Original Contribution

Ochratoxin A promotes porcine circovirus type 2 replication *in vitro* and *in vivo*

CrossMark

Fang Gan [a], Zheqian Zhang [a], Zhihua Hu [a], John Hesketh [b], Hongxia Xue [a], Xingxiang Chen [a], Shu Hao [a], Yu Huang [a], Patience Cole Ezea [b], Fahmida Parveen [a], Kehe Huang [a,*]

[a] *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, Jiangsu Province, China*
[b] *Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, UK*

ARTICLE INFO

ABSTRACT

Ochratoxin A (OTA), a worldwide mycotoxin found in food and feeds, is a potent nephrotoxin in animals and humans. Porcine circovirus-associated disease (PCVAD), including porcine dermatitis and nephropathy syndrome, is a worldwide swine disease. To date, little is known concerning the relationship between OTA and porcine circovirus type 2 (PCV2), the primary causative agent of PCVAD. The effects of OTA on PCV2 replication and their mechanisms were investigated *in vitro* and *in vivo*. The results *in vitro* showed that low doses of OTA significantly increased PCV2 DNA copies and the number of infected cells. Maximum effects were observed at 0.05 µg/ml OTA. The results *in vivo* showed that PCV2 replication was significantly increased in serum and tissues of pigs fed 75 µg/kg OTA compared with the control group and pigs fed 150 µg/kg OTA. In addition, low doses of OTA significantly depleted reduced glutathione and mRNA expression of NF-E2-related factor 2 and γ-glutamylcysteine synthetase; increased reactive oxygen species, oxidants, and malondialdehyde; and induced p38 and ERK1/2 phosphorylation in PK15 cells. Adding *N*-acetyl-L-cysteine reversed the changes induced by OTA. Knockdown of p38 and ERK1/2 by their respective specific siRNAs or inhibition of p38 and ERK1/2 phosphorylation by their respective inhibitors (SB203580 and U0126) eliminated the increase in PCV2 replication induced by OTA. These data indicate that low doses of OTA promoted PCV2 replication *in vitro* and *in vivo* via the oxidative stress-mediated p38/ERK1/2 MAPK signaling pathway. This suggests that low doses of OTA are potentially harmful to animals, as they enhance virus replication, and partly explains why the morbidity and severity of PCVAD vary significantly in different pig farms.

© 2014 Elsevier Inc. All rights reserved.

Ochratoxins were the first major group of mycotoxins identified after the discovery of aflatoxins and are produced by some fungal species of *Aspergillus* and *Penicillium* [1,2]. Ochratoxin A (OTA), which has a long biological half-life, is a worldwide mycotoxin that naturally occurs in food and feeds such as corn silage, barley, oats, rye, wheat, and other plant products [3,4]. It has been reported that the contamination levels of OTA in feed and blood vary between farms and countries [5,6]. As a consequence of its widespread occurrence in food and feeds, livestock are continuously exposed to OTA. Previous studies indicated that OTA exposure produces a potent nephrotoxin in animals [7–9]. In addition to being a nephrotoxin, OTA has hepatotoxic and immunotoxic effects [10,11]. The molecular mechanisms behind these effects of OTA include interference with antioxidant enzymes and alterations in DNA structure [12]. In particular, OTA induces depletion of

NF-E2-related factor 2 (Nrf2) and γ-glutamylcysteine synthetase (γ-GCS) mRNA levels and reduces glutathione (GSH) levels [13,14] and protein-bound sulfhydryl groups, resulting in enhanced production of reactive oxygen species (ROS). Reactive oxygen species play key roles in the regulation of many signaling pathways [15–17] such as the signal transduction pathways of p38 and extracellular signal-regulated kinases 1/2 (ERK1/2) mitogen-activated protein kinases (MAPKs), two MAPKs that are involved in the regulation of cell proliferation, differentiation, and apoptosis [18,19] and are activated in response to oxidative stress [20–22].

Porcine circovirus (PCV) is classified in the genus *Circovirus* of the Circoviridae family. Two genotypes of PCV have been recognized: PCV type 1 (PCV1) and PCV type 2 (PCV2). Although widespread in pigs, PCV1 is considered to be nonpathogenic [23]. On the other hand, PCV2 is the primary causative agent of several syndromes collectively known as porcine circovirus-associated disease (PCVAD). This cluster of diseases includes postweaning multisystemic wasting syndrome (PMWS), which is a newly emerging worldwide swine disease first reported in Canada in 1991 [24]; porcine dermatitis and nephropathy syndrome; and porcine respiratory disease complex.

The impact of PCVAD on production costs is significant, at up to $20 per pig in the United States [25]. The clinical signs of PCVAD are nonspecific and variable. According to the American Association of Swine Veterinarians, PCVAD can be subclinical or can include one or more clinical manifestations, including multisystemic disease with weight loss and high mortality, respiratory disease, porcine dermatologic and nephropathy syndrome, enteric signs including diarrhea, and reproductive disorders on an individual or herd basis [25–27]. However, not all pigs infected with PCV2 will develop PCVAD, and the severity levels differ in different pig farms. PCVAD development has been linked to animal management, presence of concurrent viral infections, stimulation of the immune system, and nutrition [28,29], suggesting that other trigger factors are associated with the disease. Our previous study indicated that oxidative stress enhanced PCV2 replication in vitro [30], which may partly explain the differences in morbidity and severity of PCVAD in pig farms. However, to date, the reason PCV2 infection is very common but PCVAD occurrence is variable between pig farms remains unresolved.

To date, little is known concerning the relationship between OTA and viral infection. Because OTA levels in feeds have been found to vary [5,6], OTA has been suggested to cause oxidative stress [9,31], and previous work has indicated that oxidative stress enhances PCV2 replication [30]. Therefore, we hypothesize that: (i) OTA promotes PCV2 replication, (ii) the differences in morbidity and severity of PCVAD in different pig farms may be related to OTA contamination level in feeds, (iii) OTA affects PCV2 replication via the oxidative stress-mediated p38/ERK1/2 MAPK signaling pathway, and (iv) low doses of OTA are potentially harmful to animals because they enhance virus replication. Thus, the objectives of the present work were to study the effects of OTA on PCV2 replication in vitro and in vivo and the signal pathway mechanisms involved.

Materials and methods

Cell culture and virus infection

Porcine kidney 15 (PK15) cells, free of PCV, were provided by the China Institute of Veterinary Drug Control. The cells were maintained in Dulbecco's minimal Eagle's medium (DMEM; Invitrogen, USA) supplemented with heat-inactivated 8% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. PCV type was determined through sequencing (Invitrogen). Stocks of PCV2 were generated using the following procedure: PK15 cells were infected with PCV2 at a multiplicity of infection (m.o.i.) of 1, when cells had reached approximately 40–50% confluence. After 1 h absorption, the inoculum was removed, and the cell monolayer was washed three times with phosphate-buffered saline (PBS). DMEM including 2% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) was subsequently added and incubation continued at 37 °C for 72 h. Next, the infected cells were subcultured in DMEM and the PCV2 was serially passaged in PK15 cells. The virus harvested at each passage was stored at −80 °C.

Cell toxicity assay

PK15 cells (3×10^3 cells/well) were cultured for 24 h in 96-well plates and then exposed to different concentrations of OTA for 48 h and subjected to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, USA). Absorbance was measured at 490 nm with a secondary wavelength of 650 nm. All tests were performed four times.

Quantitative real-time PCR

Quantitative real-time PCR was performed to determine both the number of PCV2 DNA copies and the levels of p38, Nrf2, and γ-GCS mRNA in PK15 cells. For PCV2 measurements, DNA was extracted using the TaKaRa DNA Mini kit (TaKaRa, China) and the purified DNA was used as a template for PCR amplification using 5′-TAGTATTCAAAGGGCACAG-3′ and 5′-AAGGCTACCACAGTCAG-3′ as forward and reverse primers to amplify a 117-bp fragment from the ORF2 gene of PCV2. Quantitative real-time PCR was carried out using the ABI Prism Step One Plus detection system (Applied Biosystems, USA). A recombinant pMD19 plasmid vector (TaKaRa) containing a PCV2 genome insert as a reference and a TaKaRa SYBR green real-time PCR kit were used.

Primers for analysis of p38 and β-actin (a control housekeeping gene) were designed using Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA) based on known porcine sequences. The forward primer 5′-ACAAGACAATCTGGGAGGTA-3′ and the reverse primer 5′-CACTGCAACACGTAACCC-3′ were used to amplify a 116-bp fragment for the p38 gene. The forward primer 5′-CTGCGGCATCCACGAAACT-3′ and the reverse primer 5′-AGGGCCGT-GATCTCCTTCTG-3′ were used to amplify a 147-bp fragment for the β-actin gene. The primer sequences used for Nrf2 [32] and γ-GCS [33] were obtained from published articles. Total RNA was extracted from PK15 cells using the RNAiso Plus kit (TaKaRa) according to the manufacturer's protocol. Potential DNA contamination of the extraction was eliminated using the DNA-Free kit (TaKaRa) and the RNA quality was assessed by the absorbance ratio at 260/280 nm. First-strand cDNA was synthesized and PCR was carried out using the ABI Prism Step One Plus detection system (Applied Biosystems) as described previously [34]. The relative mRNA levels of target genes were determined using the Δ cycle threshold (ΔC_t) method with β-actin serving as a reference gene. For the target genes, the $\Delta \Delta C_t$ values of all the samples were calculated by subtracting the average ΔC_t of the samples from the ΔC_t of the control samples (PK15 cells without transfection). The $\Delta \Delta C_t$ values were converted to fold differences by raising 2 to the power of $-\Delta \Delta C_t$ ($2^{-\Delta \Delta Ct}$) [34].

Indirect immunofluorescence assay (IFA)

PK15 cells were washed with PBS containing 0.1% Tween 20 (PBST) and fixed in 4% paraformaldehyde. After three washes, the cells were perforated with 0.1% Triton X-100 and then blocked in PBST containing 1% bovine serum albumin (BSA) at 37 °C for 45 min to prevent nonspecific binding. Next, the cells were incubated at 37 °C for 1 h with porcine anti-PCV2 antibody (UnivBiotech, China) diluted in PBST containing 1% BSA (PBSTB; 1:50), and after three washes with PBST, FITC-conjugated rabbit anti-pig antibody (Sigma; diluted 1:100 in PBSTB) was added and incubated for 1 h at 37 °C. After three washes, the cells were examined under a fluorescence microscope. Cells positive for PCV2 viral antigens were counted in six fields of view.

GSH and malondialdehyde (MDA) assays

Cell extracts were prepared by sonication (Sonics VCX105, USA) in ice-cold PBS and centrifuged at 12,000 rpm for 20 min to remove debris. The supernatant fluid was collected and GSH levels were determined spectrophotometrically at 412 nm by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) as described previously [35] using commercially available kits (Jiancheng, China). MDA levels were measured spectrophotometrically at 532 nm using the thiobarbituric acid reaction method as described previously [36] using commercially available kits (Jiancheng). Total protein concentration was determined using a BCA protein assay kit (Beyotime, China). The

data were expressed as nanomoles of GSH or MDA per milligram of protein.

