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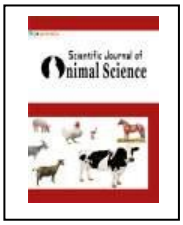

Original article

Effect of zearalenone on estrogen receptor, IGF-I and IGF-II genes expression in bovine oviduct epithelial cell culture

S. Erfani[a,*], M.T. Harkinezhad[b], H. Amanlou[a]
[a]*Department of Animal Science, Faculty of Agriculture. University of Zanjan, Zanjan, Iran.*
[b]*Research Institute of Biotechnology, University of Zanjan, Zanjan, Iran.*

**Corresponding author; Department of Animal Science, Faculty of Agriculture. University of Zanjan, Zanjan, Iran.*

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ABSTRACT

Zearalenone (ZEA) is a nonsteroidal estrogenic component that we assumed ZEA can bind to estrogen receptors and causes estrogenic responses and affect Insulin like growth factors (IGF) secretion and cause embryo growth retardation and abortion in cattle. Therefore bovine oviduct epithelial cells cultured (BOEC) in DMEM-Ham F12 medium, 1% Penicillin-erythromycin and 5% fetal bovine serum. The cells passaged for 2 times. Culture flasks divided into 4 treatments and 3 replications. Amounts of 100, 160 and 250 µl of ZEA(10000 ng/ml) added to 3 treatment of experiment and one treatment maintained as control group. After 36 hours cells detach from flasks surface and total RNA extracted. Total RNA converted into cDNA and estrogen receptor, IGF-I and IGF-II genes expression assessed with quantitative Real Time PCR. Results analyzed with SPSS statistics 20. The results show that estrogen receptor gene expression in presence of different amount of ZEA had a significant difference ($P<0.01$) with control group, but IGF-I, II gene expressions had no significant difference ($P<0.01$) between treatments and control group. It means that ZEA is agonist to estrogen receptor and it can activate estrogen receptors transcription. Although ZEA is able to activate estrogen receptors and cause alteration in function of reproductive tract. IGFs genes expressed in both treatments and control group and there was no any difference between them. ZEA is group of endocrine disrupters, but it has no effect on IGFs system

hormones.

1. Introduction

Zearalenone (ZEA) is an estrogenic mycotoxin produced by Fusarium species, such as F. graminearum and F. culmorum that induces hyperestrogenism in cows (Karami, 2008). The oviduct associated with the gamete transport synchronizes fertilization, nutrition, and development of the embryo. Many of these functions are controlled by steroid hormones such as estrogen (Reinhart, et al., 2003). Zearalenone causes estrogenic responses in dairy cattle and large doses of this toxin are related with abortions. It is recommended that zearalenone should not be more than 250 ppb in the total diet. Also ZEA interferes with implantation, gestation, ovulation, fetal development and the viability of neonatal animals (Zinedine et al., 2007). In a study (Takagi et al., 2008) dairy cow oocytes were cultured and different amount of ZEA concentrations 0, 1, 10, 100, and 1000 g/L added to media. In this way maturation rates decreased. And for further investigation, maturation of 124 oocytes examined and in 1000 g/L ZEA was arrested in metaphase I, had no effect on fertilization rate. The rate of blastocyst formation did not significantly differ among the groups. High ZEA concentration could affect meiotic competence but not the development rates and fertilization. The induction of estrogen controlled genes in tissue culture by zearalenone toxin demonstrated that this no steroidal mycotoxin acts as an agonist by activating the estrogen receptor (Mayr, 1988). Despite having a lower affinity to estrogen receptors than 17 β estradiol (10–100 times less), zearalenone and its metabolite, α-zearalanol, act with ERs (Nikov et al., 2000) to activate transcription of estrogen receptor genes in vivo (Mehmood et al., 2000) and in vitro (Kuiper et al., 1998). Insulin like growth factors (IGF-I and IGF-II) are expressed in embryos and reproductive tracts of cow and several species. IGFs have endocrine, paracrine and autocrine function that associated with cell division, blastocyst formation, implantation and embryo growth. Also, oviduct and endometrial secretions contain IGF-I and IGF-II in cattle (Ciftci, 2011). The insulin-like growth factor (IGF) system in exclusive and plays important role in every tissue. Hence it plays an essential role in embryonic and postnatal development, the IGF system is important in adult physiology. ZEA and its metabolites considered as endocrine disruptors since they would be regulating hormonal activity as well as acting as agonists or antagonists at the receptor (Penning et al., 2004). To further investigation of the role of estradiol in the insulin like growth factor system, an experiment was conducted to determine the dosage of the anastrozole as aromatase inhibitor, needed to decreases serum concentrations of estradiol-17β (E2) in maturing boars. Feeding the anastrozole to boars reduced the level of increase in serum concentrations of IGF-I. These data support a role for estradiol (E2) in the IGF system regulating components in pigs (Hilleson and Clapper, 2005).

