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Effects of *in vitro* exposure of sheep ovarian tissue to zearalenone and matairesinol on preantral follicles

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Short Communication

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Summary

The aim of this study was to evaluate the effect of 1 µmol/l zearalenone (ZEN) and 1 µmol/l matairesinol (MAT), alone or in combination, on the morphology of *in vitro*-cultured ovarian preantral follicles. Ovaries from four adult sheep were collected at a local slaughterhouse and fragmented, and the ovarian pieces were submitted to *in vitro* culture for 3 days in the presence or absence of the test compounds. The morphology of primordial and primary follicles was impaired by ZEN. The plant lignan MAT alone did not maintain the morphology of the ovarian follicles; its combination with ZEN counteracted the negative effects observed when follicles were cultured in the presence of the mycotoxin alone. However, MAT was not able to promote the *in vitro* development of the ovarian follicles.

Introduction

Although acute intoxications can be avoided in farm animals, it is not always possible to circumvent the chronic exposure to dietary contaminants. Xenoestrogens are common compounds present in the form of mycoestrogens or phytoestrogens. Zearalenone (ZEN) is a non-steroidal oestrogen-like mycotoxin of high concern to livestock reproduction because of its strong capacity to bind to oestrogen receptors and, therefore, cause reproductive, immunotoxic (Hueza *et al*., 2014), and oxidative stress effects (Qin *et al*., 2015).

Usually, feed additives with a capacity to bind (Santos *et al*., 2011; Samik and Safitri, 2017) or to biotransform (Rogowska *et al*., 2019) this mycotoxin are added to the diet to limit or minimize the livestock exposure. However, a complete decontamination is not always obtained *in vivo*, and other interventions are needed, for example the dietary supplementation with compounds able to counteract the negative effects of ZEN. For example, feed is supplemented with antioxidants.

Among the dietary phytoestrogens, matairesinol (MAT) is a plant lignan that occurs in several grains and legumes. This phytochemical is known by its anticarcinogenic, oestrogenic, antiestrogenic, and antioxidant activities (Niemeyer *et al*., 2003). Flaxseed meal, an ingredient that can be encountered in the ruminant diet, is abundant in MAT (Liggins *et al*., 2000). The positive effects of dietary inclusion of flaxseed has been already reported for the quality of milk (Otero *et al*., 2018), immunomodulation (Caroprese *et al*., 2015), and improved reproductive performance (Didarkhah *et al*., 2020) in sheep. However, it is not known if parts of these positive effects are linked to the plant lignan MAT. Therefore, the aim of the present study was to evaluate the effect of ZEN, MAT, or their combination, on the morphology and development of sheep preantral follicles. Follicular density was also evaluated because this may indicate basal fertility (Lass *et al*., 1997). For this, ovarian tissue from sheep were cultured *in vitro* in the presence of ZEN, MAT, or ZEN combined with MAT.

Materials and methods

Test compounds

Unless mentioned otherwise, substances used in the present experiment were purchased from Chem Cruz (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and stock solutions were prepared using dimethyl sulfoxide (DMSO) as a solvent. The final DMSO concentration added to the culture medium was 0.1%. The culture medium was purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Animals ethics

This study was approved and conducted according to the Animal Management and Ethical Regulation Committee of the State University of Ceará (No. 9433833/2018). Ovarian tissues were collected from a commercial abattoir.

Ovarian tissue collection

Sheep ovarian pairs were collected from four adult sheep from a local slaughterhouse immediately after slaughter. After collection, ovaries were washed once in 70% alcohol for 10 s, followed by two washes in Minimum Essential Medium (MEM) supplemented with HEPES (MEM-HEPES), penicillin (100 µg/ml) and streptomycin (100 µg/ml). Then, the ovaries were stored in sterile tubes with the same medium and transported to the laboratory at 4°C within 1 h.

Experimental design

Four sheep ovarian pairs was divided into five fragments of 3 × 3 × 1 mm, and randomly assigned to the different experimental conditions. Of these, one fragment was randomly selected to be the fresh control, and the remaining eight fragments (two per treatment) were cultured *in vitro* in non-treated culture medium or in culture medium supplemented with zearalenone (ZEN), matairesinol (MAT), or a combination of ZEN and MAT (ZEN + MAT). All supplements were used at a concentration of 1 µmol/l. Concentrations of the test compounds were selected based on previously tested levels of ZEN in combination with the xenoestrogen equivalent (1 µmol/l) (Silva *et al.*, 2019). After *in vitro* culture, ovarian fragments were fixed for histological analysis. The culture medium was separately analyzed for oxidative stress using nitrite and malondialdehyde (MDA) levels as markers.