Intracellular ROS assay

Intracellular ROS in PK15 cells were measured with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma) as described previously [37]. In brief, after the culture medium was removed, the cells were washed three times with PBS. After dilution to a final concentration of 10 μM with serum-free DMEM, DCFH-DA was added to the cells and incubated for 30 min at 37 °C. Next, cells were washed three times with PBS. The cells were resuspended in PBS and the total intracellular fluorescence intensity was measured for more than 10,000 cells of each sample by flow cytometry (FACSCalibur, Becton–Dickinson, USA). The level of total intracellular ROS, paralleled by an increase in fluorescence intensity, was calculated as the percentage of control cells without PCV2 infection.

Intracellular oxidants assay

Intracellular oxidant levels in PK15 cells were measured with MitoSOX red mitochondrial superoxide indicator (Invitrogen) as described previously [37]. Briefly, after the culture medium was removed, the cells were washed three times with PBS. MitoSOX red mitochondrial superoxide indicator, diluted to a final concentration of 4 μM with serum-free DMEM, was added to the cells and incubated for 10 min at 37 °C while being protected from light. The cells were then washed three times with PBS. The cells were resuspended in PBS and the fluorescence was measured immediately by FACSCalibur flow cytometer. The level of intracellular oxidant levels, paralleled by an increase in fluorescence, was calculated as the percentage of control cells without PCV2 infection.

Cell lysis and Western blot

PK15 cells were harvested into 80 μl of lysis buffer containing protease inhibitor (Beyotime) and were sonicated (Sonics VCX105). The lysate was centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant fluid was immediately collected for use. Protein concentration was determined using the BCA kit (Beyotime). Sixty micrograms of protein was diluted in sample loading buffer and heated at 95 °C for 5 min. The denatured proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 40 min at room temperature in Tris-buffered saline containing 5% BSA and 0.1% Tween 20, followed by overnight incubation at 4 °C in specific primary antibodies (anti-ERK1/2, anti-p-ERK1/2, anti-p38, anti-p-p38, or anti-β-actin) from the cell signaling pathway. The membranes were washed and incubated in secondary antibody (polyclonal anti-rabbit–horseradish peroxidase from Sigma) at room temperature for 1 h. Blots were visualized according to the standard enhanced chemiluminescence system (Bio-Rad, USA).

Small interfering RNA (siRNA) transfection

A p38-specific siRNA was designed using the sequence of *Sus scrofa* p38 mRNA (GenBank Accession No. XM_003356616.1) and Invitrogen BlockiT RNAi designer. ERK-specific siRNA and control siRNA sequences were obtained from published papers [35,38]. The p38-specific siRNA sequence was 5′-GCAGGAGCUGAACAAGA-CAtt-3′. The ERK1/2-special siRNA sequence was 5′-CUCCAAAG-CUCUGGAUUUAtt-3′. The control siRNA had the sequence 5′-UUCUCCGAACGUGUCACGUtt-3′. The three double-stranded RNAs were synthesized by Invitrogen. Duplexes were resuspended in RNA-free water to obtain 20 μM solutions before use. PK15 cells in

DMEM 8% FBS without antibiotics were seeded in 12-well plates at a density of 8×10^4 cells/well and incubated overnight at 37 °C. When cells were 30–50% confluent, siRNA was introduced using the X-tremeGene siRNA transfection reagent (Roche, USA) according to the manufacturer's protocol. Transfection reagent (2.5 μl) and 0.5 μg siRNA were added to each well and incubated for 5 h. The cells were then washed with DMEM and transferred to DMEM+4% FBS.

Production of OTA

Aspergillus ochraceus (No. 3.4411) for production of OTA was purchased from the Institute of Microbiology, Chinese Academy of Sciences, China, and grown on sterilized shredded corn (250 g) moistened by a 40% (v/w) addition of sterile water in 500-ml conical flasks, by incubation on a rotary shaker at 28 °C for 2 weeks. The brown granular product, which bore no obvious sign of fungal growth or sporulation, was sterilized at 100 °C for 20 min (yield 6 kg) and stored at −20 °C. A sample was analyzed by LC–MS/MS with diode array detection for OTA and found to contain ~20 μg/g corn substrate. The OTA-rich shredded corn was then homogenized into pig rations to give the required concentration of OTA in the basal diet.

Determination of OTA by LC–MS/MS

OTA extraction

OTA was extracted from corn substrate and basal diet as described previously [39] with some modification. Two grams of corn substrate or basal diet was mixed with 10 ml acetonitrile/water (80/20, v/v) in a 50-ml centrifuge tube and vortexed for 2 min and then sonicated for 1 h and centrifuged at 4000 rpm for 15 min. The supernatant fluid (5 ml) was transferred into a 10-ml centrifuge tube and dried under nitrogen at 50 °C. One milliliter of acetonitrile/water (v/v 20/80) was then added and the solution was filtered through a 0.22-μm nylon membrane before LC–MS/MS.

OTA was extracted from the serum or tissues using the method described previously [40]. Serum or tissue homogenates (1 ml) were mixed with 5 ml methanol/water (80/20) in a 10-ml centrifuge tube and vortexed for 2 min and then sonicated for 1 h and centrifuged at 4000 rpm for 15 min. Five milliliters of supernatant fluid was transferred to a 10-ml centrifuge tube and dried under nitrogen gas at 50 °C. After drying, the residue was resuspended in 500 μl acetonitrile/water (20/80, v/v) containing 10 mmol/L ammonium acetate by shaking and was then filtered as described above.

Standard solution

Accurately weighed solid portions of OTA (1.00 ± 0.01 mg) were dissolved in 1 ml of ethanol to prepare a 0.5 mg/ml stock solution and stored at −20 °C in amber glass vials. The working standard solutions of OTA were prepared by suitably diluting the stock solution with the mixture of acetonitrile/water (20/80, v/v) containing 10 mmol/L ammonium acetate and were stored at 4 °C in the dark. Accordingly, the stock solution of the IS ($[^{13}C_{20}]$OTA) was used as purchased and diluted with the aforementioned combined solution to 50 ng/ml. All working solutions were prepared immediately before use.

LC–MS/MS

The OTA concentration was measured by LC–MS/MS (TSQ Quantum Ultra, Thermo Scientific, USA) equipped with electrospray ionization (ESI). The chromatographic separation was performed on an Agilent Poroshell 120EC-C_{18} column (50 × 2.1 mm, 3.0 μm) with a flow rate of 0.3 ml/min. The mobile phases consisted of (A) water containing 0.05% formic acid and 5 mM

ammonium acetate and (B) methanol. A linear gradient elution program was applied using the following procedure. After an initial time of 1 min at 90% A, B was increased to 100% within 2 min and maintained for 0.5 min, then reduced to 10% B over 0.5 min, with an equilibration time of 1 min. The injection volume was 5 μl and the sample temperature was set at 4 °C.

The MS analysis was performed in positive ESI mode with a spray voltage of 4 kV. The vaporizer temperature was 300 °C, and the capillary temperature was 350 °C. Sheath gas pressure and aux valve flow were 30 psi and 20 arb, respectively. The collision gas pressure was 1.5 mtorr. Data acquisition and processing were performed using Xcalibur software (Thermo Scientific). Additionally, the parent ion (m/z) of OTA was 404.25 and the product ions and collision energy were 239.1 and 22 ev, respectively. The most intense production was employed as the quantifying ion, and the less intense signals were used as qualifying ions for confirmation of toxin identity [40].

Animals and feeding experiment

The study was conducted in a 300-sow pig farm. Clinical signs of PMWS were observed on the farm, and the farm was known to be enzootically infected with PCV2 based on previous serologic analysis. Sixty weanling piglets (Landrace × Yorkshire × Duroc) age 6 weeks were selected for detection of PCV2 infection by real-time PCR. PCV2 DNA copies detected by real-time PCR were 10^3–10^5 because of natural infection.

A total of 27 piglets of approximately equal body weight (10.5 ± 0.55 kg) were selected for the experiment, and the average of PCV2 DNA copies detected by real-time PCR was $10^{3.37}$. The pigs were randomly divided into three groups and kept under similar conditions of climate, ventilation, temperature, humidity, and light in different rooms. Each group had three replicates, with 3 piglets per replicate. The pigs were fed ad libitum either a good quality commercial pig ration (control) provided by the pig farm or the same ration artificially contaminated with the fermented OTA-rich shredded corn to give the desired contamination levels of OTA in the diet (75 and 150 μg/kg) for 42 days. All animals had free access to tap water. Each pig was weighed at the beginning and the end of the feeding trial. This study met the standards of the European Guidelines for Animal Welfare and the study protocol was approved by the Committee for the Care and Use of Experimental Animals at the Agriculture University of Nanjing, and the Animal Ethics Number for the study is SYXK (Su) 2011-0036.

Clinical monitoring was carried out daily. Blood samples of six pigs in each group were taken through the precaval vein on days 0, 14, 28, and 42 until the termination of the experiment. At the end of the experiment on day 42 (12 weeks of age), three piglets were euthanized and tissue samples of liver, kidney, spleen, lung, inguinal lymph nodes (ILN), and bronchial lymph nodes (BLN) were collected. The serum and tissues samples were analyzed for OTA concentrations by LC–MS/MS and for PCV2 DNA copies by real-time PCR. The tissue samples were used for sequencing the PCV2 genomic DNA.

Nucleotide sequencing of the PCV2 isolates from pigs in different groups

To evaluate whether OTA could result in base pair and amino acid changes in the PCV2 in pigs, the various full-length PCV2 PCR products were amplified and sequenced from the DNA extracted from the pigs in different groups using a method described previously [41]. Briefly, the purified PCV2 genomic DNA was amplified by PCR using the primer pair PCV2-F (920–946 nt, 5′-ATCCACGGAG-GAAGGGGGCCAGTT-3′) and PCV2-R (925–901 nt, 5′-GTGGATTGTT CTGTAGCATTCTTCCA-3′). The amplicon products with an A tail were

purified using the TaKaRa MiniBEST Agarose Gel DNA Extraction kit, cloned into the pMD19 T vector system (TaKaRa), and transformed into Escherichia coli competent cells. Positive colonies were detected using PCR. Plasmid DNA was extracted using the Axygen Plasmid Miniprep Kit (Axygen Biotechnology Co. Ltd., China) according to the manufacturer's instructions. Nucleotide sequencing was run at a commercial facility (Sangon Biotechnology Co., Ltd., China). The sequences were compared pair-wise at both the nucleotide and the amino acid levels using Lasergen and DNAMAN software.

Statistical analysis

Statistical analyses were performed by a one-way analysis of variance followed by Duncan's multiple-range tests to separate the means using the SPSS computer program for Windows (version 17.0). Results are expressed as the mean $\pm$ standard error (SE). A p value of less than 0.05 was considered statistically significant.

