Animal Reproduction Science 168 (2016) 126–137

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effects of zearalenone-diet on expression of ghrelin and PCNA genes in ovaries of post-weaning piglets

Meiling Dai[a], Shuzhen Jiang[a], Xuejun Yuan[b], Weiren Yang[a], Zaibin Yang[a,*], Libo Huang[a,*]

[a] Department of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an, Shandong 271018, PR China
[b] Department of Life Sciences, Shandong Agricultural University, Tai'an, Shandong 271018 PR China

ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 4 March 2016
Accepted 14 March 2016
Available online 17 March 2016

Keywords:
Ghrelin
Piglet
Ovary
PCNA
Zearalenone

ABSTRACT

Numerous reports have provided evidence that zearalenone (ZEN) can increase the weight of genital organs. These findings have been confirmed by many studies in which the ghrelin gene was expressed in the ovary and was implicated in the control of cells in reproductive tissues. The proliferating cell nuclear antigen (PCNA) is an important marker of cell proliferation. The present study investigates the effects of a ZEN-treated diet on the development of ovaries in post-weaning piglets by the detection of ghrelin and PCNA protein and relative abundance of mRNA using optical microscopy, immunohistochemistry and quantitative real-time (qRT-PCR). A total of 20 piglets (Duroc × Landrace × Yorkshire) weaned at 28 d, with an average body weight of 8.74 ± 0.26 kg ($P = 0.919$) were used in this study. Piglets in the control group ($n = 10$) were fed a normal basal diet, and those in the treatment group ($n = 10$) were fed a diet containing ZEN (1.04 mg/kg), for 35 d. The proportion of growing follicles and diameter of the largest growing follicle in ovaries were greater in piglets fed the diet with ZEN. The total integrated optical densities of protein and mRNA of ghrelin and PCNA were greater with the feeding of the ZEN-treatment diet. The results suggested that 1.04 mg/kg ZEN could promote the autocrine action or expression of the ghrelin gene in piglet ovary, and further accelerate the development of ovaries (follicles).

1. Introduction

Zearalenone (ZEN), also known as RAL and F-2 mycotoxin, is an estrogenic metabolite produced by some species of the *Fusarium* and *Gibberella* molds. Biomin (2011) reported that severe contamination of grain and feed with ZEN occurs in numerous areas worldwide. In China, the delayed harvesting of grain and its processing for edible oils have been found to significantly increase rates of contamination by *Fusarium*, and concentrations of mycotoxins, such as ZEN, are much greater than those reported for other countries (Li et al., 2014; Selvaraj et al., 2015). Once ingested, ZEN is absorbed and metabolized to its reduced form, alogues α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) (Zinedine et al., 2007). ZEN is a structural analogue of estrogen and has a structure similar to 17β-estradiol. Thus this mycotoxin is able to mimic the activity of naturally occurring estrogens. The biotransformation of ZEN to major glucuronide metabolites reduces the estrogenic activity (Alexopoulos, 2001; Frizzell et al., 2015), interferes with various enzymes involved in the steroid metabolism

* Corresponding authors.
E-mail addresses: yzb204@163.com (Z. Yang), huanglibo227@126.com (L. Huang).

http://dx.doi.org/10.1016/j.anireprosci.2016.03.006
0378-4320/

(Bravin et al., 2009), slightly decreases serum concentrations of progesterone, estradiol, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Chen et al., 2015), disturbs the progesterone synthesis in cultured granulosa cells (Cortinovis et al., 2013; Miksicek, 1994), induces oocyte degeneration, and reduces meiotic competence of compact cumulus oocyte complexes during *in vitro* maturation (Alm et al., 2006). Females of various species, particularly swine, are sensitive to ZEN and its metabolites (Zinedine et al., 2007) which mainly have an adverse effect on estrus (Frizzell et al., 2015). The effect of ZEN on proliferation of ovarian granulosa cells has been well established in horses (Minervini et al., 2006) and pigs (Cortinovis et al., 2014; Gajecka et al., 2011; Zhu et al., 2012). ZEN promotes the development of ovarian cells in pregnant sows as well as in the F_1-newborns (Schoevers et al., 2012). Meanwhile, the proliferating cell nuclear antigen (PCNA) provides for an effective marker to measure the proliferation of cells (Kheradmand et al., 2012; Phoophitphong et al., 2012).

