Epigenetic Disruption Analysis of Zearalenol on Different Stages of In-vitro Culture of Parthenogenetically Activated Bovine Embryos

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ABSTRACT

As a preformed form on plant tissues being consumed by the animals or during the process of biotransformation, zearalenone is converted into many intermediate compounds including alpha and beta zearalenol, which may be toxigenic to the oocyte and subsequent embryo development stages. In this study, we observed in-vitro effects of 3 μM, 30 μM alpha and 3 μM, 30 μM beta zearalenol addition compared to the negative control group on the consecutive stages of bovine oocytes in the in-vitro maturation (IVM) media along with proceeding ill effects on parthenogenetically activated bo-

vine embryos, in two steps defined in-vitro culture (IVC) media. Developmental competence of embryos was accessed by observing cleavage percentage, subsequent blastocyst formation percentage, and counting of nuclei of blastomeres in the individual blastocysts after Hoechst staining. With the increasing doses of relevant mycotoxins, we observed a decreasing trend of embryo cleavage with a significant difference (<0.05) among control to 30 μM alpha and 30 μM beta zearalenol groups. Blastocyst formation rate showed significant decrease in all mycotoxin added treatment groups compared with the control while the cell numbers of individual blastocyst were only reduced in 30 μM beta zearalenol group compared to the control group ($P<0.05$). All these results indicate that beta zearalenol causes more genetic disruption to the subsequent stages of bovine zygotes as compared to the alpha zearalenol at the same concentration.

INTRODUCTION

Fungal mycoflora may produce specific mycotoxins that are involved in the production of a variety of clinical syndromes and illnesses in animals and humans (Chandra et al., 2011; Cao et al., 2013). Fusarium is suspected to be an etiological agent for many plants (West et al., 2017), human (Al-Hatmi et al., 2016), and animal (Antonissen et al., 2014) ailments, including Degnala disease in cattle and buffaloes in indo-pak region (Dandapat et al., 2011: Kumar, 2016). It produces its effects through its mycotoxins in which Zearalenone and its derivatives including alpha and beta zearalenol are known to us (Cortinovis et al., 2013; Bordin et al., 2017; Han et al., 2017).

The structure of the zearalenone and its metabolites resemble many characteristics of steroid hormones, and can bind to the estrogen receptor (ER) as an agonist (Minervini and Dell'Aquila, 2008). Three (3)(α-) (β-) HSD is a critical factor in ovarian follicular developmental process, and by competitively inhibiting 3(α-) reduction of testosterone, estradiol, and progesterone steroids, zearalenone can cause accumulation of these active components. This process will interrupt the metabolic pathway of steroidogenesis (Olsen et al., 1989; Minervini et al., 2001; Ji et al., 2017).

Alterations in the reproductive system that are caused by the exposure of zearalenone have been reported after in vivo studies (Ji et al., 2017) that verify information about net effects in the whole of the animal's body There, the toxin may act at different organs and sites, but at the same time, these investigations reveal little information related to the involved underlying processes (Alm et al., 2002). That's why tissue and cell culture studies are of increasing importance in the assessment of all of the risks which are due to these kinds of toxic compounds (Tiemann et al., 2007). Knowledge of the structure to function relationship in cell culture may be used for the prediction of mycotoxin toxicities in the in-vivo system of body (Alm et al., 2002).