Results

Cytotoxic effects of various concentrations of OTA on PK15 cells

To assess whether any potential effect of OTA on PCV2 replication could be the result of OTA inducing cell toxicity, we examined the effects of OTA at various concentrations on cell viability. As shown in Fig. 1, over the range of concentrations used, the viability of PK15 cells was not significantly affected by OTA up to a concentration of 1 μg/ml, but at concentrations of 2.0 and 4.0 μg/ml, OTA lowered cell viability. Thus, in subsequent experiments, OTA was used at concentrations between 0.01 and 1.0 μg/ml.

OTA promotes PCV2 replication in PK15 cells

To identify the potential effect of OTA on PCV2 replication, PK15 cells at a density of 8×10^4/well in 12-well plates or 8×10^3/well in 96-well plates were incubated with PCV2 at an m.o.i. of 1 for 24 h and then in the presence of 0.01–1.0 μg/ml OTA for a further 48 h or with dimethyl sulfoxide (DMSO) alone as a solvent control group. As shown in Fig. 2, DMSO had no effect on PCV2 replication. The $\log_{10}$ PCV2 DNA copies (Fig. 2A) and the number of infected cells (Fig. 2B) significantly increased after incubation with 0.01, 0.05, 0.1, and 0.5 μg/ml OTA compared with the control group, and the maximal effects of PCV2 replication were observed in OTA at 0.05 μg/ml. No significant increase was observed in PK15 cells treated with OTA at 1.0 μg/ml. These results indicate that low doses of OTA increase PCV2 replication in PK15 cells.

OTA treatment of pigs

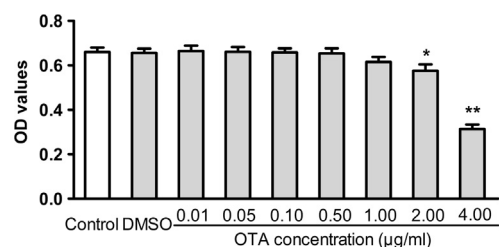The OTA concentration in corn substrate was approximately 20 μg/g, whereas the OTA concentration in the basal diet was

Fig. 1. Effect of OTA on PK15 cell viability. PK15 cells were seeded at a density of 3×10^3/well in 96-well plates and cultured with OTA for 48 h at 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, and 4.0 μg/ml. Cell viability was determined by MTT assay. Data are presented as means $\pm$ SE of four independent experiments. Significance compared with control, $*p < 0.05$ and $**p < 0.01$.
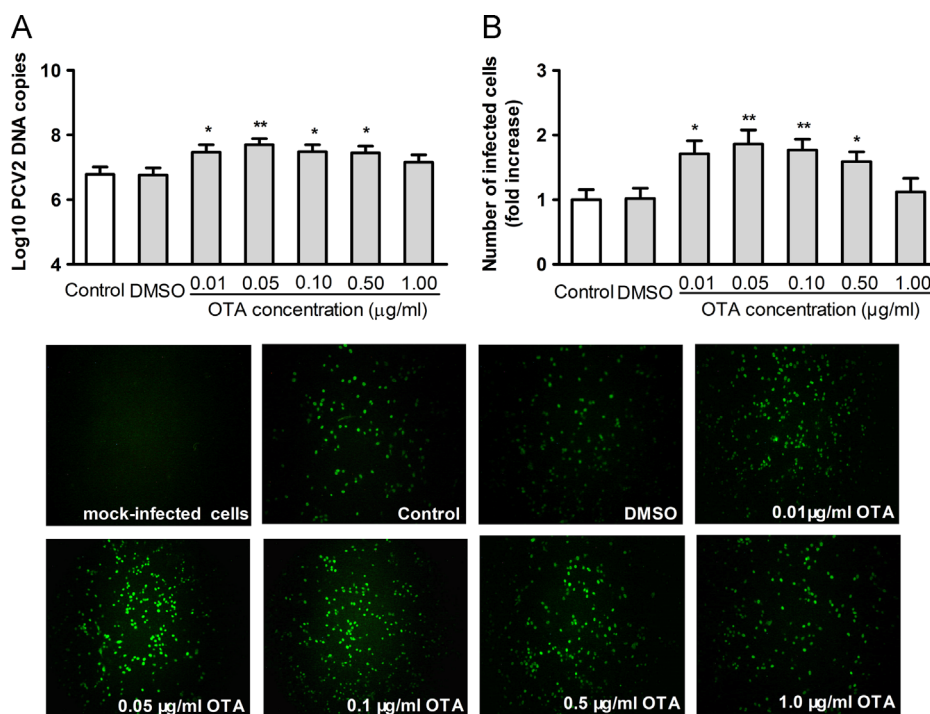
Fig. 2. OTA promotes PCV2 replication in PK15 cells. PK15 cells were infected with PCV2 for 24 h and then incubated together with OTA at concentrations of 0.01, 0.05, 0.1, 0.5, and 1.0 μg/ml for 48 h. Cells were assayed for (A) PCV2 viral DNA copies by real-time PCR and (B) the number of infected cells by IFA after a further 48 h in the presence of OTA. Data are presented as means ± SE of three independent experiments. Significance compared with control (without OTA and DMSO treatment), *$p < 0.05$ and **$p < 0.01$.

below detectable levels. Fig. 3 shows the LC–MS/MS chromatogram of the OTA standard, OTA in the corn substrate, and OTA in the basal diet.

All the pigs remained clinically healthy throughout the entire experimental period, except one pig from the 75 μg/kg OTA (low dose) group. This pig began to show clinical signs of PMWS at 10 weeks of age, including wasting, lack of response to antibiotic treatments, pallor, and dyspnea, and died at 11 weeks of age.

Serum and tissue concentrations of OTA are shown in Fig. 4A and B. Before the initial exposure on day 0 of the experiment, there was no detectable OTA or very low OTA in the serum of the pigs. On days 14, 28, and 42 postfeeding with OTA diets, significant increases in serum OTA concentrations were observed. Levels rose to 0.27–0.47 μg/ml in the 75 μg/kg OTA group and 0.40–0.69 μg/ml in the 150 μg/kg OTA group. In pigs fed the basal diet, the liver, kidney, spleen, ILN, and BLN tissues had very low OTA concentrations close to the detection limit, and there was no detectable OTA in the lung. In contrast, OTA concentrations increased significantly in the tissues from OTA-treated pigs. Levels increased from 0.05 to 0.11 μg/g in the 75 μg/kg group and from 0.08 to 0.16 μg/g in the 150 μg/kg OTA-treated group. The OTA concentration was consistent with the concentration that showed the promotion effects of OTA on PCV2 replication in the *in vitro* experiment.

PCV2 DNA copies in the serum and tissues of pigs are shown in Fig. 4C and D. Before the initial exposure on day 0 of the experiment, the mean $\log_{10}$ PCV2 DNA copies was approximately $10^{3.37}$. On days 14, 28, and 42 after feeding with OTA diets, a significant increase in PCV2 DNA copies was observed in the 75 μg/kg OTA group on day 42 compared with the control group, whereas a slight, but not statistically significant, increase in PCV2 DNA copies was observed on days 14 and 28. Significant increases in PCV2 DNA copies were observed in kidney, spleen, and lung of pigs treated with 75 μg/kg OTA and in the BLN of pigs treated with 75 and 150 μg/kg OTA. The results *in vivo* were largely consistent

with the *in vitro* data indicating that low doses of OTA promote PCV2 replication but relatively high doses of OTA do not. Thus, in subsequent experiments, the mechanisms of OTA promotion of PCV2 replication were studied on PK15 cells.

At the end of the experiment, both groups of pigs treated with basal diet plus 75 or 150 μg/kg OTA had lower final body weights (22.28 ± 0.43 kg, 22.56 ± 0.31 kg), but there were no significant differences compared with control pigs treated with basal diet (23.27 ± 0.36 kg).

The virus strains isolated from the tissues of control and OTA-treated pigs were sequenced. As shown in Fig. 5, three strains from three different groups had a genome of 1,767 nt. The strain from the 75 μg/kg OTA-treated pigs had 3 base changes at positions 257 (A), 338 (T), and 1368 (T) and the strain from the 150 μg/kg OTA-treated pigs had 4 base changes at positions 257 (A), 338 (T), 429 (C), and 1368 (T) compared with the strain from control pigs with bases at positions 257 (T), 338 (C), 429 (T), and 1368 (C). However, these base changes did not result in amino acid changes of the Rep and capsid proteins (data shown in Supplementary Fig. 1).

