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HT-2 toxin affects development of porcine parthenotes by altering DNA and histone methylation in oocytes matured *in vitro*

Yue Zhang [a], Ru-Xia Jia [a], Meng-Hao Pan [a], Yujie Lu [a], Xiang-Shun Cui [b], Nam-Hyung Kim [b], Shao-Chen Sun [a, *]

[a] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China
[b] Department of Animal Sciences, Chungbuk National University, Cheongju 361-763, Republic of Korea

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ABSTRACT

T-2 toxin is a type A mycotoxin produced by various *Fusarium* species, while HT-2 toxin is a major metabolite of T-2 toxin. Both T-2 toxin and HT-2 toxin are known to have deleterious effects on animals. Our previous work showed that HT-2 treatment caused the failure of porcine oocyte maturation. In this study, we reported that HT-2 also affected porcine embryo development. In HT-2 toxin treated group, all the percentages of embryos in 2-cell, 4-cell and blastocyst stage were significantly lower compared with those in control groups. We then explored the causes from the epigenetic modification aspect of the oocytes. The analysis of fluorescence intensity showed that 5-methyl cytosine (5 mC) level was increased after exposure to HT-2 toxin in porcine oocytes, indicating that the general DNA methylation level increased in the treated porcine oocytes. In addition, histone modifications were also affected, since our results showed that H3K4me2 and H3K9me2 levels were increased in the oocytes from HT-2-treated group. Therefore, our results indicated that HT-2 toxin decreased porcine embryo developmental competence through altering the epigenetic modifications of oocytes.

1. Introduction

T-2 toxin is a secondary metabolite which belongs to the trichothecene mycotoxin family while HT-2 toxin is the metabolite of T-2 toxin. T-2 and HT-2 toxin was reported to be produced by *Fusarium sporotrichioides*, *F. poae, F, equiseti,* and *F. acuminatum*. T-2 toxin has the greatest cytotoxicity among the trichothecenes [1] and has been implicated in several mycotoxin diseases of animals and human [2]. Several studies indicated that T-2 toxin might impair the reproduction and development in animals, especially in pigs [3–6]. It was reported that exposure of oocytes to mycotoxins would inhibit oocyte maturation and reduce the percentage of oocytes that cleaved and formed a blastocyst [7] and mycotoxin alpha-zearalenaol affected porcine pre-implantation embryonic development [8]. Moreover, ochratoxin A, one of the other mycotoxins, also inhibited mouse embryonic development [9]. Besides ochratoxin A, the exposure to zearalenone and deoxynivalenol of porcine oocytes could cause aneuploidy and abnormal embryo development [7]. Our recent work also showed that zearalenone affected developmental competence of mouse egg [10].

Trichothecene mycotoxin is widely known as an inhibitor of synthesis of DNA and protein, which further affects genetic modification. It was reported that in primary and cloned murine T cells, deoxynivaleno (one of the trichothecene) caused the increase of Interleukin-2 gene expression [11]. Meanwhile there were also studies showed that mycotoxins affected epigenetic modifications [12,13]. While DNA and histone methylations are involved in epigenetic modifications. Both of them are associated with various cellular processes, such as transcriptional repression, embryonic development, genomic imprinting and the alteration of chromosome structure [14,15]. DNA methylation is one of the most important epigenetic modification in animals [16]. Methylation erasure occurs in the pre-implantation embryo with the exception of imprinted genes and some repeated sequences which means most methylation patterns will be altered in the early embryo [17]. During mammalian embryonic development, DNA methylation is reprogrammed, while histone modification interacts with DNA methylation to produce further epigenetic regulation [18]. Previous work showed that histone methylation was important for early embryonic stem cells [19]. Among histone methylation, H3K9

* Corresponding author.
E-mail address: sunsc@njau.edu.cn (S.-C. Sun).

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methylation is an important epigenetic mark of heterochromatin formation and transcriptional silencing. H3K9 methyltransferase G9a is essential for mouse embryogenesis [20]. H3K4 methylation alters chromatin structure and serves to recruit or exclude binding of non-histone proteins to chromatin as well [21]. Our previous work showed that mycotoxins, such as aflatoxin, zearalenone, and deoxynivalenol, had toxic effects on epigenetic modifications during mouse oocytes maturation [22]. Recently we found that HT-2 toxin affected the maturation of porcine oocyte [23]. Although HT-2 treatment caused the failure of meiotic maturation in a big proportion of oocytes, one question arises that whether the oocytes which reached the MII stage with the exposure of HT-2 toxin could develop into a blastocyst.

In present study we hypothesized that HT-2 toxin might have a negative effect on the developmental competence of porcine embryo, and this might be due to the effects on epigenetics modification of oocytes. Due to the low efficiency of *in vitro* fertilization of porcine oocyte, we adopted the pathenote embryo as the model, and our results showed that HT-2 toxin disturbed the developmental competence of porcine embryos from the altered levels of DNA and histone methylation in porcine oocytes.

