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# Changes of Porcine Growth Hormone and Pituitary Nitrogen Monoxide Production as a Response to Cadmium Toxicity

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**Abstract** The present study was designed to investigate the effects of various cadmium concentrations on porcine growth hormone (GH) secretion in serum and cultured pituitary cells and to explore the possible mechanisms of cadmium toxicity. In feeding trial, 192 barrows (Duroc × Landrace × Yorkshire), with similar initial body weights, were randomly divided into four different treatment groups with three replicates for each treatment. The diets were supplemented for 83 days with 0, 0.5, 5.0, and 10.0 mg/kg cadmium (as $CdCl_2$). For the cell culture trial, dispersed pituitary cells were incubated with graded doses of cadmium (0, 5, 10, 15, or 20 μM) for 24 h. Pigs treated with 10 mg/kg cadmium had significantly decreased serum GH content. 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide assay showed that Cd toxicity was dose-dependent. Cell viability was reduced to 50% at 15 μM concentration. Administration of cadmium significantly reduced GH secretion, whereas cellular NO content and inducible nitric oxide synthase activity increased to a certain extent. These findings suggest that the decrease of GH might be related to NO production and to a change of NO signal pathway caused by cadmium.

**Keywords** Cadmium · Growth hormone · NO · iNOS activity · Pigs

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## Introduction

Cadmium is a toxic heavy metal element that has raised concern due to its accumulation in the environment. Given the persistent intake of this metal by mammals, there is likely an increase of cadmium content in the animal body. This could create a health risk for both animals and humans. More recently, cadmium accumulation in endocrine glands such as hypothalamus, pituitary, and gonads was investigated [1–4]. As a result, this accumulation leads to disorders of the endocrine system and changes of hormones secretion such as growth hormone (GH), sex hormones, gonadotropin-releasing hormone (GnRH), adrenocorticotropic hormone, and prolactin.

There is increasing evidence showing that cadmium is a neuroendocrine disruptor. Cadmium could lead to different disorders of the endocrine system because of its accumulation in different endocrine glands. The studies of our research group also found that 10.0 mg/kg cadmium significantly depressed follicle-stimulating hormone, luteinizing hormone, estradiol ($E_2$), and progesterone concentration in serum of the pigs, and cadmium accumulation in pituitary and ovary increased remarkably [5]. These results indicated that the changes of serum hormone contents resulted from cadmium exposure. It was shown that this metal could affect the activity of the hypothalamic–pituitary–gonadal axis by acting at the hypothalamus, the pituitary, the gonads, and/or the sex accessory organs [6–9]. However, information about the mechanism is scarce.

Nitrogen monoxide (NO) is a cellular signal molecule. It has many well-known physiological functions [10, 11] and plays regulatory functions in the pituitary [12, 13]. This study was designed to investigate the effects of cadmium on the concentration of porcine serum GH, then observe its effect on cellular NO and GH contents and their relation in vitro.

## Materials and Methods

### Chemical and Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Gibco (Eggenstein, Germany). Cadmium chloride for cell culture, 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and other reagents were obtained from Sigma Chemical (St. Louis, MO, USA). Cadmium chloride was dissolved and diluted with purified water to an original stock solution, at least 100 times as concentrated as that added to the cell cultures. To eliminate contaminants, the original stock solutions were sterile-filtered with a 0.22-μm filter before use. The final concentration was obtained by dilution in culture medium.

### Animals and Treatments

All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University. One hundred and ninety-two barrows (Duroc × Landrace × Yorkshire), with similar initial body weights at 27 kg, were randomly divided into four different treatment groups with three replicates (pens) for each treatment. The pigs received the same corn–soybean basal diet and supplemented with 0, 0.5, 5.0, and 10.0 mg/kg cadmium (as $CdCl_2$). The content of cadmium in basal diets was 0.11 mg/kg. Experimental diets were formulated to meet or exceed the nutrient requirement for growing and finishing pigs recommended by the NRC [14]. The basal diets for pigs weighing 27–40 kg contained 64.7% corn, 20%