In vitro *culture*

Fragments of ovarian cortex were individually cultured *in vitro* in 24-well plates each containing 1 ml of culture medium. Culture medium consisted of α-MEM supplemented with 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 2 mg/l glutamine, 2 mg/l hypoxanthine, and 1.25 mg/ml bovine serum albumin. For the experimental group, ovarian fragments were cultured in the absence or presence of the test compounds, as mentioned above. Dimethyl sulfoxide (DMSO) at a concentration of 0.1% was used as the dilution vehicle, and was also added to the non-supplemented culture medium. Fragments were then cultured for 3 days at 39°C in humidified air with a 5% CO_2 atmosphere.

Histological analysis

Ovarian fragments were fixed in 4% paraformaldehyde and then dehydrated in increasing ethanol concentrations. Subsequently, these fragments were embedded in paraffin and cut into 7-µm sections, mounted under glass slides and stained with Periodic Acid-Schiff (PAS) and haematoxylin. For morphological evaluation, slides were examined under an optical microscope (Nikon, Japan) at ×400 magnification. The analyzed preantral follicles were classified into primordial (oocyte surrounded by a layer of squamous granulosa cells), primary (oocyte surrounded by a layer of cubic granulosa cells) or secondary (oocyte surrounded by two or more layers of cubic granulosa cells) (Sales *et al.*, 2016). To avoid double counting, follicles were counted only in sections where their oocyte nucleus was observed. Follicles were classified as morphologically normal or degenerated considering the following characteristics: absence or presence of pycnotic bodies, cytoplasmic retraction and granulosa cell organization, as described by Santos *et al.* (2006).

Density of preantral follicles

The density of preantral follicles, per development group or as total, was calculated as the total number of follicles, per class, divided by the total tissue volume and expressed as the number of follicles/mm^3 of ovarian tissue (Asadi-Azarbaijani *et al.*, 2017). The volume of the analyzed ovarian sections was calculated by summing the number of sections of area multiplied by the thickness of each section.

Nitrite and MDA quantification in culture medium

For the evaluation of nitrite and MDA levels, 200 µl of culture medium from each treatment were collected and stored in a freezer at −80°C for later measurement. For nitrite levels, a protocol already established by Green *et al.* (1981) applied. In summary, 100 µl of Griess reactive (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine hydrochloride, H_3PO_4 in 1%, 1:1:1:1 distilled water) was added to 100 µl of the culture medium. Then the samples were incubated at room temperature for 10 min. A standard curve was drawn with various sodium nitrite concentrations (ranging from 0.25 to 2.378 µM), and the samples were evaluated at the 560 nm wavelength of using a microplate reader (Metertech Inc., Taipei, Taiwan). The blank (negative control) was prepared by adding 100 µl of Griess reactive to 100 µl of the base medium.

To assess MDA levels, the thiobarbituric acid reactive substances (TBARS) method was used. For this, 100 µl of the culture medium was used and incubated in a water bath at 37°C for 1 h. Subsequently, 80 µl of 35% perchloric acid was added and centrifuged at 14,000 rpm at 4°C for 10 min. Thereafter, 50 µl of thiobarbituric acid was added to the supernatant and the mixture was incubated at 100°C for 30 min (Draper and Hadley, 1990). After cooling to room temperature, the measurement was performed by absorbance at a wavelength of 532 nm in a microplate reader (Metertech Inc., Taipei, Taiwan).

Statistical analysis

The ovarian fragments were randomly assigned to treatment conditions prior each experiment. Statistical analysis was conducted with the GenStat statistical software (GenStat for Windows 20th Edition, VSN International, Hemel, Hempstead, UK; https://www.vsni.co.uk/downloads/genstat/). Data were compared using analysis of variance (ANOVA). The null hypothesis was that there was no treatment effect on the response parameter. Treatment means were compared according to Tukey's test after a significant treatment effect was confirmed by ANOVA. Data are presented as mean ± standard error of the mean (SEM). The *P*-value of the statistical model is given per response parameter. Effects with $P \leq 0.05$ were considered to be statistically significant.