Ghrelin is a relatively newly discovered member of the endocrine system (Rak-Mardyla, 2013; Rak-Mardyla et al., 2012, 2015). Studies on various animals including rats (Matsubara et al., 2004), chickens (Sirotkin et al., 2006) and buffaloes (Gupta et al., 2015) have demonstrated that ghrelin affects reproductive function of animals. However, little information is available of the effect on ovarian development in post-weaning piglets when there are minimal amounts of ZEN contaminating the diets that are being consumed by post-weaning piglets. In the present study, post-weaning piglets were fed the diet containing a small dose of ZEN (1.04 mg/kg) to develop an animal model to analyze the actions of ZEN in modulating relative abundance of ghrelin and PCNA protein and mRNA in the piglet ovary.

2. Materials and methods

2.1. Preparation of zearalenone diet

Purified ZEN (Fermentek, Israel) was dissolved in acetic ether and then poured onto talcum powder. A ZEN premix was prepared by blending ZEN-contaminated talcum powder with ZEN-free corn, which was subsequently mixed with a corn-soybean meal to make the experimental diet (Jiang et al., 2011). Two diets were prepared in one batch, then stored in covered containers prior to feeding. A composite sample of each experimental diet was sampled for analysis of ZEN and other mycotoxins by Inspection and Quarantine Center of Shandong Entry & Exit Inspection and Quarantine Bureau (Shandong, China) before and at the end of the feeding experiment. The detection limit for these mycotoxins was 1.0 μg/kg for aflatoxin (AFL), 0.1 mg/kg for ZEN, 0.1 mg/kg for deoxynivalenol (DON) and 0.25 mg/kg for fumonisins (FUM) (European-Commission, 2006). Analyzed ZEN concentrations were 0 and 1.04 ± 0.03 mg/kg in the control and ZEN-treated experimental diet, respectively. The dose of ZEN used in the present study was based on results of Jiang et al. (2011) and Chen et al. (2015). The FUM, AFL and DON were not detected in diets of the control and treatment group.

2.2. Experimental design, animals, and management

Piglets used in all experiments were cared for in accordance with the guidelines for the care and use of laboratory animals described by the Animal Nutrition Research Institute of Shandong Agricultural University and the Ministry of Agriculture of China.

A total of 20 post-weaning healthy piglets (Duroc × Landrace × Yorkshire) were selected, aged approximately d 28 with an average body weight of 8.74 ± 0.26 kg (mean ± SD) ($P = 0.919$; $F_{1,18} = 0.036$) without restriction of feed or water. Piglets were randomly allocated into two groups after 7 d of adaptation in the Agricultural Experiment Station of Shandong Agricultural University (Tai'an, Shandong, China). Control group piglets ($n = 10$) were fed a normal basal diet (Table 1). Piglets in the treatment group ($n = 10$) were fed a ZEN-treated (1.04 mg/kg) diet, for 35 d. Diets used in the study were isocaloric and isonitrogenous, the only difference being the amount of ZEN. All nutrient concentrations were formulated to meet or exceed minimal requirements according to the NRC (1998). Representative samples of feed were taken at the beginning and end of the experimental period for nutrient analyses, according to the methods described by the AOAC (2012).

2.3. Samples collection

Piglets were fasted for 12 h on the last day of the experiment before being euthanized. The ovaries were then rapidly isolated from the surrounding fat and tissue. One of each pair was stored at −80 °C, the other was fixed promptly in Bouin's fluid for 24–48 h using graded alcohol concentrations for dehydration as well as xylene, and was conventionally embedded in paraffin. Then, 5 μm sections were cut on a Leica microtome (RM 2235, Germany), mounted on poly-L-lysine coated glass slides, and dried overnight at 37 °C prior to routine staining for histological examination and immunohistochemical analysis.

2.4. Histological examination

Sections of ovary tissue were dewaxed and rehydrated using xylene and graded alcohol concentrations before being stained with hematoxylin for 1 min. Sections were then flushed with water until no further color could be washed out. Subsequently, sections were differentiated to wipe off the hematoxylin of the cytoplasm dyeing with hydrochloric acid alcohol, submerged in tap water until sections turned blue, and incubated in eosin for 1 min. Sections were subsequently dehydrated and cleared with xylene, then sealed in clear resin.

2.5. Measure the proportion of follicle in ovary and the diameter of the largest growing follicle

To determine the proportion of different follicles, two entire sections with an intermediate distance of 100 μm (10 stained sections per group) were observed at a magnification of 100× (Olympus BX41, Japan). Follicles were counted and classified as primordial or growing follicles.