OTA induces oxidative stress in PK15 cells

To understand the increase in oxidative stress by OTA, Nrf2 mRNA, γ-GCS mRNA, GSH, ROS, oxidants, and MDA levels were measured in PK15 cells. Cells at a density of 2×10^5/well in six-well plates were inoculated with PCV2 at an m.o.i. of 1 for 24 h and then in the presence of OTA at concentrations of 0.01, 0.05, 0.1, and 0.5 μg/ml or DMSO alone for 48 h. As shown in Figs. 6 and 7, DMSO alone had no effects on the above parameters. Regardless of whether PK15 cells were infected, OTA treatments significantly decreased Nrf2 mRNA (Fig. 6A), γ-GCS(H) mRNA (Fig. 6B), γ-GCS (L) mRNA (Fig. 6 C), and GSH levels (Fig. 6D) and increased ROS (Fig. 7A), oxidants (Fig. 7B), and MDA levels (Fig. 7C) compared with the control groups, and OTA at 0.05 μg/ml had the maximum
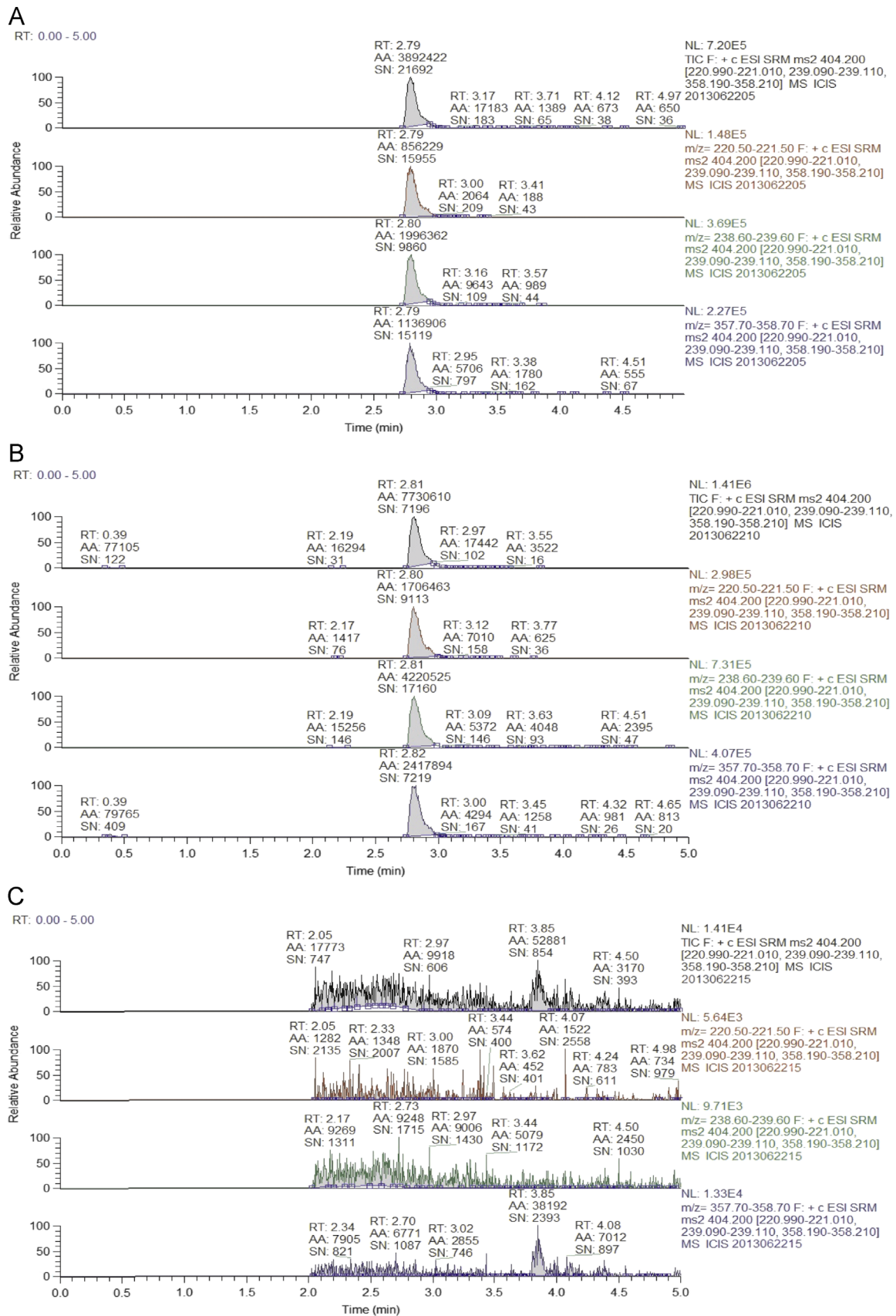
Fig. 3. LC–MS/MS chromatograms. (A) OTA standard (10 μg/ml), (B) OTA in corn substrate, and (C) OTA in basal diet.
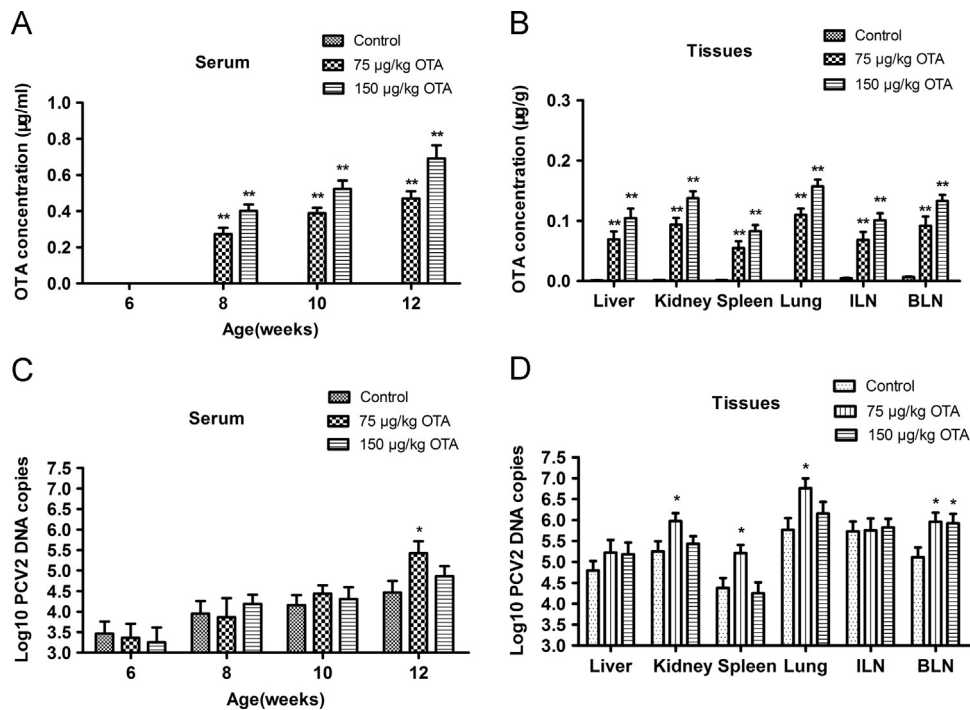
Fig. 4. OTA concentration and PCV2 DNA copies. (A, B) OTA concentration in (A) serum and (B) liver, kidney, spleen, lung, ILN, and BLN from pigs fed either basal diet (control) or basal diet with added OTA at 75 and 150 μg/kg. (C, D) PCV2 DNA copies in (C) serum and (D) liver, kidney, spleen, lung, ILN, and BLN from the same pigs were assessed by real-time PCR. Data are presented as means ± SE. Significance compared with control, $*p < 0.05$ and $**p < 0.01$.

effects within limits. These results suggest that low doses of OTA induce cellular oxidative stress.

OTA promotes p38 and ERK1/2 phosphorylation in PK15 cells

Next, we investigated whether OTA could induce p38 and ERK1/2 phosphorylation in PK15 cells. PK15 cells at a density of 2×10^5/well in six-well plates were inoculated with PCV2 at an m.o.i. of 1 for 24 h, and then OTA was added at concentrations of 0.01, 0.05, 0.1, and 0.5 μg/ml. After incubation together for 48 h, the cells were harvested and p38 and ERK1/2 phosphorylation was analyzed by Western blotting. As shown in Fig. 8, exposure of the cells to OTA at these concentrations significantly increased p38 and ERK1/2 phosphorylation levels in PK15 cells, compared with the control group. The maximum effects on the phosphorylated forms of p38 (p-p38) and ERK1/2 (p-ERK1/2) were observed with 0.05 μg/ml OTA. Levels of the total forms of each MAPK (p38 and ERK1/2) remained unaltered after OTA exposure. Thus, the data indicate that OTA induces p38 and ERK1/2 phosphorylation in PCV2-infected PK15 cells.

Both p38 and ERK1/2 mediate promotion of PCV2 replication by OTA-induced oxidative stress in PK15 cells

Because it has been reported that oxidative stress can activate the p38 and ERK1/2 MAPK signaling pathway [42,43], we investigated whether OTA is able to promote PCV2 replication by activation of p38 and ERK1/2 MAPK in response to the oxidative stress in PK15 cells. To address this question we assessed the effects of *N*-acetylcysteine (NAC) on PCV2 replication, oxidative stress, and p38 and ERK1/2 phosphorylation. As shown in Fig. 9, we found that NAC could significantly decrease the number of PCV2 DNA copies (Fig. 9A) and the number of infected cells (Fig. 9B), compared with the control group. In addition, NAC abrogated the effects of OTA on PCV2 replication. OTA decreased the mRNA expression of Nrf2, γ-GCS(H), and γ-GCS(L) (Fig. 9C) and GSH levels (Fig. 9D) and increased ROS (Fig. 9D) and oxidant levels

(Fig. 9E) in PCV2-infected PK15 cells. However, NAC reversed the changes induced by OTA. NAC did not abolish the effects of OTA on the expression of p38 and ERK1/2, but on their phosphorylation (Fig. 9F). These results indicate that the mechanism by which OTA promotes PCV2 replication may be through an oxidative stress-mediated p38 and ERK1/2 signaling pathway.

ERK- and p38-specific inhibitors attenuate OTA-promoted PCV2 replication in PK15 cells

To understand the correlation between p38 and ERK1/2 signaling pathways and OTA-promoted PCV2 replication, we tested the effects of the selective inhibitors SB203580 (p38-specific inhibitor) and U0126 (ERK-specific inhibitor) on OTA-stimulated PCV2 replication. As shown in Fig. 10, SB203580 significantly decreased PCV2 DNA copies (Fig. 10A) and the number of infected cells (Fig. 10B), compared with the control group, and attenuated the promotion of PCV2 replication induced by 0.05 μg/ml OTA (Fig. 10A and B). A similar effect on PCV2 DNA copy number and the number of infected cells was observed when cells were treated with U0126 (Fig. 10A and B). SB203580 and U0126 had no effects on the oxidative stress indices mentioned above (Fig. 10C, D, and E). SB203580 and U0126 inhibited p38 and ERK1/2 phosphorylation, respectively (Fig. 10F). These results indicate that inhibition of the p38 and ERK1/2 signaling pathways attenuate OTA-promoted PCV2 replication in PK15 cells.

