotoxin, found in corn, wheat, barley, sorghum and oats, as well as other foods. ZEN is produced by numerous species and subspecies of *Fusarium* in the presence of high humidity and low temperatures (10–15 °C). ZEN is suspected to cause infertil-

ity and hyperestrogenism in cows [2] and induce feminization of young bulls due to decreased testosterone production [3]. Diet with about 660 ppb of ZEN can result in poor consumption, decreased milk production, diarrhea and poor reproductive performance [4]. Moreover, the large doses of such toxin are associated with early abortions [5].

Takagi et al. [6] detected ZEN and its metabolites in 6 of 32 normal follicles and 7 of 20 cystic follicles. They also found that the maturation rate of bovine oocytes decreased after culturing oocytes in maturation media containing various ZEN concentrations in a dose-dependent manner. It impairs the immunity through reduction of serum immunoglobulins (IGA) in cows fed a contaminated diet [7,8]. Moreover, it was reported that dairy herds with low fertility had higher levels of blood zearalenone [9]. The plasma ZEN concentration was 1.7–5.5-fold higher compared to the follicular fluid (FF) concentration in dairy cows receiving a contaminated diet

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[10]. Thus, it can be deduced that oviductal cells can be exposed to ZEN via FF or the blood circulation and consequently may affect the immunological environment of the oviduct during the pre-ovulatory stage. The mechanisms of action of ZEN can occur via two different pathways; the classical receptor-mediated pathway or the alteration of non-genomic, epigenetic cellular mechanism [11,12].

ZEN, but not estradiol (E2), decreases sperm motility and spontaneous acrosome reactions (ARs) while increasing chromatin decondensation in sperm [13]. Sperm binding to the oviductal epithelium forms a sperm reservoir, maintains the viability of sperm cells, as well as regulating sperm capacitation and hyperactivation [14]. Moreover, the properly timed and the gradual release of sperm from the reservoir ensures that oocytes will be fertilized shortly after ovulation and prevents polyspermy [15,16].

Recently, we reported a novel anti-inflammatory response of BOEC to sperm binding *in vitro* [17]. Sperm binding to BOEC induced expression of anti-inflammatory cytokines (*TGFB1* and *IL10*) and production of PGE_2 [18,19]. PGE_2 is considered to be a potent immune modulator and plays an essential role in the pre-ovulatory oviduct as it suppresses sperm phagocytosis by neutrophils [17,19]. ZEN has been shown to be immunotoxic at high concentrations [20–22], but its role in inflammation is still not fully understood [23].

Considering the action of ZEN as a potent endocrine disruptor [24], and its adverse effect on sperm, this study was undertaken to investigate the immunological impact of ZEN on BOEC in response to sperm co-culture, and its effect on the local PGE_2 production in BOECs *in vitro*. Additionally, we compared such effects with E2 effect.

2. Material and methods

2.1. Collection and classification of oviducts

Female reproductive tracts were opened and macroscopically examined (to ensure that they are free of inflammation, pus, and abnormal color) in a local slaughterhouse (Hokkaido Livestock Co., Doto Plant Tokachi Factory; 1–1, Kita 2-chome, Nishi 24-jo, Obihiro, Hokkaido, Japan). The phase of the oestrous cycle was identified as previously reported [25], based on the appearance, and colour of the corpus luteum, and the follicular diameter as preovulatory (days 19–20), postovulatory (days 1–2), and mid-luteal (days 10–12). The ovary was judged to be in the preovulatory phase (days 19–20) if it contained at least one large follicle > 10–15 mm in diameter and a regressed corpus luteum (<1 cm in diameter, firm in consistency), with no vasculature visible on its surface.