Another study evaluated the uterine insulin like growth factor (IGF) system with using an endocrine disruptor in early pregnancy with exposure to exogenous estrogen on days 9 and 10 of gilts gestation. Results demonstrate that early exposure of pregnant gilts to estrogen causes loss of uterine IGFs during the concepts elongation. Timing for the release of uterine IGFs during early concepts development in porcine may play an important role in the ability to attach and survive embryo during the pregnancy (Ashworth et al., 2005).

We have conduct an examination on the IGF family since this family is one of the most important growth factor families in embryo early development. The distribution of both mRNAs encoding IGF-I and IGF-II has been recently detected in bovine oviduct monolayer (Xia et al., 1996). Therefore, The present study had done to investigate the expression of mRNAs encoding estrogen receptor, IGF-I and IGF-II gene in bovine monolayer oviduct epithelial culture in presence of zearalenone toxin. We suggest that, zearalenone affect oviduct estrogen receptor, IGF-I and IGF-II genes expressions and it could be related to embryo abortion.

2. Materials and methods

2.1. Bovine oviduct cell culture

Oviduct was obtained from pregnant cow that slaughtered at a nearby commercial abattoir. The fetus, ampulla and isthmus removed and a section of oviduct cut out.

For establishment of monolayer cultures, oviduct contents were squeezed into a 35-mm Petri dish. Oviduct tissue sample has been washed with phosphate buffer saline (PBS) and culture medium two times. The epithelial cells were isolated by scratching the both surface of tissue with plastic scraper. The cells were suspended in T-25 culture flask containing of 4 ml culture medium (Gibco), 10% fetal bovine serum (FBS) (Sigma) and 50 U/ml Penicillin-Erythromycin. By 48 h, cells observed under invert microscope (AUSTRIA micros) and approximately 20% of the surface of flask was covered by attached cells. The cultures were maintained by removal of the old medium and addition of 4 ml of fresh culture medium every 48 h. By week 1 the monolayer was confluent. After that, the cells passage into 4 culture flasks. In fact, these 4 flasks were the treatments of experiment. After one week the cells covered the whole surface of flasks. Then the cells of each treatment had passage into 3 flasks. Therefore, in this way the 12 replication of experiment had prepared.

2.2. Treatment with zearalenone and incubation

Zearalenone toxin with 10000 ng/ml concentration prepared from Faroogh laboratory. Added 100, 160 and 250 µl zearalenone to replications of treatments 1, 2 and 3 respectively. The replications of treatment 4 were the control group. Cells with zearalenone incubated for 36 hour.

2.3. Freezing the cells

Adding 200 µg EDTA-Trypsin to flasks and incubate for 2 minutes, cells isolated from surface of flasks. Cells centrifuged (eppendorf- 5810R) in 106 × g for 5 minutes at room temperature to sediment. Above liquid removed and added 1 ml fetal calf serum and cells suspended. Then cells transferred to cryovials (2ml- IMEC) and stored at -80°C until use.