ERK- and p38-specific siRNAs inhibit OTA-promoted PCV2 replication in PK15 cells

We used p38 siRNA and ERK siRNA to silence the expression of the p38 and ERK signaling pathways, and the extent of p38 and ERK knockdown was evaluated by determination of p38, p-p38, ERK1/2, and p-ERK1/2 after PK15 cells were transfected with p38-specific, ERK-specific, or a control siRNA (ssiRNA). As shown in Fig. 11, transfection of PK15 cells with corresponding siRNA resulted in significant decreases in p38 and ERK1/2 expression and their

```
                                         ......
Control pigs.seq                  GAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATT      240
75 µg/kg OTA-treated pigs.seq     GAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATT      240
150 µg/kg OTA-treated pigs.seq.seq GAGGAAGGACGAACACCCCACCTCCAGGGGTTCGCTAATT     240
Consensus                         gaggaaggacgaacaccccacctccaggggttcgctaatt


Control pigs.seq                  TTGTGAAGAAGCAAACTTTTAATAAAGTGAAATGGTATTT      280
75 µg/kg OTA-treated pigs.seq     TTGTGAAGAAGCAAACATTTAATAAAGTGAAATGGTATTT      280
150 µg/kg OTA-treated pigs.seq.seq TTGTGAAGAAGCAAACATTTAATAAAGTGAAATGGTATTT     280
Consensus                         ttgtgaagaagcaaac tttaataaagtgaaatggtattt


Control pigs.seq                  CGGTGCCCGCTGCCACATCGAGAAAGCGAAAGGAACGGAT      320
75 µg/kg OTA-treated pigs.seq     CGGTGCCCGCTGCCACATCGAGAAAGCGAAAGGAACGGAT      320
150 µg/kg OTA-treated pigs.seq.seq CGGTGCCCGCTGCCACATCGAGAAAGCGAAAGGAACGGAT     320
Consensus                         cggtgcccgctgccacatcgagaaagcgaaaggaacggat


Control pigs.seq                  CAGCAGAATAAAGAATACTGCAGTAAAGAAGGCAACTTAC      360
75 µg/kg OTA-treated pigs.seq     CAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTAC      360
150 µg/kg OTA-treated pigs.seq.seq CAGCAGAATAAAGAATATTGCAGTAAAGAAGGCAACTTAC     360
Consensus                         cagcagaataaagaata tgcagtaaagaaggcaacttac


Control pigs.seq                  TGATCGAATGTGGAGCTCCTAGATCTCAAGGACAACGGAG      400
75 µg/kg OTA-treated pigs.seq     TGATCGAATGTGGAGCTCCTAGATCTCAAGGACAACGGAG      400
150 µg/kg OTA-treated pigs.seq.seq TGATCGAATGTGGAGCTCCTAGATCTCAAGGACAACGGAG     400
Consensus                         tgatcgaatgtggagctcctagatctcaaggacaacggag


Control pigs.seq                  TGACCTGTCTACTGCTGTGAGTACCTTGTTGGAGAGCGGG      440
75 µg/kg OTA-treated pigs.seq     TGACCTGTCTACTGCTGTGAGTACCTTGTTGGAGAGCGGG      440
150 µg/kg OTA-treated pigs.seq.seq TGACCTGTCTACTGCTGTGAGTACCTTGCTGGAGAGCGGG     440
Consensus                         tgacctgtctactgctgtgagtaccttg tggagagcggg

                                         ......
Control pigs.seq                  ATAGGTTAGGGCATTGGCCTTTGTTACAAAGTTATCATCT      1360
75 µg/kg OTA-treated pigs.seq     ATAGGTTAGGGCATTGGCCTTTGTTACAAAGTTATCATCT      1360
150 µg/kg OTA-treated pigs.seq.seq ATAGGTTAGGGCATTGGCCTTTGTTACAAAGTTATCATCT     1360
Consensus                         ataggttagggcattggcctttgttacaaagttatcatct


Control pigs.seq                  AGAATAACAGCAGTGGAGCCCACTCCCCTGTCACCCTGGG      1400
75 µg/kg OTA-treated pigs.seq     AGAATAATAGCAGTGGAGCCCACTCCCCTGTCACCCTGGG      1400
150 µg/kg OTA-treated pigs.seq.seq AGAATAATAGCAGTGGAGCCCACTCCCCTGTCACCCTGGG     1400
Consensus                         agaataa agcagtggagcccactcccctgtcaccctggg

                                         ......
Control pigs.seq                  CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGT      1760
75 µg/kg OTA-treated pigs.seq     CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGT      1760
150 µg/kg OTA-treated pigs.seq.seq CTTGGATACGTCATAGCTGAAAACGAAAGAAGTGCGCTGT     1760
Consensus                         cttggatacgtcatagctgaaaacgaaagaagtgcgctgt


Control pigs.seq                  AAGTATT........................            1767
75 µg/kg OTA-treated pigs.seq     AAGTATT........................            1767
150 µg/kg OTA-treated pigs.seq.seq AAGTATT........................           1767
Consensus                         aagtatt
```

Fig. 5. Sequence alignment of a fragment of the virus genomic sequence from control group and OTA-treated pigs.

phosphorylation (Fig. 11A and B). Transfection of PK15 cells with corresponding siRNA had no effects on the oxidative stress indices mentioned above (Fig. 11C, D, and E).

To address whether p38 and ERK play key roles in the PCV2 replication promotion induced by OTA, PK15 cells were cultured overnight and then transfected with p38, ERK, or a control siRNA. After 5 h of transfection treatment, the medium was removed, and fresh basal medium and OTA were added for a 48-h culture. As shown in Fig. 12, a significant decrease in PCV2 DNA copies (Fig. 12A) and the number of infected PK15 cells (Fig. 12B) in cells
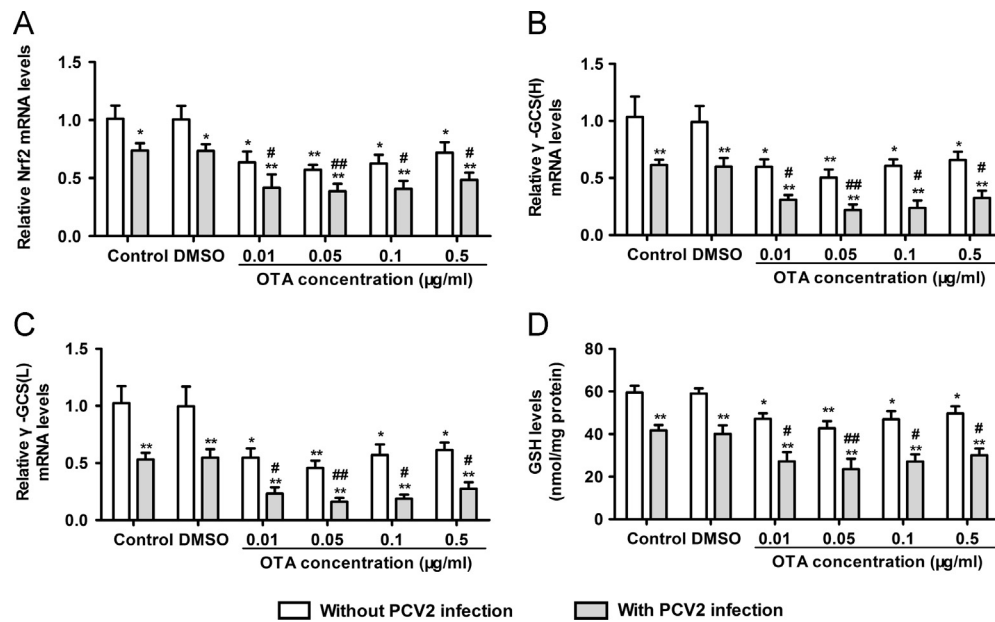
Fig. 6. Effects of OTA on Nrf2 and γ-GCS mRNA levels and reduced GSH levels in PK15 cells. PK15 cells were incubated with or without PCV2 for 24 h before OTA treatment. Cells were harvested after a further 48 h in the presence of OTA. The (A) Nrf2 and (B, C) γ-GCS mRNA levels and (D) reduced GSH levels were assayed as described under Materials and methods. Data are presented as means ± SE of three independent experiments. Without PCV2 infection, significance compared with control, $*p < 0.05$ and $**p < 0.01$. With PCV2 infection, significance compared with control, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.
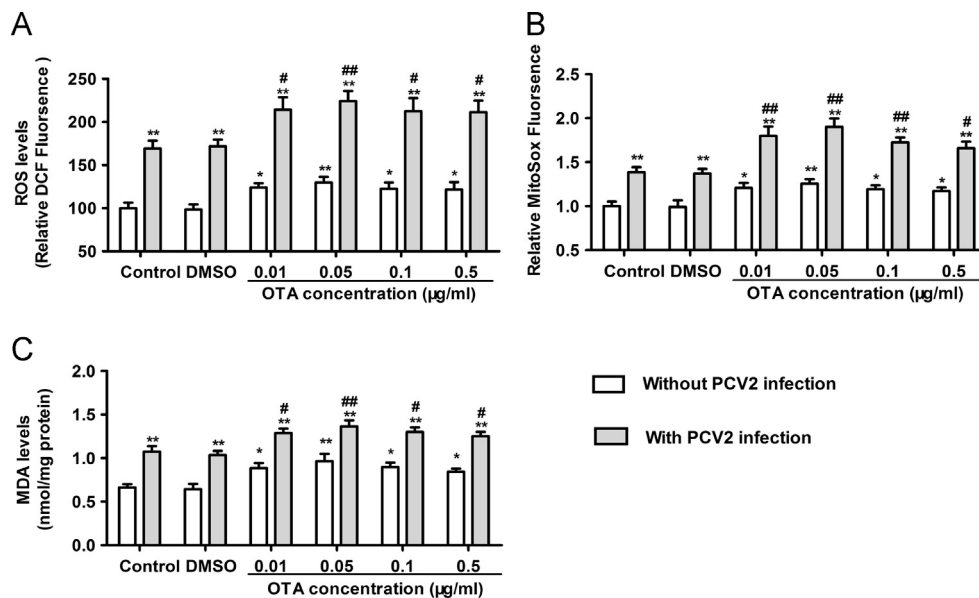

Fig. 7. Effects of OTA on ROS, oxidants, and MDA levels in PK15 cells. PK15 cells were incubated with or without PCV2 for 24 h before OTA treatment. Cells were harvested after a further 48 h in the presence of OTA. Levels of (A) ROS, (B) oxidants, and (C) MDA were assayed as described under Materials and methods. Data are presented as means ± SE of three independent experiments. Without PCV2 infection, significance compared with control, $*p < 0.05$ and $**p < 0.01$. With PCV2 infection, significance compared with control, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

treated with the p38 or ERK siRNA without or with OTA was observed compared with the control or OTA group, respectively. Overall, these results demonstrate that the p38 and ERK1/2 signaling pathways are necessary for the promotion of PCV2 replication induced by OTA in PK15 cells.

Discussion

It has been reported that OTA contamination levels in feed and in pig blood vary considerably between farms and countries [44]. It is also known that PCV2 infection is very common, but the morbidity and severity of PCVAD vary in different pig farms. The