For isolation of BOEC, only healthy oviducts (five oviducts for each set) in the pre-ovulatory phase were selected. Oviducts were ligated at both ends, and immersed in phosphate buffer saline without Ca^{2+} or Mg^{2+} ($\text{PBS}^{-/-}$) supplemented with 0.3% gentamicin (Sigma-Aldrich, Steinheim, Germany) and amphotericin B (Sigma-Aldrich, Steinheim, Germany), kept in the refrigerator of the slaughter house until the next day (according to Japanese rules), and then transported in the early morning to the laboratory. In the laboratory, oviducts were cut, separated from the surrounding connective tissue, rapidly rinsed in 70% ethanol for disinfection, and rinsed three times with $\text{PBS}^{-/-}$. Epithelial cells were isolated and cultured as previously described [26] with minor modifications. Briefly, BOEC from several cows were mechanically dislodged, pooled, purified, and cultured in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, Gibco, Grand Island, USA) supplemented with 2.2% NaHCO_3 , 0.1% gentamicin, 1% amphotericin, and 10% heat-inactivated fetal calf serum (FCS) (BioWhittaker, Walkersville, MD, USA) in 6-well culture plates

(NalgeNunc International, Roskilde, Denmark) for 4 or 5 days at 38.5°C in 5% CO_2 in air until a monolayer was formed. The cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA) for 5 min, re-plated in 12-well culture plates at a density of 3×10^4 cells/mL, and cultured until sub-confluence. The BOEC monolayer from the second passage was primed with 100 pg/mL E2 (Sigma-Aldrich, Steinheim, Germany) and 1 ng/mL P4 (Sigma-Aldrich, Steinheim, Germany). The concentrations of these steroid hormones were maintained at levels similar to their physiological levels in the bovine oviduct during the pre-ovulatory period *in situ* [25].

2.2. Sperm preparation

Frozen 0.5 mL semen straws were obtained from three highly fertile Holstein bulls from the Genetics Hokkaido Association (Hokkaido, Japan). Nine straws (three straws from each bull) were thawed in a water bath at 38°C for 30 s, pooled, and washed 3 times using a modified Tyrode's albumin, lactate, and pyruvate medium (Sp-TALP) [27]. Sperm were then separated using a swim-up procedure [28]. Briefly, four 0.25 mL aliquots of sperm suspension were layered under 1 mL aliquots of Sp-TALP medium in 15 mL Falcon tubes. After 1 h of incubation at 39°C , the top 0.85 mL from each tube was aspirated, pooled and centrifuged at 1000g for 10 min. The pellet was washed by centrifugation in fresh Sp-TALP, and the final pellet was reconstituted in 0.5 mL of Sp-TALP for the future use. The progressive motility of the recovered sperm was assessed by visual examination under a light microscope equipped with a stage warmer (> 90%). Acrosome integrity was assessed in a sample using a dual staining procedure with Trypan Blue supravital stain and Giemsa stain, as described by Kovacs and Foote [29]. These swim-up sperm were used in the subsequent experiments.

2.3. Exposure of BOEC to ZEN (with and without sperm co-culture)

The sub-confluent BOEC monolayers (after second passage) were washed twice and cultured in medium supplemented with 0.1% FCS in combination with zero, 1, 10, 100 or 1000 ng/mL ZEN (MP Biomedicals, Germany). Another group of BOEC was exposed to E2 (Sigma-Aldrich, Steinheim, Germany; 5 ng/mL that equivalent to 100 ng/mL ZEN that may bind to ERs by 5%) and then incubated at 38.5°C in 5% CO_2 for 24 h. Then BOECs were co-cultured with swim-up spermatozoa (2×10^5 sperm/mL) for another 24 h. The BOEC group without any addition of ZEN and without culturing with sperm served as a control. Finally, the medium was collected and cells were trypsinized for 5 min, washed twice with $\text{PBS}^{-/-}$ and resuspended in 300 μL $\text{PBS}^{-/-}$. Cell viability was estimated using Trypan blue staining. The remaining cells were washed again by centrifugation at 300g for 10 min at 4°C , lysed with Trizol (Invitrogen, Carlsbad, USA), and stored at -80°C until RNA extraction.

2.4. Quantification of PGE_2 concentration

PGE_2 concentrations were measured in BOEC-conditioned media collected at the end of the experiment (48 h) by using a competitive enzyme immune assay (EIA), as previously described [25]. Briefly, a 96-well ELISA plate (Corning, NY, USA) was coated with 100 μg of anti-rabbit IgG (Seikagaku, Tokyo, Japan) which was achieved after incubation with polyclonal antibody solution (100 μL ; 1:600,000) for 24 h at 4°C . The following day, plates were decanted and 15 μL of standards or samples were incubated with anti- PGE_2 -HRP (1:40,000) overnight at 4°C . The coefficient of variance within and between assays was 7.3 and 11.4%, respectively.