2.4. RNA isolation

RNA isolation kit (cinnapure RNA purification kit- PR891620) purchased from CinnaClonCompany. Cells thawed in 37 °C and centrifuged in 956× g for 2 minutes at room temperature. The above liquid removed and cells had washed with PBS. According to kit protocol 400 µl lysate solvent added to 1.5 ml micro tube containing of cells. Vortex for 10 seconds and pipetting to homogenized appropriately. Then 300 µl precipitation solvent added to cells. By pipetting the mix, 700 µl of this mix transferred to purification columns and centrifuged in 956 × g for 2 minutes. 2 ml microtube under the columns removed and columns had been placed in new 2 ml micro tubes. 400 µl of WASH-I solvent added into columns and centrifuged. The liquid under the columns removed and added 400 µl of WASH-II solvent to columns and centrifuged. Liquid under columns removed and this stage done for two times. Follow that prewarmedRNase free water at 37 °C for 3 minutes, added in center of columns and incubated at 37 °C for 3 minutes. Columns centrifuged in 956 × g for 2 minutes. The columns removed and RNA stored at -80 °C.

2.5. CDNA synthesis

Total RNA isolated from oviduct cells, was reverse transcribed into cDNA with 2-step RT-PCR kit purchased from Sinaclon Company. The first mixture consisted of 1µl oligo d(t), 1µl 10mM dNTPS,4µl Nuclease- free Water per sample to the final volume 10 µl, prepared into 1.5 ml microtube. 12 µl of mixture transferred into 12 (number of replications) 0.2 ml micro tubes and 4 µl of total RNA added into mixture. Samples incubated in 65 °C for 5 minutes in PCR. Then had cooled on ice for 2 minutes. Also the second mixture consists of 2 µl 10X Buffer M-MuLV, 0.5µl M-MuLV Reverse Transcriptase and 7.5 µl Nuclease- free Water per sample prepared. 10 µl of mixture added to samples and the RT reaction was carried out at 42 °C for 60 min and followed by a denaturation step at 85 °C for 5 min, and stored at -20 °C until future investigation.

2.6. Quantitative Real-Time PCR (qPCR) for estrogen receptor and IGFs genes in oviduct cells

Mix containing of 4µlHotTag Eva GreenqPCR mix(no Rox- BT11102c),0.16 µl forward and reverse primers and 7.68 µl of nuclease free water per sample prepared. 12 µl of mix transferred to 12 micro tubes (0.2 ml). Then 8 µl of cDNA added to mix. For each gene, prepared separate mix. Then expression of each gene observed on Real-Time PCR with 95°C for 15 minutes to cDNA denaturation, 55-58°C (depending on primers Tm) for 60 seconds to annealing and 72°C for 20 seconds to elongation. Expression of Housekeeping gene (GAPDH) had obtained from RT-PCR too.

2.7. Statistical analyses

By putting threshold for every gene cycles, we obtain CT for every treatment. The CT data corrected with data released from GAPDH gene and then analyzed with paired samples t-test on SPSS.

3. Results

3.1. Effect of zearalenone on oviduct epithelial cell viability

By adding zearalenone(10000 ng/ml) to cells, the flasks exposure with 100 µl toxin (A) show a few dead and isolated cells, flasks with 160 µl toxin (B) show more dead cells and flasks with 250 µl toxin (C) have been observed all cells isolated from flask surface. The flasks with no toxin (D) show any dead cells.

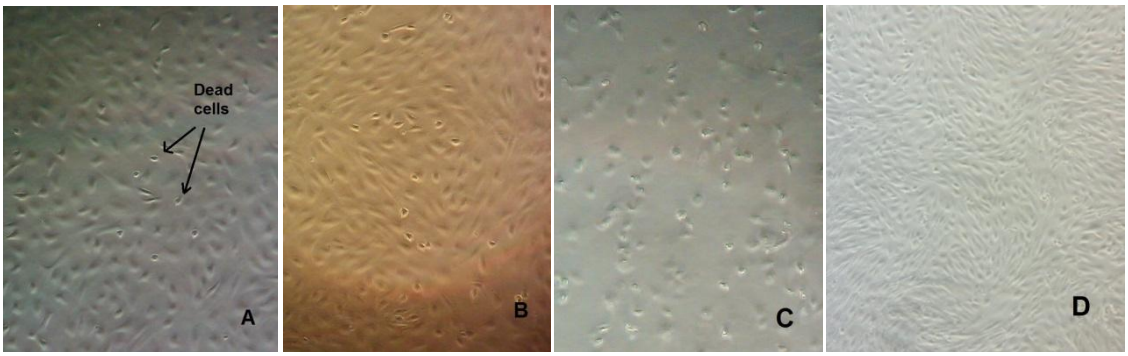


Fig.1. Bovine oviduct epithelial cells response to zearalenone toxin. Figure (A) illustrates that treatment received 100 microliter zearalenone, a little dead and float cells (flash in the picture) observe in the medium culture. 160 microliter zearalenone to cells of treatment 2 (B) result in more dead cells in culture flasks and 250 microliter zearalenone in the flasks of Treatment 3 (C) show all cells quietly dead and floated in medium. control group (D) received no zearalenone toxin and all cells was alive and attached to culture flasks surface. (100x magnification).