present work is the first to investigate the relationship between OTA concentration and PCV2 replication in vitro and in vivo. The in vitro data show that an increase in PCV2 replication is induced by low doses of OTA in PK15 cells. To verify the results in vitro, we used PCV2-positive infected pigs as models and found that a low dose of OTA could increase PCV2 replication in the blood and tissues of pigs, but a relatively high dose could not. The OTA concentrations that promoted PCV2 replication in blood and tissues were consistent with those in PK15 cells. Thus, we investigated the mechanism of OTA promotion of PCV2 replication in PK15 cells as an in vitro model and found that OTA affects PCV2 replication through oxidative stress-mediated p38 and ERK1/2 MAPK signaling pathways. The work indicates that OTA may act
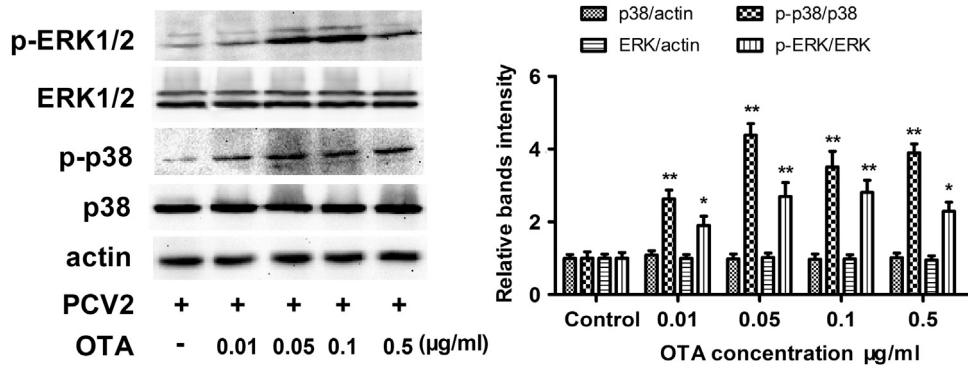
Fig. 8. OTA activates p38 and ERK1/2 MAPK in PK15 cells. PK15 cells at a density of 2×10^5/well in six-well plates were inoculated with PCV2 for 24 h and then OTA was added at concentrations of 0.01, 0.05, 0.1, and 0.5 μg/ml, and incubation continued for 48 h. After harvest, the cell proteins were extracted and subjected to Western blotting as described under Materials and methods. Data are presented as means ± SE of three independent experiments. Significance compared with control, $*p < 0.05$ and $**p < 0.01$.
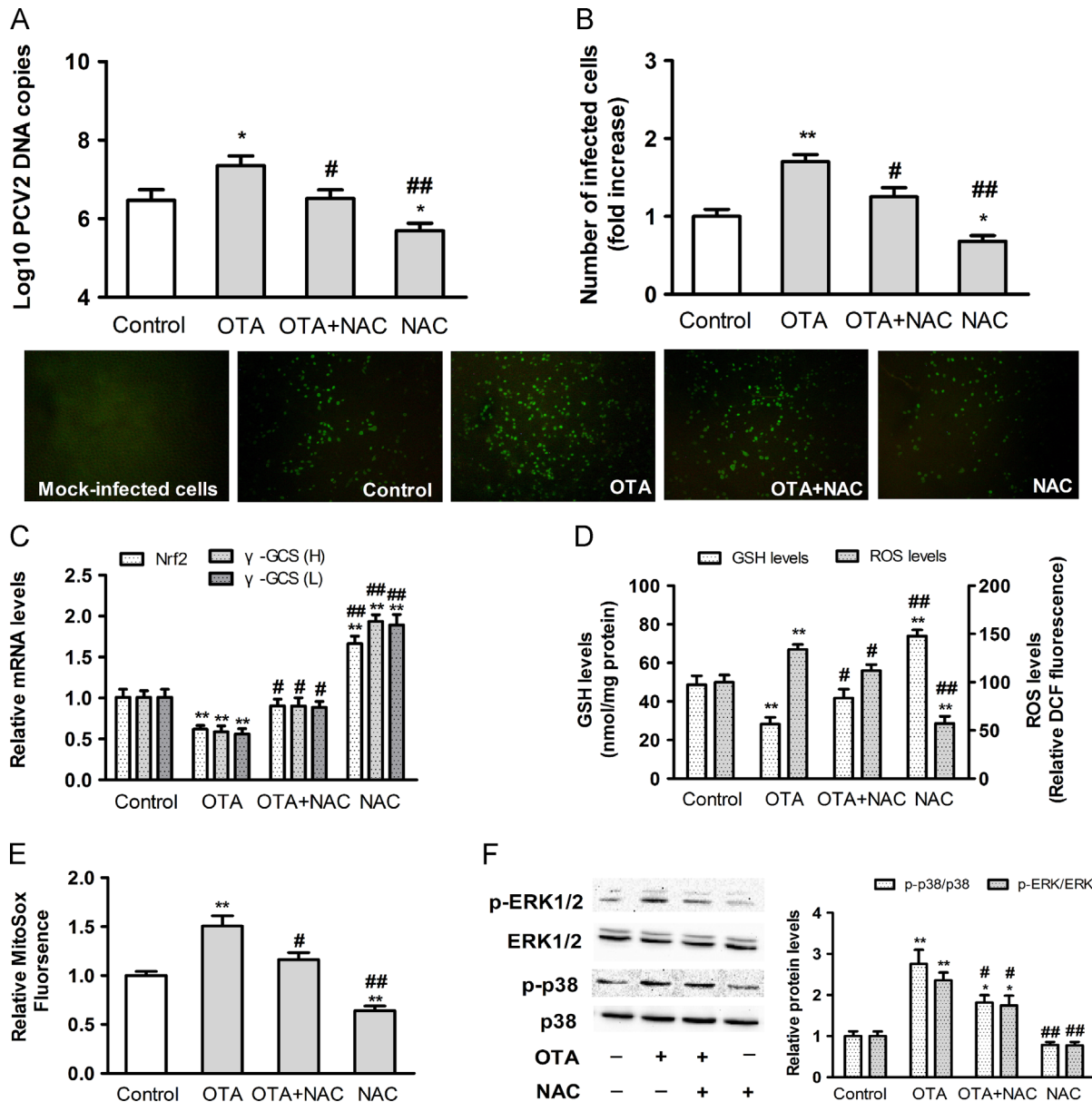

Fig. 9. Effects of OTA and/or NAC on PCV2 replication, oxidative stress, and p38 and ERK1/2 phosphorylation in PCV2-infected PK15 cells. PK15 cells were inoculated with PCV2 for 24 h and then with OTA (0.05 μg/ml), NAC (4 mM), or OTA and NAC together before the cells were incubated for an additional 48 h. Cells were harvested and assayed for (A) PCV2 DNA copies, (B) the number of infected cells, (C) Nrf2 and γ-GCS mRNA levels, (D) GSH and ROS levels, (E) oxidant levels, and (F) p38 and ERK1/2 phosphorylation as described under Materials and methods. Data are presented as means ± SE of three independent experiments. Significance compared with control, $*p < 0.05$ and $**p < 0.01$. Significance compared with OTA, $\#p < 0.05$ and $\#\#p < 0.01$.
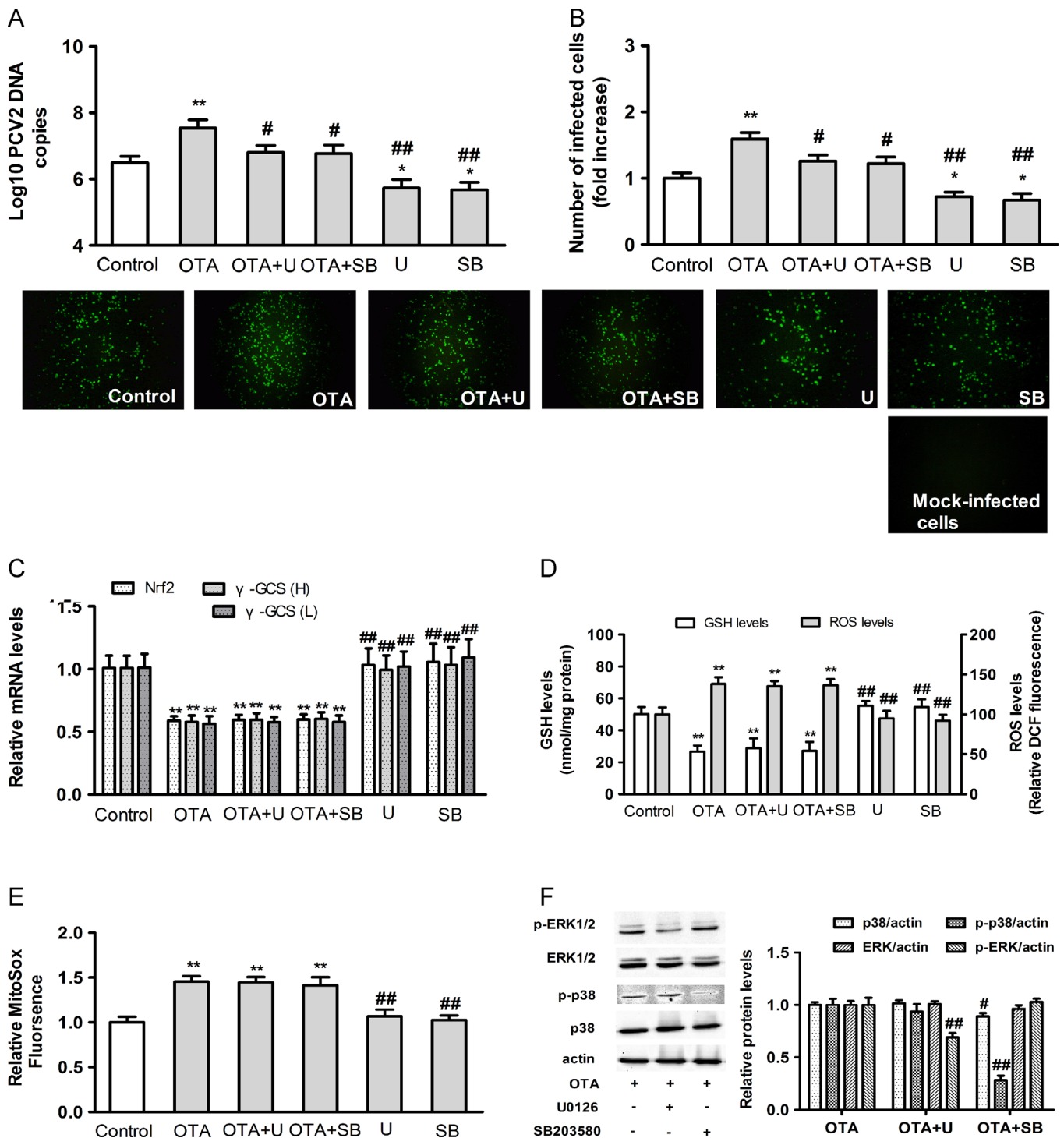
Fig. 10. SB203580 and U0126 reverse PCV2 replication promotion induced by OTA in PK15 cells. PCV2-infected cells were incubated with OTA (0.05 μg/ml) with or without SB203580 (10 μM) or U0126 (10 μM). Cells were assayed for (A) PCV2 viral DNA copies and (B) the number of infected cells, (C) mRNA levels of Nrf2 and γ-GCS, (D) GSH and ROS levels, (E) oxidant levels, and (F) p38 and ERK1/2 phosphorylation levels as described under Materials and methods. Data are presented as means ± SE of three independent experiments. Significance compared with control, $*p < 0.05$ and $**p < 0.01$. Significance compared with OTA, $\#p < 0.05$ and $\#\#p < 0.01$.

as an important trigger for PCV2 infection, which could partly explain why the morbidity and severity of PCVAD vary between pig farms. In addition, the results suggest that low doses of OTA may be harmful to animals by enhancing replication of the virus.