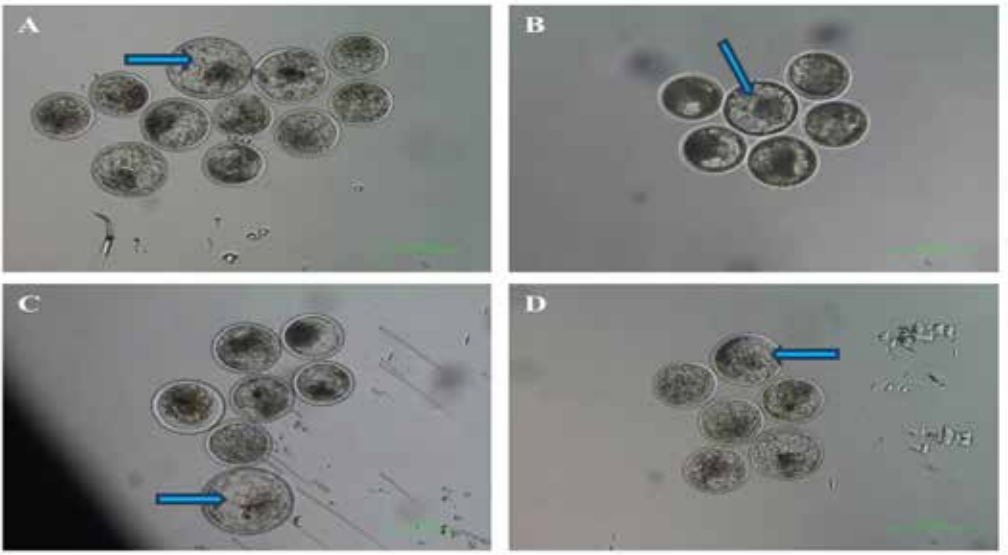
The effects of zearalenol have been studied in the in-vivo systems compared to in-vitro systems, especially in the sensitive species of animals (Minervini and Dell'Aquila, 2008). Cattle and Buffalo all around the world are facing challenges of Fusarium contaminated feeding like contaminated rice straw in scarcity seasons, which can produce several ailments in them (Benkerroum, 2016; Kumar, 2016). Effects of zearalenone and its derivatives have been minimally studied on IVM and IVC systems in bovine. In this work, we analyzed subsequent variations caused by alpha and beta zearalenol on bovine in-vitro culture system after parthenogenesis for the observations of zygote cleavage and blastocyst formation rates by assuming an interruption in the steroidogenesis and cell division mechanism interruption due to these mycotoxins.

MATERIALS AND METHODS

Chemicals

DMSO (Dimethyl Sulfoxide , Serva-20386, Heidelberg, Germany), Alpha Zearalenol (Santa Cruz Biotechnology, SC-202388), and Beta Zearalenol (Sigma, Deisenhofen Germany. Z-2000) were purchased from

Figure. 1. *Microscopic view of blastocysts formed in the IVC two step media, in the Control and experimental groups after parthenogenetic activation. Arrows indicating blastocoel formed at or after 7th day of parthenogenesis. (A) Control group (B) 3 µM alpha zearalenol (C) 30 µM alpha zearalenol and (D) 3 µM beta zearalenol.*



respective company being mentioned here. All other reagents were taken from Sigma-Aldrich, except those mentioned separately.

Oocytes Collections and in vitro Maturation (IVM)

Bovine ovaries from a local slaughterhouse were taken in normal saline with a temperature of 35 °C. Cumulus oocyte complexes (COC,s) from antral follicles with dense layers of cumulus cells (2-6 mm in diameter) and homogenous cytoplasm were selected and washed with HEPES buffered tissue culture medium- 199 (TCM-199; Gibco). In-vitro maturation of these oocytes in five different groups (Table. 1) were carried out for 22 hrs at 39 °C in an incubator having 5% CO2 and 90 % humidity in four well plates separately. IVM medium was having TCM-199 supplemented with 10% FBS (Gibco), 1 µg/mL β-Estradiol, 10 ng/mL epidermal growth factor α (EGFα), 100 µM Cysteamine, 0.9 mM Sodium pyruvate, 1% Penicillin-Streptomycin (v/v, Gibco), 5 µg/mL follicular stimulating hormone (FSH) and respective doses of added alpha or beta zearalenol in each group.

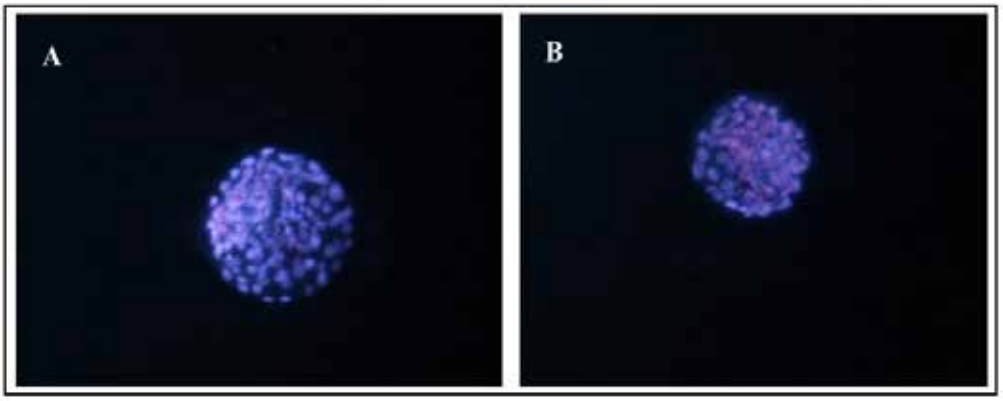
Oocytes Activation by Parthenogenesis

After 22 hrs of culture in the in-vitro maturation media, all of the COC,s were denuded by removing cumulus cells using 1.2 mg/mL hyaluronidase in the HEPES-buffered TCM-199 medium. All the cumulus-free oocytes after denuding were activated with the addition of 5-10 µM Calcium ionomycin in HEPES-buffered TCM-199 solution for 4 minutes followed by stabilization of these oocytes in 1.9 mM 6-dimethylaminopurine (6-DMAP) containing medium for 4 hrs. Before and after the use of Calcium ionomycine, all the oocytes were washed in the HEPES buffered TCM-199 medium one and two times respectively.