3.2. Expression of genes

After isolating cells from culture flasks, treatment interaction with toxins was detected for quantitative Real Time-PCR analysis of oviduct estrogen receptor, IGF-I, IGF-II gene expression in bovine.

Table 1
bovine PCR primer sequences used for quantitative Real Time-PCR

Gene	Forward primer/revers primer	Fragment size(bp)	Anealing temperature(ºC)
EstR	F- 5' GCCTCAGGCTACCATTACGG 3'	247	61.89
	R- 5'CATCATCTCTCTGGCGCTTG3'	247	62.05
IGF-I	F- 5' CAGATAGAGCCTGCGCAATG 3'	221	62
	R- 5'ATGGGCATCTTCACCTGCTT 3'	221	61.95
IGF-II	F- 5' GCTGGTGCTTCTTGCCTTCT 3'	193	61.99
	R- 5' TCGGAAGCAACACTCTTCCA 3'	193	61.92

In Real-Time PCR, we obtain cycles of estrogen receptor, IGF-I, IGF-II genes and the CT had obtained (table 2).

Table 2
data obtained from Real Time-PCR for GAPDH, estrogen receptor, IGF-I and IGF-II genes.

Treatment	GAPDH	Estrogen Receptor	IGF-I	IGF-II
1	23.72	33.62 ± 1.81	30.35 ± 1.81	26.77 ± 1.81
1	25.15	27.88 ± 0.38	29.93 ± 0.38	26.37 ± 0.38
1	26.43	27.34 ± 0.9	29.51 ± 0.9	26.84 ± 0.9
2	26.82	26.96 ± 1.29	29.69 ± 1.29	27.59 ± 1.29
2	24.43	25.83 ± 1.1	30.20 ± 1.1	25.89 ± 1.1
2	19.57	26.64 ± 5.96	30.07 ± 5.96	25.76 ± 5.96
3	26.90	25.42 ± 1.37	28.88 ± 1.37	25.07 ± 1.37
3	25.66	26.37 ± 0.13	29.13 ± 0.13	25.93 ± 0.13
3	25.27	26.45 ± 0.26	28.61 ± 0.26	25.95 ± 0.26
Control	27.06	0 ± 1.53	29.04 ± 1.53	27.03 ± 1.53
Control	27.39	0 ± 1.86	29.48 ± 1.86	26.11 ± 1.86
Control	28.00	0 ± 2.47	29.18 ± 2.47	27.46 ± 2.47

Mean=25.53.

The CT data had corrected with housekeeping gene (GAPDH) and data analyzed with t-test (P<0.01) as shown in below tables.

Table 3
Paired Samples Test for estrogen receptor gene and control group

		Paired Differences				tdf	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	99% Confidence Interval of the Difference		
					Lower	Upper	
Pair 1	Treatment1 – control 12.767	31.99667	4.34074	2.006 a	2.50613	7.12374	
Pair 2	Treatment2 – control 12.634	30.35333	4.16140	2.006 a	2.40258	6.50806	
Pair 3	Treatment3 – control 25.896	27.62000	1.84738	2.001 a	1.06658	17.03433	

adifferences at P<0.01 are significant.

The results show that in (P<0.01) estrogen receptor gene expression between treatments and control group (without zearalenone) has significant difference. Therefore, Zearalenone imitates the effect of mammalian female estrogen hormone and induces estrogen receptors.

Table 4
Paired Samples Test for IGF-I gene and control group.