Results and Discussion

Figure 1 depicts the percentages of morphologically normal primordial, primary and secondary preantral follicles, as well as the density of these follicles in the ovarian tissue before and after culture. *In vitro* culture significantly decreased the percentage of

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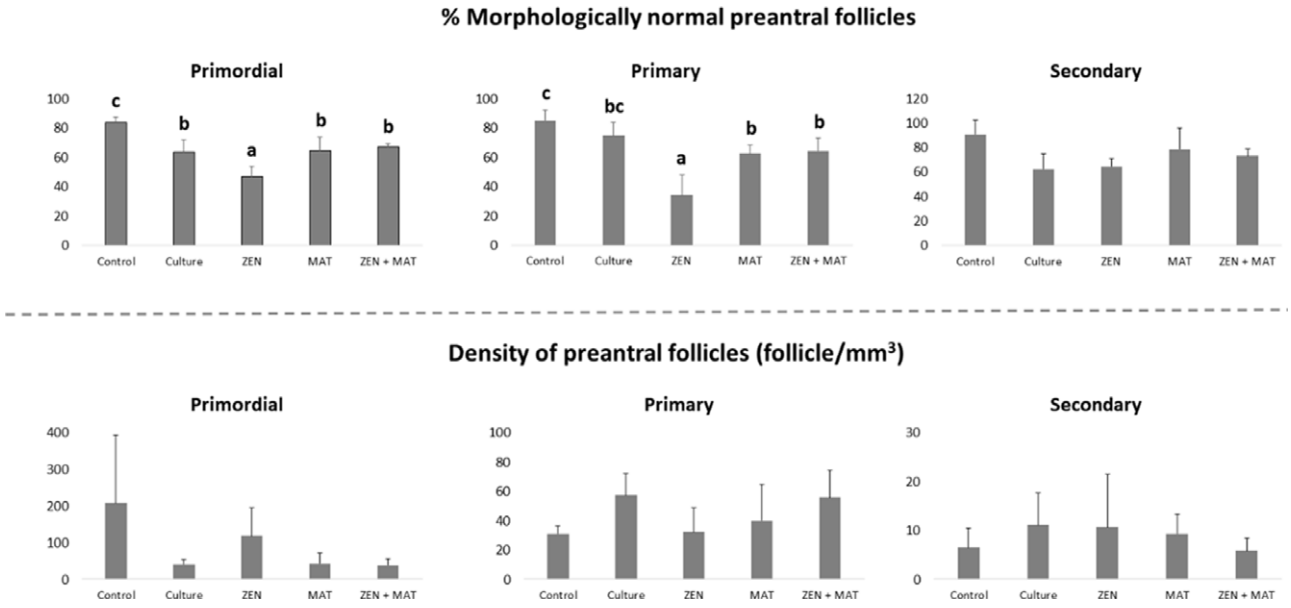

Figure 1. Mean (± standard error of the mean) percentages of morphologically normal preantral follicles per class, and the density of primordial, primary, and secondary ovarian follicles. [a–c]Different letters indicate significant differences among treatments ($P \leq 0.05$).