In vitro Culture (IVC) of Embryos.

The parthenogenetically produced zygotes were cultured in two steps chemically defined culture media (Lim et al., 2007) in a humidified incubator at 38.5 °C temperature having 5 % CO2 and 5 % O2 in air. In a 60mm petri plate, 5-7 embryos were cultured in each of 30 µL drop covered with mineral oil. In two steps, chemically defined culture media embryos were cultured in the

Figure 2. *Microscopic view of representative blastocysts (for counting of blastomeres per blastocyst) visualized under UV light after staining with Hoechst in the control and experimental groups. (A) Control group and (B) 3 µM alpha zearalenol.*



D1 medium for the first 4 days, and then for culture to the later stage, shifted in D2 medium till 7th day post-parthenogenesis. After the second day of parthenogenesis, embryos were checked for cleavage ratio in all the groups. Blastocyst formation was accessed on the basis of expansion and formation of blastocoel according to the international codes for developing different stages of embryo, starting from 5 to 9 for early compact to hatched blastocyst stage respectively.

Blastocysts Staining for Cell Counting

At 7th or 8th day of post parthenogenetic activation, all of the embryos that developed into blastocysts (accessed on the basis of expansion and blastocoel formation) were subjected to blastomeric nuclei staining by initially incubating in Triton-X solution (having 1 % Triton-X in PBS solution) for half minute at room temperature. They were subsequently and immediately transferred to the next solution of Hoechst solution (absolute alcohol with 25 µg/mL bisbenzimide (Hoechst-33258) stored them for 10-12 hrs at 4 ^{o}C temperature. After 12 hrs of this incubation, stained blastocysts were mounted onto a slide glass in glycerol drops and the total blastomeric nuclei were counted under stereomicroscope.

Statistical Analysis

Variation among experimental groups was analyzed with one way analysis of variance (ANOVA) applying Turkey's Multiple Comparison Test using GraphPad Prism 5 software (GraphPad Software, Inc., USA). Differences were taken as significant when $P<0.05$.

RESULTS

Cleavage Percentage After Parthenogenesis

After 22 hrs of IVM media culture, all of the oocytes (with or without 1st polar body) were subjected for parthenogenetic activation. After 2 days of parthenogenesis embryos were observed for cleavage in each separate group. Significant difference ($P<0.05$) between control and other two more concentrated groups viz. 30 µM alpha and 30 µM beta zearalenol was seen, with a pronounced difference between control and 30 µM beta zearalenol groups (Table. 1).

Blastocyst Formation Rates

After the 7th day of parthenogenetic activation, blastocyst formation rates in each individual experimental group were analyzed. Presence of blastocoel and the expanded mass of blastomeres were taken as inclusion criteria for blastocysts formation initially, which was confirmed after staining and blastomeric counting in individual blastocysts. Significantly higher numbers of blastocysts were observed in the control group (many fold) compared to all the rest four mycotoxin containing groups. The least numbers

of blastocysts were observed in 30 µM beta Zearalenol group showing this concentration to be the most lethal in the formation of blastocysts (Table.1 and Fig. 1).

Counting of Blastomeres per Blastocyst

The number of cells in each blastocyst were counted after Hoechst staining, showing a significant difference in the blastomeric counting between control and 30 µM beta zearalenol group (Table. 1 and Fig. 2).

DISCUSSION

Fusarium produces its effects on reproductive system through its mycotoxins in which Zearalenone and its derivative, including alpha and beta zearalenol, are known (Minervini and Dell-Aquila, 2008; Cortinovis et al., 2013; Bordin et al., 2017; Han et al., 2017).

To sustain development at an early embryonic stage, it is necessary for the oocyte to provide many important proteins and transcripts of maternal origin. "Maternal to embryonic transition" is a mechanism in which a large number of maternal macromolecules are assembled, utilized, and degraded. Embryonic and zygotic transcripts and proteins are produced to control and manage of early embryonic development (Wang et al., 2012; Ji et al., 2017). At this critical stage, if environmental contaminants may get involved, then they can influence this process of transition in early embryonic development.