The ED50 values were 260 pg/mL and the ranges of the standard curves for these assays were 20–2000 pg/mL.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from BOEC using Trizol reagent (Invitrogen, Carlsbad, USA) as previously described [30]. Extracted RNA was detected by ultraviolet (UV) spectroscopy (optical density, 260 nm) and the concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at 260 and 280 nm absorbance values. The total extracted RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at -80°C until cDNA production. A DNase treatment step was performed using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA and other contaminations. The extracted RNA (2 μL) was incubated for 30 min at 37°C with 1 μL of the $10 \times$ RQ1 RNase-free DNase reaction buffer and 2 μL of the 1 $\mu\text{g}/\mu\text{L}$ RNase-free DNase.

To terminate the reaction, 1 μL of the RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65°C . First-strand cDNA synthesis was conducted according to the commercial protocol described in the SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The mixture was prepared using 2 μL of the total RNA extracted from the sample (BOEC), 1.5 μL of 50 ng/ μL random primer (Invitrogen, Carlsbad, CA, USA), 1.5 μL of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA), and 12 μL of H_2O to obtain a total volume of 18 μL per sample.

This mixture was then incubated at 65°C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second mixture, which consisted of 3 μL of 0.1 M dithiothreitol (Invitrogen, Carlsbad, CA, USA), 1.5 μL of 40 units/ μL RNase in Ribonuclease Inhibitor (Promega, Madison, WI, USA), and 6 μL of $5 \times$ First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), was added to each tube. The samples were incubated for 2 min at 42°C , and 0.2 μL of 200 units/ μL SuperScript II Reverse Transcriptase were added to each tube. The thermal cycler was programmed at 25°C for 10 min, 42°C for 50 min, and then 70°C for 15 min. The synthesized cDNA was stored at -30°C .

2.6. Real-time polymerase chain reaction (real-time PCR)

To quantify the mRNA expression of target genes (*IL10*, *TGFB1*, *TNF*, *IL1B*, *PTGES*, and β -actin), we used the synthesized cDNA to perform real-time PCR using a QuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) by an iCyclerIQ (Bio-Rad Laboratories, Tokyo, Japan). The amplification program was performed with an initial activation step (15 min at 95°C), followed by 40 cycles of PCR (15 s denaturation at 95°C , 30 s annealing at

55 – 58°C , and 20 s extension at 72°C). The calculated cycle threshold (Ct) values were exported to Microsoft Excel to be analyzed. Ct values were normalized using β -actin as the internal standard using the Delta–Delta comparative threshold method [31] to quantify the fold change between the samples. The expression of β -actin was stable in all experiments and cross treatments; no significant variations in β -actin expression were detected. Specific primers for each gene were designed using PRIMER EXPRESS software (Perkin-Elmer, Boston, MA) as shown in Table 1.

2.7. Statistical analysis

Data obtained from ZEN experiment were statistically analyzed using general linear model (G.L.M.) procedure of SAS System [32] as follows: $Y_{ij} = \mu + S_i + Z_j + S_iZ_j + E_{ij}$, where: Y_{ij} = The individual observation, μ = Overall mean, S_i = effect of sperm co-culture ($i = 1, 2$), Z_j = Effect of ZEN treatment ($j = 1, 2, 3, 4$ and 5), S_iZ_j = Fixed interacting effect between sperm co-culture and ZEN treatment; E_{ij} = The total random error. The significant differences between treatment means were determined by using Duncan's new multiple ranges tests [33]. While the results obtained from E2 treatment were analyzed by the least square analysis of variance in a 2×2 factorial model as follows: $Y_{ij} = \mu + S_i + H_j + S_iH_j + E_{ij}$, where: Y_{ij} = The individual observation, μ = Overall mean, S_i = effect of sperm co-culture ($i = 1, 2$), H_j = Effect of E2 treatment ($j = 1$ and 2), S_iH_j = Fixed interacting effect between sperm co-culture and E2 treatment; E_{ij} = The total random error.