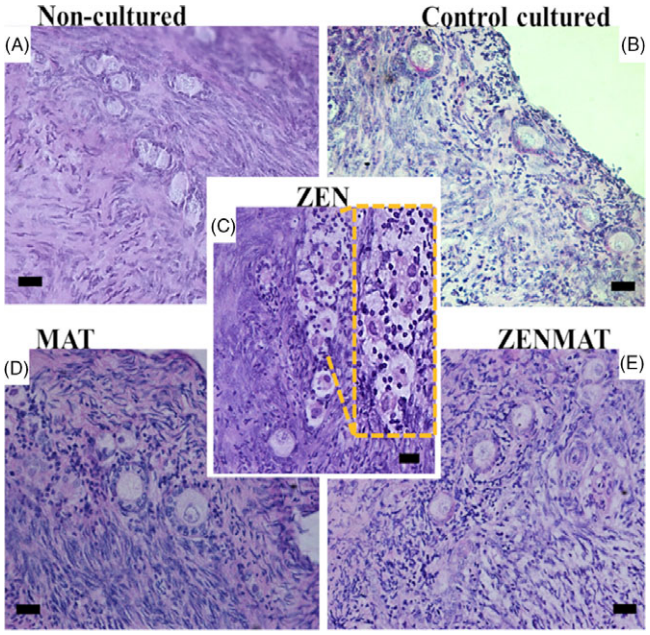

Figure 2. Representative images showing histological aspects of morphological normal preantral follicles before culture (A) and after culture in the absence of xenoestrogens (B), degenerated follicles when cultured in a medium supplemented with 1 µmol/l ZEN (C), and normal follicles when cultured in a medium supplemented with MAT alone (D) or ZEN + MAT (E). Bars, 50 µm. Staining: Periodic Acid-Schiff (PAS) and haematoxylin.

morphologically normal primordial follicles when compared with a non-cultured control. Although *in vitro* culture is not able to keep the rate of morphologically normal follicles similar to that of fresh tissue, the culture of ovarian tissue remains an indicated method to evaluate the effect of toxins in complex structures such as ovarian follicles (Santos *et al.*, 2013). The lowest percentages of morphologically normal primordial follicles were observed when the ovarian fragments were exposed to ZEN. All the other treatments resulted in percentages of morphologically normal primordial follicles similar to a non-supplemented cultured control. Similarly, the lowest percentages of morphologically normal primary follicles were observed when the ovarian fragments were exposed to ZEN, whereas ovarian tissue culture in the presence of MAT or ZEN + MAT resulted in percentages of morphologically normal primary follicles similar to the non-supplemented cultured control, but lower than the fresh (non-cultured) control.

The present culture period of 3 days is sufficient to achieve activation and initial development of preantral follicles. This was shown previously for the shorter culture period of 24 h (Abir *et al.*, 1999, 2001; Brito *et al.*, 2013). Although MAT maintained the rates of morphologically normal primordial and primary follicles that were also exposed to ZEN, no increase in follicular activation was observed, as shown by the unchanged density of growing (primary and secondary) ovarian follicles. This plant lignan inhibits angiogenesis by suppressing hypoxia-inducible factor-1a (HIF-1a) and its target gene, vascular endothelial cell growth factor (VEGF; Lee *et al.*, 2012). VEGF is expressed in granulosa cells and oocytes from preantral follicles (Brito *et al.*, 2018) and stimulates the growth of preantral follicles (Hunter *et al.*, 2004).

Regardless of the treatment, *in vitro* culture did not affect the percentages of normal secondary follicles, which may also be linked to an inhibitory effect of MAT on VEGF, or by its limited antioxidant activity at the tested concentration. This is supported by the fact that none of the treatments affected the levels of nitrite or MDA in the culture medium (data not shown). Exposure to ZEN may result in oxidative stress and cell membrane damage, which can be characterized by an increase in nitric oxide production and lipid peroxidation (Barbasz *et al.*, 2019). Such an effect can be determined in cell culture suspensions, in which an increase in nitrite levels is indicative of oxidative stress and membrane lipid peroxidation will result in increased MDA levels.

Representative images of preantral follicles from each treatment are depicted in Fig. 2. It was noted that ovarian follicles exposed to ZEN presented vacuolization of the oocyte, whereas this effect was not present when the culture medium contained ZEN + MAT.

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Ooplasm vacuolization caused by ZEN has been linked to autophagy (Schoevers *et al.*, 2012). However, there is a lack of information regarding the mechanisms behind the counteracting effect of MAT against ZEN.

In conclusion, we demonstrated the effects of two xenoestrogens on the morphology of sheep preantral follicles. As showed in previous studies, ZEN impairs the morphology and developmental capacity of preantral follicles (Schoevers *et al.*, 2012; Silva *et al.*, 2019). This oestrogenic mycotoxin is known by its capacity to induce oxidative stress and to decrease the antioxidant capacity of granulosa cells (Qin *et al.*, 2015). Therefore, the use of antioxidant compounds appears to be one method to alleviate the negative effect of ZEN. Although MAT protected the primordial and primary follicles from morphological damage caused by ZEN, this plant lignan was not able to promote follicular development *in vitro*. One must bear in mind that a substantial proportion of MAT is metabolized by the host's intestinal bacteria into the mammalian lignans enterodiol and enterolactone. Therefore, further studies should consider the interaction of ZEN with these metabolites.

Conflicts of interest. The authors declare no conflict of interest to disclose.

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