		Paired Differences				Tdf	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	99% Confidence Interval of the Difference		
					Lower	Upper	
Pair 1	Treatment1 – control 3.768	3.08000	1.41587	2.064 b	.81746	5.03312	11.19312
Pair 2	Treatment2 – control 1.867	4.63000	4.29454	2.203 b	2.47946	19.97821	29.23821
Pair 3	Treatment3 – control 1.869	1.18000	1.09380	2.203 b	.63151	5.08760	7.44760

bdifferences at P<0.01 are not significant.

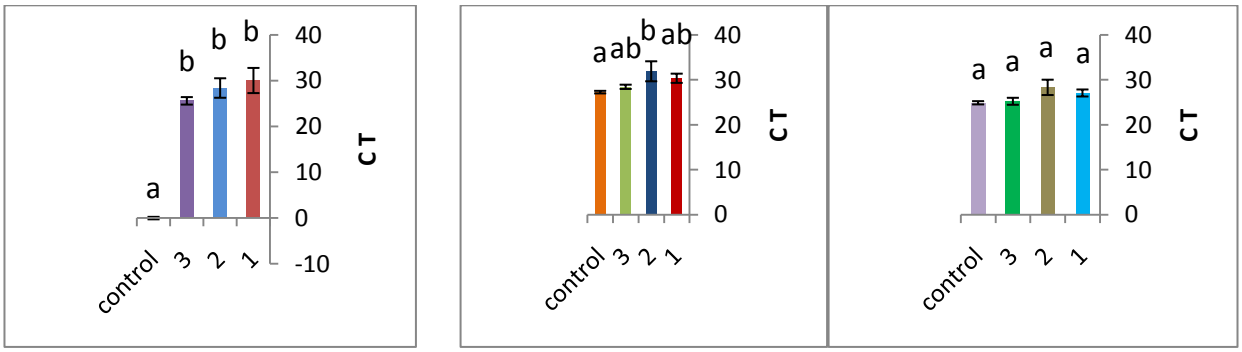
The results for IGF-I gene expression asses shows that the differences between treatments and control group are not significant.

Table 5
Paired Samples Test for IGF-II gene and control group.

		Paired Differences				tdf Sig. (2-tailed)
		Mean	Std. Deviation Error Mean	Std.	99% Confidence Interval of the Difference	
					Lower Upper	
Pair 1	Treatment1–control 3.424	2.1767 2.076 b	1.10120	.63578	-4.13331	8.48664
Pair 2	Treatment2–control 1.961	3.42333 2.189 b	3.02348	1.74561	-13.90154	20.74821
Pair 3	Treatment3–control .303	.32333 2.790 b	1.84625	1.06593	-10.25588	10.90254

bdifferences at P<0.01 are not significant.

Results in IGF-II gene (P<0.01) demonstrated that gene expression in presents of toxin zearalenone have no significant difference with control group. It means that zearalenone as a hormone disrupter, has no effect on IGF-I and IGF-II gene expression.



(EstR) (IGF-I) (IGF-II)

Fig. 2. Difference of estrogen receptor, IGF-I and II genes expression between treatments and control group. The CT obtain from qPCR for every replication of treatments data analyzed with SPSS 20 and diagrams attained. Diagrams demonstrate that by adding 100,160 and 250 microliter zearalenone(ZEA) to treatments1, 2 and 3 respectively, Estrogen receptor gene expressed increased dose-dependently but control group didn't expressed at all and clearly shows that difference is significant(P<0.01). Evaluation of IGF-I and II genes shows treatments (receiving 100, 160 and 250 microliter ZEA) and control group expressed in same amount and there isn't any significant difference (P<0.01) between them.

The results of three investigate genes expression displayed on diagrams as shown above. The diagrams illustrated the difference between treatments. In diagram of estrogen receptor (EstR) expression assay we observed that cell culture media containing ZEA in concentration 100, 160 and 250 µl, increased the estrogen receptor gene expression dose-dependently and distinct there is significant difference (P<0.01) between treatments and control group. But in diagrams of IGF-I and IGF-II genes expression assay, three amounts of ZEA had no effect on IGFs gene expression. Treatments and control group expressed in same amount and there isn't any significant difference (P<0.01) between them.