3. Results

The main effects of sperm as well as ZEN significantly influenced the gene expression of the majority of measured cytokines and PGE_2 production (Table 2). There was no significant influence of interactions between sperm co-culture and ZEN exposure to BOEC on *IL10* and *TNF*, while the interactions were significant in all other gene expression and PGE_2 production throughout the present study (Table 2–4).

As expected and in line with our previous findings, sperm (at zero level from ZEN) suppressed the expression of pro-inflammatory cytokine genes (*TNF* and *IL1B*) and up-regulated the expression of anti-inflammatory cytokine genes (*TGFB1*) in BOEC. In sperm-BOEC co-cultures, the production of PGE_2 was stimulated (Table 3, 4).

A significant pro-inflammatory shift of cytokine expression was detected in BOEC after exposure to ZEN (Table 2). At higher concentrations (100 and 1000 ng/mL), ZEN induced the expression of the pro-inflammatory cytokines *TNF* and *IL1B* in BOEC (Table 2). ZEN disturbed the down-regulation of *IL1B* in BOEC generated by sperm in a dose-dependent manner (Table 3).

Table 1
Primers used to amplify specific bovine transcripts.

Gene		Sequence of nucleotide (5' \Rightarrow 3')	Accession No.
β actin	F	TCACCAACTGGGACGACATG	AY141970.1
	R	CGTTGTAGAAGGTGTGGTGCC	
<i>TNF</i>	F	CAAAAGCATGATCCGGGATG	NM.173966.3
	R	TTCTCGGAGAGCACCTCTC	
<i>IL1B</i>	F	AATCGAAGAAAGGCCGTCT	NM.174093.1
	R	ATATCCTGGCCACCTCGAAA	
<i>TGFB1</i>	F	CTTCTTCAAATGCAGCATTGG	NM.001166068.1
	R	GGGTCTGGGTGATACAACGAA	
<i>IL10</i>	F	GAGATGCGAGCACCTGTCT	NM.174088.1
	R	GGCTGGTTGGCAAGTGGATA	
<i>IL8</i>	F	CCAATGGAACGAGGTCTGC	NM.173925.2
	R	CCTTCTGCACCCACTTTTCT	
<i>PTGES</i>	F	AAAATGTACGTGGTGCCGT	NM.174443.2
	R	CTTCTCCGAGCCTCACTT	

Table 2The main effects of sperm and different ZEN concentrations on gene expression of measured cytokines and PGE₂ production by BOEC.

Treatment	TGFB	IL10	PTGES	TNF	IL1B	IL8	PGE ₂ (ng/mL)
Main effect of sperm							
BOEC	1.11 ± 0.04	1.17 ± 0.10	1.37 ± 0.07	1.33 ± 0.08	1.22 ± 0.07	2.60 ± 0.26	62.08 ± 10.56
Sperm + BOEC	1.57 ± 0.07	1.41 ± 0.10	1.53 ± 0.05	1.03 ± 0.06	1.10 ± 0.07	3.96 ± 0.38	107.46 ± 16.52
F test	**	N.S.	*	**	N.S.	**	**
Main effect of ZEN (ng/mL)							
Zero	1.40 ± 0.12 ^a	1.45 ± 0.11	1.40 ± 0.09 ^b	0.84 ± 0.08 ^b	0.76 ± 0.06 ^c	1.14 ± 0.05 ^d	39.58 ± 13.96 ^{cd}
1	1.28 ± 0.08 ^a	0.98 ± 0.14	1.10 ± 0.06 ^c	0.97 ± 0.07 ^b	1.01 ± 0.08 ^b	1.83 ± 0.11 ^c	55.08 ± 10.03 ^d
10	1.45 ± 0.07 ^a	1.21 ± 0.13	1.36 ± 0.11 ^b	1.07 ± 0.07 ^b	1.17 ± 0.08 ^b	3.91 ± 0.40 ^b	72.29 ± 7.98 ^b
100	1.42 ± 0.13 ^a	1.23 ± 0.21	1.73 ± 0.10 ^a	1.47 ± 0.14 ^a	1.41 ± 0.11 ^a	4.09 ± 0.43 ^b	82.01 ± 9.85 ^b
1000	1.04 ± 0.07 ^b	1.48 ± 0.18	1.72 ± 0.05 ^a	1.67 ± 0.12 ^a	1.59 ± 0.09 ^a	5.43 ± 0.51 ^a	174.90 ± 22.92 ^a
F test	**	N.S.	**	**	**	**	**

Results are presented as the mean of the fold expressions for the measured cytokines and PGE₂ production ± SEM of four independent experiments performed in triplicate. *, ** and N.S.: Significant, highly significant and not significant at the 0.05 and 0.01 probability levels. a, b, c, d means in the same column with different superscripts are significantly different (P < 0.05), as determined by Duncan's multiple range test.