soybean meal, 0.3% fish meal, 8% wheat bran, 3% yeast, 1% stone meal, 1.5% calcium phosphate, 0.4% food salt, 0.2% lysine, and 1% mineral/vitamin premix; that for pigs weighing 40–80 kg contained 66.9% corn, 19% soybean meal, 0.3% fish meal, 10% wheat bran, 1.1% stone meal, 1.2% calcium phosphate, 0.4% food salt, 0.2% lysine, and 1% mineral/vitamin premix; and that for pigs weighing 80 kg or over contained 68.1% corn, 13% soybean meal, 15% wheat bran, 1.2% stone meal, 1.2% calcium phosphate, 0.4% food salt, 0.2% lysine, and 1% mineral/vitamin premix. The feeding trial lasted for 83 days after a 7-day adaptation period. All pigs were housed in an open-front pig barn with a concrete floor, and the size of the pens used was 350×400 cm. A dry/wet feeder with two waterers were allocated in each pen and the pigs were allowed ad libitum access to feed and water.

### Serum Sample Preparation

At the end feeding trial, four pigs from each replicate were randomly selected, fasted for 12 h (water was provided ad libitum), and then humanly killed by exsanguination. Blood samples were centrifuged at 2,200×*g* for 10 min and serum was separated and packed in Eppendorf tubes, snap-frozen in liquid nitrogen, and then stored at −70°C until subsequent analysis.

### Pituitary and Cell Dispersion

Pituitary glands were obtained from propubertal (5–6 months) Duroc × Landrace × Yorkshire pigs in a local slaughterhouse. The animals were killed after electrical stunning and immediately decapitated; the pituitary glands were then carefully removed and transferred to sterile cold DMEM culture medium supplemented with 0.3% fetal bovine serum (FBS), 0.58% Hepes, 0.22% sodium bicarbonate, 1% antibiotic–antimycotic solution, pH 7.4. When the glands were transported to the laboratory, they were washed three times with fresh medium, posterior parts were discarded, and anterior pituitary cell dispersion was carried out as described in detail previously [15, 16] with minor modifications. In brief, anterior pituitaries were cut into 1–2-$mm^3$ fragments, decanted, rinsed with MEM, and then exposed sequentially to 0.2% trypsin (type I), 0.1% collagenase (type V), 0.1% soybean trypsin inhibitor (type I), 4 μg/ml deoxyribonuclease (type I), and $Ca^{2+}$/$Mg^{2+}$-free salt solution containing EDTA (2 and 1 mM). A final step of mechanical dispersion rendered monodispersed pituitary cells. Cell viability was always above 95% as estimated by the Trypan blue exclusion test.

### Cell Culture and Experimental Treatments

Dispersed cells were plated at a density of $10^5$ cells/ml of DMEM supplemented with 10% FBS into plates and cultured in a humid atmosphere of 95% air/5% $CO_2$ at 37°C. For the cell activity experiment, cells were seeded onto 96-well tissue culture plates. For cytochemical studies, cells were seeded onto 24-well tissue culture plates. After 48 h of culture, medium was replaced with fresh DMEM–FBS. The cells were treated with different concentrations of cadmium for various times.

Six replicate wells per experiment were tested for each agent. At the end of the treatment period, to evaluate GH release, medium samples were collected and centrifuged at 12,500×*g* for 5 min to remove cells and cellular debris, and supernatants were stored at −70°C until hormone assay. To measure cellular NO production and inducible nitric oxide synthase (iNOS) activity, the cell layer was solubilized by adding 1 ml/well of a buffer consisting of 137 mM NaCl, 8 mM sodium phosphate (pH 7.4), 1 mM phenylmethylsul-phonylfluoride,

1%(v/v) Triton X-100, 0.5%(w/v) sodium deoxycholate, and 0.1% sodium dodecyl sulfate. After 15 min of incubation under continuous agitation, the cell lysate was centrifuged and the liquid extract was stored at −70°C until assay. The results presented in this study correspond to three independent experiments. Each experiment was carried out separately using 4–6 pooled pituitary glands and dispersed together. To avoid variability between experiments, GH secretion and intracellular content from the same experiment were analyzed in the same assay.