In the present *in vitro* experiment, the viability of PK15 cells was significantly lowered by OTA at a concentration above 1.0 μg/ml, consistent with previous studies [45,46]. In contrast, at OTA concentrations of 0.01–0.5 μg/ml, no cytotoxicity was observed, but PCV2 replication was significantly enhanced, particularly at 0.05

μg/ml. Therefore, the promotion of PCV2 replication induced by low doses of OTA seems specific and unrelated to cell toxicity. However, we found that OTA at 1.0 μg/ml lowered cell viability and caused no significant increase in PCV2 replication. We speculate that the effects of different OTA concentrations are related to both the association of the extent of PCV2 replication with oxidative stress and the cell's physiological state. It is known that virus replication requires a cell to be in a good physiological state. When a cell's physiological state is not affected by OTA, oxidative stress could promote virus
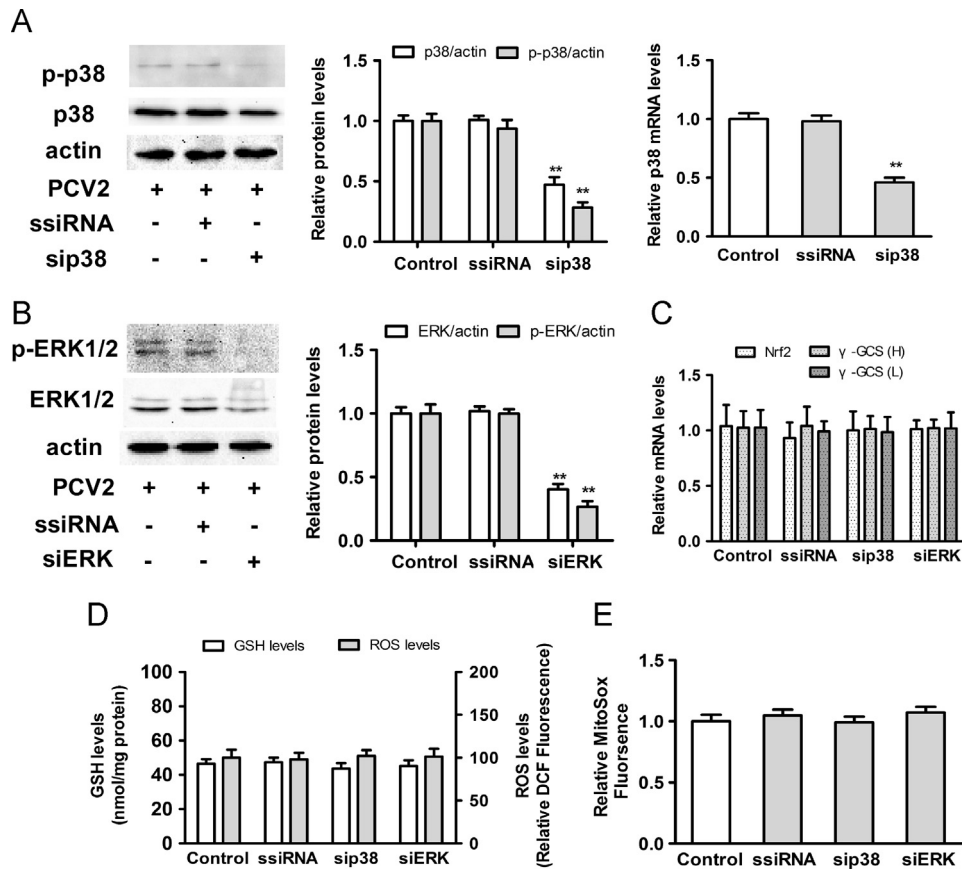
Fig. 11. Efficiency of p38 and ERK1/2 knockdown by siRNA in PK15 cells. PCV2-infected cells were incubated with or without p38 or ERK siRNA. Cell samples were assayed for (A) p38 mRNA levels and actin, p38, and p-p38 protein expression; (B) ERK1/2 and p-ERK1/2 expression; (C) mRNA levels of Nrf2 and γ-GCS; (D) GSH and ROS levels; and (E) oxidant levels. Data are presented as means $\pm$ SE of three independent experiments. Significance compared with control, $^{**}p < 0.01$.

replication, but when the cell's physiological state is lowered by OTA, although OTA could induce oxidative stress, it could not promote PCV2 replication.

In the present experiment in vivo, diets containing 75 and 150 µg OTA/kg were used to feed pigs for 42 days. The lower level was higher than the recommended dose limits of 50 µg OTA/kg set by the European Commission [47] and lower than the recommended dose limits of 100 µg OTA/kg set by China [48], whereas the higher level was higher than the recommended dose limits set by both the European Commission and China. However, both levels were much lower than those used in many studies that have investigated the effects of OTA on pigs [49,50]. It has been reported previously that OTA depressed appetite and reduced growth rate, but these effects were usually observed with feed contamination levels of OTA higher than 200 µg/kg. Previous works have indicated that pigs fed a contaminated diet at 800 µg OTA/kg presented with a significant reduction in weight gain after 1 year [51]. The diets containing 75 and 150 µg OTA/kg used in the present study have a relatively low OTA content, consistent with our finding that low doses of OTA were better than higher doses in promoting PCV2 replication in vitro, and were unlikely to affect appetite or body weight. The present in vivo study showed that a wide distribution of OTA was observed in various pig tissues after OTA treatment, with the tissue distribution in the following order: lung > kidney > BLN > liver > ILN > spleen. Interestingly, this tissue distribution of OTA was different from that observed in both pigs [52] and rats treated with 0.2 mg/kg bw, but was similar to the results of the tissue distribution of OTA in rats treated with the lower dose of 0.1 mg/kg bw [40]. The response of tissues to OTA has been linked to a number of factors, such as the length of feeding exposure, the dose, and the type of feed contamination. The ranges of OTA concentrations were 0.27–0.47 µg/ml in

serum and 0.05–0.11 µg/g in tissues of the 75 µg/kg OTA treatment group and 0.40–0.69 µg/ml in serum and 0.08–0.16 µg/g in tissues of the 150 µg/kg OTA treatment group, which was consistent with the concentration that produced the promotional effects of OTA on PCV2 replication in the in vitro experiment. Essentially, OTA increased PCV2 DNA copies in tissues such as the kidney, spleen, and lung of pigs treated with 75 µg/kg OTA and in BLN of pigs treated with 75 and 150 µg/kg OTA. The same effect was found only on day 42 in the serum of pigs treated with 75 µg/kg OTA. The PCV2 replication promotion induced by OTA in tissues was greater than that in serum, which may be because OTA concentrations in tissues were much lower than they were in serum. Overall, the results in vivo were largely consistent with the in vitro data indicating that low doses of OTA promote PCV2 replication but high doses of OTA do not. Overall, the results in vitro and in vivo suggest that OTA increases PCV2 replication, which may then trigger PCVAD development.

The severity of PCVAD has increased in recent years in China, resulting in significant economic losses in the swine industry. PCV2 is the major swine pathogen associated with PCVAD. Genetic variation among PCV2 isolates has been reported in recent years, which could enhance virulence and pathogenicity of the virus [53,54]. To assess whether OTA could cause PCV2 mutation, the entire PCV2 gene was cloned and sequenced in different treatments in the present study. The results showed that treatments with 75 or 150 µg/kg of OTA in the diet resulted in base changes, but did not result in amino acid changes. Thus, OTA treatment did not enhance the virulence of the virus in pigs, although OTA could increase PCV2 replication.

It has previously been reported that OTA treatment increases production of ROS and MDA and leads to depletion of GSH [31,55]. In addition, oxidative stress was reported to increase virus
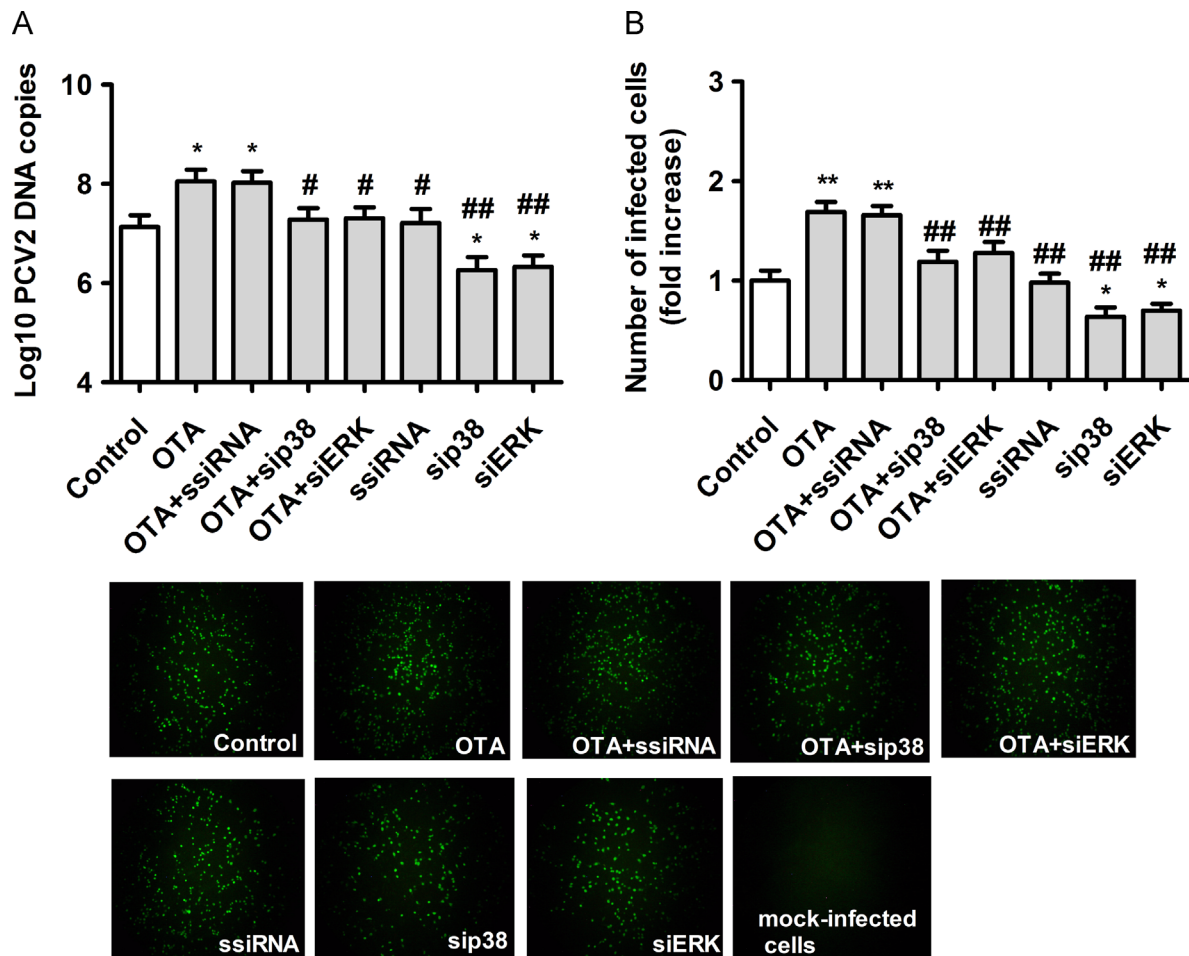
Fig. 12. p38- and ERK-specific siRNA reverses the promotion of PCV2 replication induced by OTA in PK15 cells. PCV2-infected cells were transfected with either p38- or ERK-specific siRNA or a control siRNA and incubated with OTA (0.05 µg/ml) for 48 h. Cells were assayed for (A) PCV2 viral DNA copies by real-time PCR and (B) the number of infected cells by IFA. Data are presented as means ± SE of three independent experiments. Significance compared with control, $*p < 0.05$ and $**p < 0.01$. Significance compared with OTA, $\#p < 0.05$ and $\#\#p < 0.01$.

replication [30,56]. In the present study, 0.01–0.5 µg/ml OTA significantly decreased GSH levels and increased ROS, oxidants, and MDA levels in PK15 cells with or without PCV2 infection. However, this effect is not dose-dependent. Cells exposed to higher concentrations of OTA (0.1 and 0.5 µg/ml) had a smaller fall in GSH than cells exposed to lower concentrations (0.01 and 0.05 µg/ml), and 0.05 µg/ml OTA had the maximum fall in GSH. It has also been reported that GSH depletion induced by buthionine sulfoximine showed a non-dose-dependent effect [60]. GSH synthesis is a two-step enzymatic reaction. First, in the rate-limiting step, γ-glutamylcysteine (γ-GC), which is regulated by Nrf2 [57], is formed by the action of glutamate–cysteine ligase. Second, γ-GC is coupled with glycine by the action of GSH synthetase [58,59]. We determined the mRNA levels of GSH pathway genes such as Nrf2 and γ-GCS (H and L) and found that there were higher mRNA levels of Nrf2 and γ-GCS at 0.1 and 0.5 µg/ml OTA than at 0.01 and 0.05 µg/ml OTA, respectively. We believe that the effects of OTA on ROS, oxidants, and MDA levels may be secondary to the changes in GSH levels in the present study. Such changes in oxidative stress may explain why 0.05 µg/ml OTA has a maximal promotional effect on PCV2 replication.