Table 3Interaction between ZEN exposure and sperm co-culture on gene expression of *TGFB* (anti-inflammatory cytokine), *IL1B* (pro-inflammatory cytokine) and *IL8* (chemokine) in BOEC.

	ZEN (ng/mL)	TGFB	IL1B	IL8
BOEC	Zero	1.02 ± 0.09 ^c	1.02 ± 0.05 ^{de}	1.08 ± 0.06 ^d
	1	1.06 ± 0.08 ^c	0.96 ± 0.1 ^e	1.77 ± 0.40 ^c
	10	1.26 ± 0.10 ^{bc}	0.97 ± 0.0 ^e	2.81 ± 0.23 ^c
	100	1.17 ± 0.07 ^c	1.51 ± 0.18 ^{ab}	2.85 ± 0.40 ^c
	1000	1.05 ± 0.10 ^c	1.74 ± 0.14 ^a	4.73 ± 0.66 ^b
Sperm + BOEC	Zero	1.78 ± 0.16 ^a	0.50 ± 0.05 ^f	1.22 ± 0.06 ^d
	1	1.52 ± 0.12 ^{ab}	1.07 ± 0.14 ^{cde}	1.89 ± 0.20 ^c
	10	1.64 ± 0.08 ^a	1.36 ± 0.02 ^{bc}	5.02 ± 0.48 ^{ab}
	100	1.76 ± 0.24 ^a	1.31 ± 0.01 ^{bcd}	5.32 ± 0.37 ^{ab}
	1000	1.03 ± 0.12 ^c	1.44 ± 0.09 ^{ab}	6.04 ± 0.73 ^a
F test		**	*	**

Results are presented as the mean of the fold expressions for the measured cytokines and PGE₂ production ± SEM of four independent experiments performed in triplicate. N.S. and **: not significant and highly significant at the 0.01 probability level. a, b, c, d, e means in the same column with different superscripts are significantly different (P < 0.05), as determined by Duncan's multiple range test.

Table 4Interaction between ZEN exposure and sperm co-culture on gene expression of *PTGES* and PGE₂ release by BOEC.

	ZEN (ng/mL)	PTGES	PGE ₂ (ng/mL)
BOEC	Zero	1.08 ± 0.05 ^b	11.16 ± 2.65 ^f
	1	1.07 ± 0.09 ^b	42.61 ± 3.2 ^e
	10	1.55 ± 0.21 ^a	64.65 ± 9.04 ^d
	100	1.75 ± 0.22 ^a	66.67 ± 10.72 ^d
	1000	1.58 ± 0.07 ^a	125.30 ± 3.34 ^{bc}
Sperm + BOEC	Zero	1.72 ± 0.11 ^a	68.00 ± 8.33 ^d
	1	1.13 ± 0.08 ^b	67.54 ± 18.3 ^{8 cd}
	10	1.19 ± 0.05 ^b	79.93 ± 1.12 ^c
	100	1.71 ± 0.07 ^a	97.34 ± 11.61 ^c
	1000	1.86 ± 0.05 ^a	224.50 ± 12.52 ^a
F test		**	**

Results are presented as the mean of the fold expressions for the measured cytokines and PGE₂ production ± SEM of four independent experiments performed in triplicate. Averages having the same letter are not significant at 5% level according to Duncan's multiple range test. **: highly significant at the 0.01 probability level. a, b, c, d, e, f means in the same column with different superscripts are significantly different (P < 0.05), as determined by Duncan's multiple range test.

ZEN induced the expression of *IL8* in BOECs. In BOEC co-cultured with sperm, ZEN (at 10, 100 and 1000 ng/mL) induced the expression of *IL8* (Table 3).