### Cell Viability Assay

Chemically induced cell death was quantitatively monitored using MTT as described previously with a slight modification [17]. This assay measures the conversion of MTT to formazan by deshydrogenase enzymes in the intact mitochondria of living cells. In brief, cells were seeded in 96-well microtiter plates and incubated for 24 h in the presence of 0, 5, 10, 15, or 20 μM cadmium in the medium. Cells without cadmium, which were used as control, were incubated for the same periods of time. After incubations, 50 μl of 5 mM MTT solution was added to each well, and the cells were incubated at 37°C in the dark for another 4 h. Thereafter, MTT was removed and the formazan crystals were dissolved in 200 μl of dimethyl sulfoxide (DMSO). The plate was gently shaken for 3 min. Optical density was determined at 570 nm using an ELISA Microplate Reader (Bio-Rad model 660). The data of the survival curves are expressed as the percentage of control (DMSO-treated).

### Determination of GH in Serum and Culture Medium

Cells were incubated with 0, 5, 10, 15, or 20 μM cadmium in the medium for 24 h. A radioimmunoassay (RIA) was used to measure GH concentration in serum and culture medium. Samples were thawed in room temperature and measured with RIA kit (Northern Immune Technic Institute, Isotopes Company, Beijing, China) in a γ-counter (Packard 8500, Downers Grove, IL, USA), according to the manufacturer's instructions.

### NO Assay

NO production was measured in pituitary cell incubated with 0, 5, 10, 15, or 20 μM cadmium in the medium for 24 h. The stable products of NO oxidation (nitrite and nitrate) were evaluated using a colorimetric nitric oxide assay kit (Jiancheng Biotech, Nanjing, China), according to the manufacturer's instructions. Nitrate concentration relative to the standard curve was determined using an aqueous solution of sodium nitrate, in the presence or absence of $CdCl_2$, to evaluate if the reaction and nitrite/nitrate ratio were affected by cadmium used in the experiments. Nitrite alone was measured by using the Griess reagents (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid), and the absorbance was read at 540 nm. Backgrounds of nitrite plus nitrate values corresponding serum-free medium were subtracted from the experimental values.

### iNOS Activity

Cellular iNOS activity was determined after cells' exposure with 0, 5, 10, 15, or 20 μM cadmium in the medium for 24 h. It was assessed indirectly by measuring the level of L-

**Table 1** Effect of Cadmium Levels on Serum GH

| Item | Cadmium (mg/kg) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 5.0 | 10.0 |
| GH (ng/ml) | 1.67±0.44 | 1.63±0.41 | 1.52±0.37 | 0.87±0.24* |

Data represent means ± SD, $n=12$

*$P<0.05$ vs. control

citrulline that is converted by iNOS from L-arginine. iNOS activity and protein content of cells were determined using assay kit following the instructions of the manufactory (Jiancheng Biotech). Activity of iNOS was expressed as units/milligram protein.

### Statistical Analysis

Values are presented as mean ± SD with six replicates per cell experiment and 12 replicates for serum GH determination. Comparisons of different groups were performed by analysis of variance (one-way analysis of variance) followed by Student–Newman–Keuls test. A $P$ value <0.05 was considered statistically significant.

## Results

### Serum GH Level

Table 1 showed the levels of serum GH. Compared with the control, serum GH level of the pigs fed the diet supplemented with 10.0 mg/kg cadmium were decreased significantly ($P<0.05$), whereas no changes were found in serum GH levels in other treatments ($P>0.05$).

### Cytotoxicity of Cadmium

Figure 1 shows the viability rate of anterior pituitary cells after incubation with different levels of cadmium for 24 h. The results indicated that cadmium toxicity was dose-dependent. MTT assay indicated that the viability percentage of pituitary cell with 10 μM cadmium in the culture medium was under 90% and was about 50% when treated with 15 μM cadmium. It was found to be very toxic to the cells when treated with 20 μM cadmium.