4. Discussion

Zearalenone (ZEA) is one of the fusarial toxins which have been known as important contaminants in foodstuff for animal nutrition. Generally, due to its chemical structures, these fusarial toxins constitute a broad range of toxicological effects (Bouaziz, 2009). Some studies indicate that ZEA at high level increase the accuracy of abortions in dairy cattle; these levels are not found just in naturally contaminated feeds (Hovingh, 2009). Ruminants such as cattle are less sensible to the mycotoxins present in moldy feeds. This is because of rumen microflora action and the liver metabolism to mycotoxin inactivation. But some mycotoxins are resisting to rumen degradation and liver pathways which cause intoxication and infertility (Rossi, 2009). Experimental studies (zwierzchowski et al., 2006) in vivo and in vitro have shown that ZEA is substrate for some enzymes involved in steroid metabolism. They evaluated the influence of low dose of zearalenone (200 microg/kg b.w.), consumption per oz. for 7 days on sexual behavior, concentration of the examined chemical and its metabolite and estrogens in immature gilts. The study revealed that zearalenone used at low dose causes the accuracy of apparent sexual behavior in sexually immature gilts with the immature reproductive system. Therefore ZEA causes reproductive problems and changes in genital organs, decrease in the rate of viability of embryos in gestating cattle, decrease in the amounts of luteinizing hormone (LH) and progesterone produced affecting the morphology of uterine tissues, decrease in milk production, feminization of young males due to decreased testosterone production, infertility and prenatal morbidity (Guerre et al., 2000). Onset of parturition, progesterone becomes the important hormone to maintain the pregnancy and progesterone at the start of pregnancy would be dominant. When we added zearalenone toxin to treatments due to its estrogenic characterization, the ZEA induced the estrogen receptor gene and expressed in different amount. This result is accordance to Meyer and Kuiper statements that expressed, ZEA is agonist to estrogen receptor and it can activate ER's transcription. Although ZEA has non-steroidal structure, it is able to activates estrogen receptors and cause alteration in reproductive tract. Considering the interaction of ZEA with estrogen receptors and the involvement of enzyme pathways, ZEA is group of endocrine disrupters (Gremmels and malekinejad, 2007). Insulin like growth factors are important for embryo development and IGF-I and IGF-II mRNAs were included in gestate cattle oviduct cells. The study that conducted by Ramper and Claper (2002), reported that injection of estradiol (E2) in women increase the density of IGF-I content in serum and decrease in serum E2, decreasing the IGF-I either In another experiment that Michels et al. (1993) conducted, declare that consumption of estradiol, increase the expression of interior pituitary IGF-I in rats and pork which the uterus of them cut out and immature pigs. By using ZEA as an estrogenic component in our experiment we expected that amount of IGF-I, II genes expression in cultured cells has difference between treatments and control group but ZEA has no effect on IGF-I and IGF-II gene transcription. Because IGF-I and II genes expressed in control group and treatments similarly. This results are in contrast with Hilleson statement that he indicate that anastrazole as an estrogenic factor increase the IGF-I transcription and increase serum IGF-I level in cattle. So, despite of that, ZEA is group of endocrine disrupters, it has no effect on IGFs system hormones. In other hand, similar to Katagi and et al. report, ZEA in various amount has no effect on cultured oocyte development rate either in present study we didn't observe any effect of zearalenone on IGF-I and IGF-II gene expression as a development and growth factor in oviduct epithelium cell culture.

Watson et al. (1999) by evaluating of bovine oviduct cell culture reported that mRNA encoding IGFs family could be seen in day 8 of culture, synchronize with this result in our study IGFs mRNA observed even after two times passaging the cells. Also Watson declare that IGF-II mRNA in bovine oviduct cell culture significantly release more than IGF-I. Contrast with Watson et al. (1999), in our experiment regarding to table 2, IGF-I gene expression was quietly more than IGF-I but this difference was not significant.

5. Concluction

In this study we evaluate the effect of various dose (0 [control], 100,160 and 250 µl) of zearalenone on Estrogen receptor, IGF-I and IGF-II gene expression in bovine oviduct epithelial cell culture. Finally, by assessing genes expression with Real Time PCR we observed that zearalenone toxin can induce Estrogen receptor gene but this toxin has not any effect on IGF-I and IGF-II genes expression. Therefore zearalenone by inducing ERs receptors may interfere in hormonal balance in bovine gestation period and cause abortion.

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The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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