ZEN did not affect the expression of *IL10* in BOEC (Table 2). At 1000 ng/mL, ZEN suppressed the effect of sperm on BOEC expression of *TGFB* (Table 3).

PTGES expression and PGE₂ production were induced in BOECs after ZEN exposure (Table 4). ZEN suppressed the expression of *PTGES* stimulated by sperm in BOEC at low concentrations (1 and 10 ng/mL), but there was no effect at higher concentrations (100

and 1000 ng/mL). At the highest concentration (1000 ng/mL), ZEN induced higher production of PGE₂ in sperm co-cultures (Table 4).

Unlike ZEN, E2 induced *TGFB* and *IL10* and had no effect on *TNF* and *IL1B* expression in BOEC. Furthermore, in sperm co-culture, E2 did not affect the anti-inflammatory cytokines (*TGFB* and *IL10*) in BOEC (Table 5). E2 suppressed *PTGES* expression in sperm co-culture, while there were no significant effects for E2 on the production of PGE₂ by BOEC (Table 5).

4. Discussion

Cereal grains used as feedstuffs for farm animals are frequently contaminated by the Fusarium toxin ZEN [34]. ZEN has been associated with infertility problems in dairy cows [4,35,36]; it decreased the conception rate from 87 to 62% of the treated heifers without any detectable lesions [37]. High ZEN concentration (300–1000 ng/mL) adversely affected the maturation rate and meiotic competence of the bovine oocytes [6,38]. ZEN acts as an endocrine disruptor and exerts strong estrogenic effects due to its competition with 17-β-estradiol for binding to cytosolic estrogen receptors in the hypothalamus, mammary gland, pituitary gland, uterus, and the oviduct [12,39].

In the current study, it was demonstrated that the high levels of ZEN (at 100 and 1000 ng/mL) induced the expression of pro-inflammatory cytokine genes (*TNF* and *IL1B*) and inhibited the effect of sperm on BOEC. These results are in agreement with Jia et al. [23], who found that ZEN induced mRNA expression of pro-inflammatory cytokine genes (*TNF*, *IL1B* and *IL6*) in the kidney in a dose-dependent manner in rats fed a diet treated with 0.3–146 mg/kg ZEN. Moreover, ZEN increased the respiratory burst of monocytes and production of pro-inflammatory cytokines (*TNF*

Table 5The main effect of E2 (5 ng/mL) and its interactions with sperm co-culture on the gene expression of measured cytokines and PGE₂ production by BOEC.

Treatment	TGFB	IL10	PTGES	TNF	IL1B	IL8	PGE2 (ng/mL)
Main effect of E2							
BOEC (-E2)	1.40 ± 0.12	1.45 ± 0.11	1.40 ± 0.09	0.84 ± 0.08	0.76 ± 0.06	1.14 ± 0.05	39.58 ± 13.30
BOEC (+E2)	1.71 ± 0.10	1.93 ± 0.17	1.14 ± 0.06	0.95 ± 0.04	0.92 ± 0.08	2.06 ± 0.14	45.25 ± 13.59
F test	*	*	*	NS	NS	**	NS
Interaction							
BOEC							
-E2	1.02 ± 0.09 ^b	1.05 ± 0.07 ^b	1.08 ± 0.05 ^b	1.11 ± 0.11 ^a	1.02 ± 0.05 ^a	1.08 ± 0.06 ^b	11.16 ± 2.65 ^b
+E2	1.62 ± 0.06 ^a	1.91 ± 0.23 ^a	1.09 ± 0.10 ^b	0.94 ± 0.06 ^a	0.91 ± 0.10 ^a	2.00 ± 0.04 ^a	15.00 ± 0.36 ^b
Sperm + BOEC							
-E2	1.78 ± 0.16 ^a	1.89 ± 0.10 ^a	1.72 ± 0.11 ^a	0.59 ± 0.03 ^b	0.49 ± 0.04 ^b	1.22 ± 0.06 ^b	68.00 ± 8.33 ^a
+E2	1.80 ± 0.19 ^a	1.94 ± 0.26 ^a	1.21 ± 0.07 ^b	0.96 ± 0.06 ^a	0.94 ± 0.14 ^a	2.11 ± 0.27 ^a	75.49 ± 2.97 ^a
F test	*	*	*	**	*	NS	NS

Results are presented as the mean of the fold expressions for the measured cytokines and PGE₂ production ± SEM of four independent experiments performed in triplicate. E2/BOEC: group of BOEC that exposed to estrogen, -E: the group of BOEC not exposed to estrogen while +E: The group of BOEC exposed to estrogen.