### Effect on GH Secretion of Pituitary Cell

To analyze the effect of cadmium on GH release of cultured pituitary cells, dispersed cells were incubated with various concentrations of cadmium for 24 h. Figure 2 illustrates the change of GH concentration in the culture media in response to cadmium treatment. As shown, administration of cadmium levels significantly reduced the secretion of GH. Moreover, GH contents were seriously decreased with 15 or 20 μM cadmium exposure.

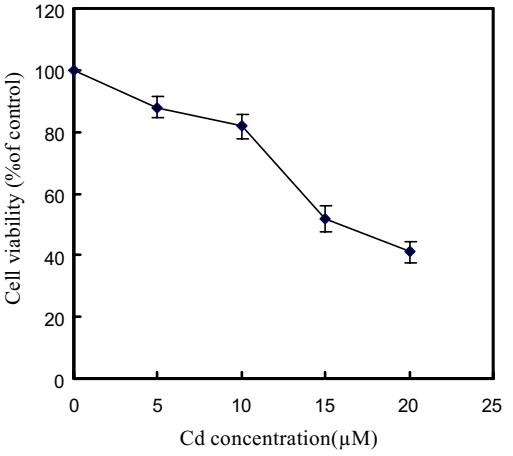


**Fig. 1** Effects of various cadmium concentrations on pituitary cell viability. The viability was determined by MTT assay after 24 h of culture. A parallel culture without cadmium served as control. *Points* represent mean ± SD, $n=6$

## NO Production

To test whether NO production was affected by cadmium in pituitary cell culture, medium was removed and cells were lysed after incubating with various concentrations of cadmium for 24 h. Changes of cellular NO content are shown in Table 2. As shown, when various levels of cadmium were added in the culture medium, the NO content increased. The results indicate these effects were concentration-dependent.

## iNOS Activity

Cellular iNOS activity was determined to investigate whether it was related to NO production. The activities of cellular iNOS are shown in Table 2. As shown, the iNOS activities in the pituitary cell increased when the culture medium contained different concentrations of cadmium. When 15 μM cadmium was added in the culture medium, iNOS activity was increased greatly.

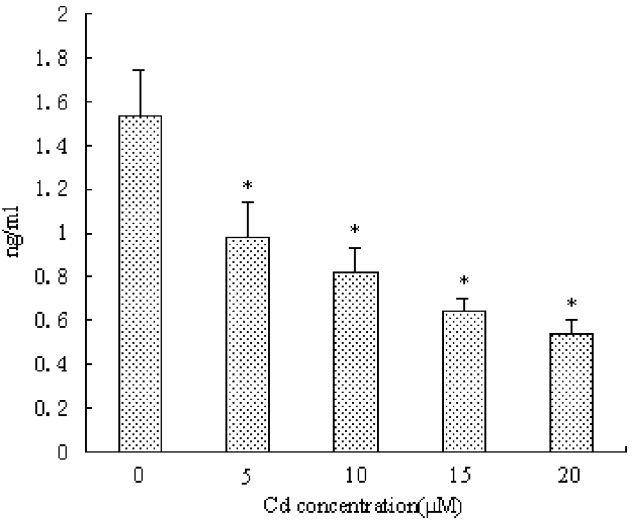


**Fig. 2** Different concentrations of cadmium effect on GH release. Pituitary cells were incubated with or without cadmium for 24 h. The *columns* represent means ± SD, $n=6$. *Asterisks* represent $P<0.05$ vs. control

**Table 2** Effects of Cadmium on NO Production and iNOS Activity

| | $CdCl_2$ (μM) | | | | |
|---|---|---|---|---|---|
| | 0 (control) | 5 | 10 | 15 | 20 |
| NO (μmol/g Pr) | 0.10±0.02 | 0.73±0.07* | 1.63±0.18* | 6.64±0.43* | 7.17±0.77* |
| iNOS (U/mg Pr) | 2.11±0.27 | 2.76±0.35* | 3.49±0.33* | 7.91±0.54* | 8.39±0.92* |