2. Materials and methods

2.1. Antibodies and chemicals

Mouse monoclonal *anti*-5-methyl cytosine (5 mC) antibody was purchased from Abcam (CAT.NO:ab10805, Cambridge, UK). Rabbit monoclonal *anti*-H3K9me2 antibody (CAT.NO: 4658), rabbit polyclonal *anti*-H3K4me2 antibody (CAT.NO: 9725) were obtained from Cell Signaling Technology (Beverly, MA, USA) while HT-2 toxin was from J&K Chemical Ltd. (CAT.NO:610451, Shanghai, China). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise indicated.

2.2. Oocyte harvesting and in vitro maturation

The experiments were conducted in accordance with the Animal Research Institute Committee guidelines of Nanjing Agricultural University, China (SYXK Su2017-0007). This study was specifically approved by the Committee of Animal Research Institute, Nanjing Agricultural University, China. The ovaries of Duroc pigs were obtained from prepubertal gilts at a local slaughterhouse and transported to our laboratory within 2 h in 0.9% physiological saline at 35 °C in a thermos bottle. After washing with sterile saline, follicular fluids with cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm antral follicles using a 10 ml disposable syringe with an 18 G needle. COCs with an intact and compact cumulus were selected for maturation. The medium used for maturation was an improved TCM-199 supplemented with 75 μg/ml of penicillin, 50 μg/ml of streptomycin, 0.5 μg/ml of FSH, 0.5 μg/ml of LH, 10 ng/ml of the epidermal growth factor (EGF), and 0.57 mM cysteine. HT-2 toxin was dissolved in DMSO to the concentration of 1 mM. Then it was diluted with a maturation medium. We chose 100 nM as a final concentration because the rate of cleavage decreased significantly in this dose. We added the same concentration of DMSO as the control. In both control and HT-2-treated groups, about 80 oocytes were cultured in 500 μl of maturation medium covered with 200 μl of mineral oil at 38.5 °C in a humified 5% CO_2; atmosphere in a four well culture dish (Nunc, Oskide, Denmark) respectively. After 44–48 h, the oocytes reached MII stage. We used stereo microscope to observe the polar body, and the oocytes with polar body were as MII oocytes (maturated oocytes). At least three replicates were used for each treatment and no less than 30 oocytes were examined for each replicate.

2.3. Parthenogenetic activation and embryo culture

After maturation, the cumulus cells were removed by gentle, repeated pipetting in a hyaluronidase solution (1 mg/ml) for 2–3 min. For activation, denuded oocytes with visible polar bodies were gradually equilibrated in activation medium comprised of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$, lined up in an activation chamber filled with activation medium. The oocytes which reached MII stage were then electrically activated in a BTX microslide 0.5-mm fusion chamber (model 450; BTX, San Diego, CA, USA) using a single direct current pulse of 0.86 kV/cm for 80 ms, followed by chemical activation with 10 mg/mL cycloheximide and5 mg/ml cytochalasin B in PZM-3 medium for 4 h. The parthenogenetically activated oocytes were cultured in PZM-3medium in a 4-well plate at 38.5 C in a 5% CO2 atmosphere at maximum humidity in an incubator. We collected 2-cell embryos, 4-cell embryos, blastocyst embryos at 24 h, 48 h, 7 days respectively after parthenogenetic activation.

2.4. Immunofluorescentstaining

After the cumulus cells were removed by repeated pipetting, the denuded oocytes were used for observations. The oocytes were first fixed with 4% paraformaldehyde for 30 min and then permeabilized with 1% Triton X-100 at room temperature for at least 8 h. After blocking with 1% BSA-supplemented phosphate-buffered saline (PBS) for 1 h, the oocytes were stained with different primary antibodies (H3K9me2, H3K4me2 both at 1:200) at room temperature for 1 h.

In order to detect 5 mC fluorescent signals, the zona pellucida of oocytes was removed with 0.05% pronase in PBS. These specimens were then treated with 2 N HCl to be denatured at room temperature for 30 min, neutralized with 100 mM Tris-HCl at pH 8.5 for 10 min. Next, these oocytes in control and HT-2-treated groups were transferred into PBS that contained 0.05% Tween-20 at 4 °C to be incubated overnight or be incubated at room temperature for 1 h followed by three times washing in PBS that contained 1% BSA. Other staining steps were the same as it was in the paragraph above. And the antibodies used for 5 mC staining was anti-5-methyl cytosine diluted at 1:500. Samples were mounted on glass slides and examined under a confocal laser-scanning microscope (Zeiss LSM 700 META, Jena, Germany).

2.5. Fluorescence intensity analysis

Control and treated oocytes were mounted on the same glass slide for fluorescence intensity. We used the Confocal Laser-scanning Microscope to do the immunofluorescence analysis (Image J software, NIH) and the same settings were used to normalize across the replicates. At least three replicates were used for each treatment and no less than 30 oocytes were examined for each replicate. A region of interest (ROI) was defined for the chromosome of oocyte and the average fluorescence intensity perunit area within the ROI was determined. Independent measurements using sized ROIs were taken, the average values of all measurements were used to determine the final average intensity between control and treatment groups. The control oocyte fluorescence was taken as 1.

2.6. Statistical analysis

At least three replicates were used for each treatment and no less than 30 oocytes were examined for each replicate. Data were evaluated by Student's t-test and expressed as mean + SEM through GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). A p-value of <0.05 was considered statistically significant.