**highly significant at the 0.01 probability level.

^{ab}Means in the same column with different superscripts are significantly different at $P < 0.05$, as determined by Duncan's multiple range test.

and IL1B) in piglets fed a ZEN (316 ppb) contaminated diet for 18 days [40]. Previously, it was also shown that ZEN (100 nM) induced *IL1B* transcription in macrophages through interaction with estrogen receptors [41]. In the present study, E2 increased the expression of pro-inflammatory cytokines only in the sperm co-culture group. Similarly, a significant increase of TNF and IL1B appeared following the exposure of ovariectomized mice to E2 [42]. Therefore, ZEN may intensify the estrogenic response of BOEC to up-regulate the expression of such pro-inflammatory cytokines. Taking into consideration that the basic E2 supplementation (0.1 ng/mL) in our culture and the subsequent non-physiological high level of E2 treatment (5 ng/mL), we cannot exclude the possibility that E2 affected the sperm action on BOEC and induced the inflammatory reaction *in vitro*.

IL8 is a pro-inflammatory chemokine that regulates the attraction and trafficking of neutrophils during the acute phase of inflammation [43]. ZEN increased the expression of *IL8* in BOEC. Notably, in the sperm co-culture group, ZEN at 10, 100 and 1000 ng/mL upregulated the expression of *IL8*. It was reported that deoxynivalenol (another Fusarium-mycotoxin) increased IL8 secretion 10- to 15-fold in human intestinal epithelial cells [44]. The current results showed a significant effect of E2 on *IL8* expression without any interaction between E2 exposure and sperm co-culture.

We did not detect any effect of ZEN on the expression of anti-inflammatory cytokines (*TGFB1* and *IL10*) in BOEC. Nevertheless, in presence of sperm, ZEN (at 1000 ng/mL) disrupted the anti-inflammatory effect of sperm on BOEC by suppressing *TGFB1* expression. Interestingly, E2 upregulated the expression of both cytokines and did not affect the BOEC gene expression induced by sperm co-culture. Similar to our results, peritoneal cells collected from E2-treated mice showed an increase in *TGFB1* and *IL10* expression [45].

It has been reported that sperm binding to BOEC induces *PTGES* expression and enhances the secretion of PGE₂ [17,18]. The concentrations of PGE₂ found in pre-ovulatory oviducts suppress sperm phagocytosis and control the pro-inflammatory cytokine expression (*TNF* and *IL1B*) in BOEC [17,19]. On the other hand, PGE₂ stimulates acute local inflammation [46]. For example, PGE₂ concentration is increased in women suffering from endometriosis [47]. In the present study, ZEN increased the expression and production of PGE₂ in BOEC. In the sperm co-culture group, the high level of ZEN (1000 ng/mL) increased the PGE₂ production more than three-fold compared to control incubations (without exposure to ZEN). Similar results were obtained in intestinal cell cultures exposed to various concentrations of DON [48]. There was no effect of E2 (5 ng/mL) on the production of PGE₂ by BOEC.

Overall, ZEN at higher levels (100–1000 ng/mL) induced a pro-inflammatory response in BOEC through stimulation of *TNF*, *IL1B*

and *IL8* mRNA expression and increased the production of PGE₂. It seems that oviductal cells exposed to ZEN recognize sperm as foreign cells to be excluded. While, E2 did not affect the pro-inflammatory cytokine (*TNF* and *IL1B*) expression in case of sperm absence and had no effect on PGE₂ production by BOEC. It could be concluded that ZEN can act as an inflammatory inducer in BOEC with some different effects when compared with E2 in presence and absence of sperm. It is therefore clear that ZEN has a negative impact on the bovine oviduct in response to sperm, thus hindering successful fertilization.

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