After an analysis of a direct effect of alpha zearalenol and beta zearalenol in bovine IVM medium and IVC medium untill blastocyst formation (in control and all the treatment groups), indicating that the development of the early embryos untill blastocysts were negatively affected in a dose depending way. Exposure of added zearalenol in IVM and IVC media led to a significant decrease in the zygotic cleavage rates, and blastocyst formation rates between the control and all the zearalenol treatment groups, especially in the 30 µM beta zearalenol group. According to (Alm et al., 2002), 15µM alpha zearalenol started de-

Table 1. *Effect of alpha and beta zearalenol with various concentrations on the cleavage rate, blastocyst formation rate and blastomeres per blastocyst of bovine zygotes in vitro.*

Groups (concentration in µM)	Concentration of Zearalenol	Number of oocytes cultured	Number of zygotes showed cleavage (%)*	Number of blastocyst formed (%)*	Average number of Blastomeres per blastocyst.*
Control	0.1 % DMSO	342	218(63.74) a	74(21.64) a	128 a
3 µM Alpha	3 µM Alpha	327	170(51.99) ab	27(8.26)b	125 a
30 µM Alpha	30 µM Alpha	323	146(45.2) bc	17(5.26)b	115 ab
3 µM Beta	3 µM Beta	323	162(50.15) ab	18(5.57)b	116 ab
30 µM Beta	30 µM Beta	316	115(36.39)bc	8(2.53)b	96 bc

** Superscripts (abc) showing significant difference in a column (P<0.05).*

creasing porcine blastocyst formation rates and total cell numbers of blastocysts compared with the control group where in-vivo produced zygotes were cultured in-vitro (26.5±9.2vs. 61.9±10.0 and 15.2±1.9vs. 48.2±1.9; P<0.05) while in our work which is related to bovine oocytes 30 µM beta zearalenol showed very significant difference in blastocyst formation rates and decreased number of blastomeric nuclei count compared with the control group. Total cell count and apoptotic cell rates in blastocyst were important characteristics which are used for the evaluation of the quality of in-vitro culture system and in-vitro embryos (Pomar et al., 2005).

These findings are contrary to the findings of (Wang et al. 2012), where 10 µM alpha zearalenol showed the same kind of effects on porcine zygotes. In a previous study, it was reported that when in-vitro derived embryos being cultured in NCSU-23 medium, the total blastomeric nuclei counting started decreasing after an addition of 15 µM alpha zearalenol and it also started decreasing in the blastocyst formation rates compared with the control group (Alm et al., 2002).

Alterations in the reproductive system, which are caused by the exposure of zearalenone, have been reported (Bordin et al., 2017; Han et al., 2017) after in vivo studies that give informations about net effects in whole of the animal's body where toxin may act at different organs and sites but at the same time these investigations reveal little information related to the involved underlying processes in them (Alm et al. 2002). Thus,the tissue and cell culture studies are of increasing importance in the assessment of all the risks which are due to these kinds of toxic compounds (Tiemann et al., 2007). Knowledge of the structure to function relationship in cell culture may be used for the prediction of mycotoxin toxicities in the in-vivo system of body (Alm et al., 2002). The effects of zearalenol have been widely studied in the in-vivo systems compared to in-vitro systems, especially in the sensitive species of animals (Minervini and Dell'Aquila, 2008; Ji et al., 2016; Ji et al., 2017). In this study, we tried to analyze in-vitro effects of zearalenol (comparing both of its alpha and beta forms) on in-vitro maturation of bovine oocytes and subsequent effects on in-vitro culture for bovine zygotes untill blastocysts formation, including their cell numbers, after parthenogenesis. According to our findings, beta zearalenol is more lethal for bovine embryogenesis compared to the same doses of alpha isomer in the body, and it is also reported by (Malekinejad et al., 2006) that more predominant metabolite in bovine granulosa and hepatocyte cells is the beta zearalenol. This indicates that the toxic effects of zearalenol are multiplied after its ingestion in the bovine as compared to the porcine.

DMSO (0.1% v/v) was used as a vehicle to completely dissolve alpha and beta forms of zearalenol in both the in-vitro maturation (IVM) media and in-vitro culture (IVC) media. According to our results, and according to Wang et al., (2012), it was noticed that there were no ill effects after adding 0.1% DMSO on maturation rates, parthenogenetic activation, cleavage percentage of zygotes, and blastocyst formation percentages.

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CONFLICT OF INTEREST

There is no conflict of interests between any person or organization related to this research work.

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