Pituitary cells were incubated in the presence of different concentrations of cadmium for 24 h. Data represent means ± SD, $n=6$

*$P<0.05$ vs. control

## Discussion

In the feeding trial, 10.0 mg/kg cadmium significantly decreased serum GH content, porcine weight gain, and efficiency of feed utilization (data not shown). Cadmium toxicity in animals is a function of dose and duration of exposure. Tolerances will be highly dependent on total accumulated body burden. The present results show the accumulative toxicity of 10.0 mg/kg cadmium during longer exposure. However, the mechanism involved is not clear. The decrease of GH level and the change of endocrine function that resulted from cadmium accumulation in pituitary may be one of the reasons that resulted in this adverse effect.

The cell culture experiment was conducted to investigate the effect of cadmium on cellular NO content and iNOS activity in vitro. The results showed that 5, 10, 15, and 20 μM cadmium decreased GH content in various degrees. The result indicates the negative effect of cadmium on pituitary function, which is the same as what other studies suggested in rats [18–20]. Moreover, the cytotoxic effect is due to induction of apoptosis [20], which agreed with some studies regarding other tissues [21, 22]. MTT assay is the most sensitive cytotoxicity assay that is mainly based on the enzymatic conversion of MTT in mitochondria by succinate dehydrogenase. The results of MTT assay suggest that the early signs of cadmium toxicity could be based on the impairment of respiratory chain in mitochondria [23].

There is increasing evidence showing that cadmium affects pituitary hormone secretion, such as prolactin and gonadotropins [20, 24, 25]. However, little literature was found to evaluate the effects of cadmium on GH in pigs. The present study indicates that 10 mg/kg cadmium significantly reduced serum GH level, and various concentrations of cadmium also decreased GH content in culture medium. Previous reports showed that acute administration of the metal decreased plasma GH levels, whereas its treatment during 14 days increased the circulating values of the hormone in rats [26]. The different results may be due to animal species and different experimental conditions.

As a signaling agent, NO plays a critical role in the cell. The function of NO is usually concerned with innate immunity and general mammalian physiology. NO is synthesized in situ by both constitutive and inducible NO synthases and plays many physiological and regulatory roles in the anterior pituitary [12, 13, 27]. The present results suggest that different cadmium levels increased NO content in culture medium, which agreed with other reports [28, 29]. NO may function as a mediator of cadmium cytotoxicity [30]. Therefore, the increased NO production induced by cadmium may directly have an effect on GH secretion. At the same time, the change of NO production caused by cadmium may affect the cellular NO signal pathway, which may indirectly act on GH secretion. On the other hand, cadmium may directly affect GH gene expression and GH production. The cellular response to cadmium is dependent on the cell line, metal, its concentration, and the duration of incubation. More research is needed to elucidate the mechanism of these interactions.

The present results also suggest that iNOS activity increased after cadmium treatment, which indicates that the increase of NO production might be due to the increase of iNOS activity. Increase of the amount of iNOS protein caused by cadmium was found in peritonel macrophages [29]. It is known that cadmium can stimulate the expression of early genes such as *c-fos*, *c-jun*, *c-myc*, and the genes coding metallothioneins, glutathione, and stress protein [31]. Ramirez et al. [29] suggested that increase of iNOS activity may be ascribed to direct or indirect action of cadmium on iNOS gene expression. However, the present result shows that iNOS activity was not increased greatly when the pituitary cell was treated with 20 μM cadmium. Cadmium can inhibit the binding of transcriptional nuclear factor kappa beta (NF-κβ) to DNA [32], whereas the activation of NF-κβ is essential for iNOS gene expression [33]. These interactions may explain the result in the present study.

The effects of cadmium on GH secretion are complex. Some studies suggested that biogenic amines such as dopamine, serotonin, and norepinephrine; amino acids such as GABA, aspartic acid, and glutamic acid; and neuropeptides (TRH, GnRH, CRH, and GRF) might be related to the secretion of pituitary hormone in case of cadmium exposure [2]. Much research is needed to elucidate the mechanism on change of GH secretion caused by cadmium.

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