Because previous work has indicated that oxidative stress increases PCV2 replication [30], we propose that OTA promotes PCV2 replication by changing the intracellular redox status. Thus, the antioxidant NAC, a free radical scavenger that is able to elevate GSH levels and decrease ROS levels [61] and inhibit replication of several

viruses [62], was used in the present work. We observed that NAC could prevent Nrf2 and γ-GCS mRNA levels decrease, prevent the GSH depletion, prevent ROS and oxidant production, and prevent PCV2 replication induced by 0.05 µg/ml OTA. This suggests that NAC could decrease the oxidative stress induced by OTA and thus abrogate the PCV2 replication promotion. These findings are consistent with previous work showing that NAC strongly decreased PCV2 replication in PK15 cells [35]. Overall, our results strongly support the hypothesis that OTA-mediated PCV2 replication is associated with oxidative stress.

Several previous studies have investigated the roles of MAPK signal pathways in viral replication. It has been reported that viruses such as borna disease virus, coronavirus, and PCV2 manipulate the p38, ERK, or JNK signal pathways to regulate viral replication [38,63–66]. In addition, it has been reported that OTA disturbs MAPK signal pathways, with ERK1/2 and p38 being activated by OTA and JNK being negatively regulated [67]. However, the signal mechanism by which OTA promotes PCV2 replication has not been previously investigated.

The present work provides several pieces of evidence that p38 and ERK1/2 are involved in the OTA-mediated promotion of PCV2 replication. First, OTA was found to induce p38 and ERK1/2 phosphorylation in PCV2-infected PK15 cells, consistent with recent observations that OTA can activate p38 and ERK1/2 signaling pathways in human gastric epithelium cells [67]. Second, either the p38 inhibitor SB203580 or the ERK1/2 inhibitor U0126 significantly

inhibited PCV2 replication and reversed the PCV2 replication promotion induced by OTA at 0.05 μg/ml, consistent with previous studies that PCV2 replication in PK15 cells was significantly reduced by either SB202190 or U0126 [65,66]. Third, the promotion of PCV2 replication induced by OTA was blocked by the p38 siRNA or ERK siRNA. This observation is consistent with an earlier report that p-ERK1/2 and Cap proteins were significantly decreased in PCV2-infected PK15 cells transfected with ERK siRNA [38]. Because the present data show that OTA depletes GSH levels, elevates ROS production, and promotes p38 and ERK1/2 phosphorylation, we hypothesize that OTA promotes PCV2 replication by altering the p38 and ERK1/2 signaling pathways, inducing oxidative stress. Indeed, we found that supplementation of NAC significantly abrogated the p38 and ERK1/2 phosphorylation induced by OTA at 0.05 μg/ml. These results indicate that OTA-induced oxidative stress exerts effects on PCV2 replication by activating p38 and ERK1/2 signaling pathways. However, our results do not exclude the possible involvement of other signaling pathways and mechanisms by which OTA promotes PCV2 replication.

Conclusion

In conclusion, the present work indicates that low doses of OTA, but not relatively high doses, increased PCV2 replication *in vitro* and *in vivo*. In addition, low doses of OTA increased PCV2 replication by oxidative stress, which, in turn, activated p38 and ERK1/2 MAPK signaling pathways in PK15 cells. Thus, our work suggests that OTA may be an important trigger for PCV2 replication and that varying levels of OTA in pig feed may partly explain why the morbidity and severity of PCVAD vary among different pig farms. The observed effects provide a scientific basis for the prevention and control of the prevalence of PCVAD. Moreover, low doses of OTA may still be harmful to animals by enhancing virus replication.

Authors' contributions

Study concept and design were done by K.H. Acquisition of data was done by F.G., Z.Z., H.X., S.H., Y.H., P.E., F.P., and K.H. Analysis and interpretation of data were done by F.G., K.H., and X.C. Drafting of the manuscript was done by F.G. and K.H. Critical revision of the manuscript for important intellectual content was done by K.H., F.G., and J.H. Statistical analysis was done by F.G., Z.H., and K.H., K.H. and J.H. obtained the funding. All authors read and approved the final manuscript.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.12.016.

References

[1] Creppy, E. E. Human ochratoxicosis. *J. Toxicol.-Toxin Rev* **18**:277–293; 1999.

[2] Pitt, J. I. Penicillium viridicatum, Penicillium verrucosum, and production of ochratoxin A. *Appl. Environ. Microbiol.* **53**:266–269; 1987.

[3] Keller, L.; González Pereyra, M.; Keller, K.; Alonso, V.; Oliveira, A.; Almeida, T.; Barbosa, T.; Nunes, L.; Cavaglieri, L.; Rosa, C. Fungal and mycotoxins contamination in corn silage: monitoring risk before and after fermentation. *J. Stored Prod. Res.* **52**:42–47; 2013.

[4] Bansal, J.; Pantazopoulos, P.; Tam, J.; Cavlovic, P.; Kwong, K.; Turcotte, A. -M.; Lau, B. -Y.; Scott, P. Surveys of rice sold in Canada for aflatoxins, ochratoxin A and fumonisins. *Food Addit. Contam.* **28**:767–774; 2011.

[5] Speijers, G.; Van Egmond, H. Worldwide ochratoxin A levels in food and feeds. In: Creppy, E. E., Castegnaro, M., Dirheimer, G., editors. *Human Ochratoxicosis and Its Pathologies.* London: Colloque Inserm John Libbey Eurotext; 1993. p. 85–100.

[6] Martins, H. M.; Almeida, I.; Camacho, C.; Costa, J. M.; Bernardo, F. A survey on the occurrence of ochratoxin A in feeds for swine and laying hens. *Mycotoxin Res* **28**:107–110; 2012.

[7] Bennett, J. W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **16**:497; 2003.

[8] Hennemeier, I.; Humpf, H. U.; Gekle, M.; Schwerdt, G. The food contaminant and nephrotoxin ochratoxin A enhances Wnt1 inducible signaling protein 1 and tumor necrosis factor-α expression in human primary proximal tubule cells. *Mol. Nutr. Food Res.* **56**:1375–1384; 2012.

[9] Marquardt, R. R.; Frohlich, A. A. A review of recent advances in understanding ochratoxicosis. *J. Anim. Sci.* **70**:3968–3988; 1992.

[10] Harvey, R. B.; Elissalde, M. H.; Kubena, L. F.; Weaver, E. A.; Corrier, D. E.; Clement, B. A. Immunotoxicity of ochratoxin A to growing gilts. *Am. J. Vet. Res.* **53**:1966–1970; 1992.

[11] Stoev, S.; Denev, S.; Dutton, M.; Nkosi, B. Cytotoxic effect of some mycotoxins and their combinations on human peripheral blood mononuclear cells as measured by the MTT assay. *Open Toxinol. J* **2**:1–8; 2009.

[12] Boesch-Saadatmandi, C.; Loboda, A.; Jozkowicz, A.; Huebbe, P.; Blank, R.; Wolffram, S.; Dulak, J.; Rimbach, G. Effect of ochratoxin A on redox-regulated transcription factors, antioxidant enzymes and glutathione-S-transferase in cultured kidney tubulus cells. *Food Chem. Toxicol.* **46**:2665–2671; 2008.

[13] Liu, J.; Wang, Y.; Cui, J.; Xing, L.; Shen, H.; Wu, S.; Lian, H.; Wang, J.; Yan, X.; Zhang, X. Ochratoxin A induces oxidative DNA damage and G1 phase arrest in human peripheral blood mononuclear cells in vitro. *Toxicol. Lett.* **211**:164–171; 2012.

[14] Boesch-Saadatmandi, C.; Wagner, A. E.; Graeser, A. C.; Hundhausen, C.; Wolffram, S.; Rimbach, G. Ochratoxin A impairs Nrf2-dependent gene expression in porcine kidney tubulus cells. *J. Anim. Physiol. Anim. Nutr.* **93**:547–554; 2009.

[15] Jang, H. J.; Kim, Y. M.; Tsoyi, K.; Park, E. J.; Lee, Y. S.; Kim, H. J.; Lee, J. H.; Joe, Y.; Chung, H. T.; Chang, K. C. Ethyl pyruvate induces heme oxygenase-1 through p38 mitogen-activated protein kinase activation by depletion of glutathione in RAW 264.7 cells and improves survival in septic animals. *Antioxid. Redox Signaling* **17**:878–889; 2012.

[16] Rahman, I.; Biswas, S. K.; Jimenez, L. A.; Torres, M.; Forman, H. J. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxid. Redox Signaling* **7**:42–59; 2005.

[17] Usatyuk, P. V.; Vepa, S.; Watkins, T.; He, D.; Parinandi, N. L.; Natarajan, V. Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxid. Redox Signaling* **5**:723–730; 2003.

[18] Garrington, T. P.; Johnson, G. L. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* **11**:211–218; 1999.

[19] LaChapelle, A. M.; Ruygrok, M. L.; Toomer, M.; Oost, J. J.; Monnie, M. L.; Swenson, J. A.; Compton, A. A.; Stebbins-Boaz, B. The hormonal herbicide, 2, 4-dichlorophenoxyacetic acid, inhibits *Xenopus* oocyte maturation by targeting translational and post-translational mechanisms. *Reprod. Toxicol.* **23**:20–31; 2007.

[20] Kurata, S-i. Selective activation of p38 MAPK cascade and mitotic arrest caused by low level oxidative stress. *J. Biol. Chem.* **275**:23413–23416; 2000.

[21] Satoh, T.; Nakatsuka, D.; Watanabe, Y.; Nagata, I.; Kikuchi, H.; Namura, S. Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. *Neurosci. Lett.* **288**:163–166; 2000.

[22] Matsuzawa, A.; Ichijo, H. Stress-responsive protein kinases in redox-regulated apoptosis signaling. *Antioxid. Redox Signaling* **7**:472–481; 2005.

[23] Allan, G. M.; McNeilly, F.; Cassidy, J. P.; Reilly, G. A.; Adair, B.; Ellis, W. A.; McNulty, M. S. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. *Vet. Microbiol.* **44**:49–64; 1995.

[24] Segales, J.; Sitjar, M.; Domingo, M.; Dee, S.; Del Pozo, M.; Noval, R.; Sacristan, C.; De las Heras, A.; Ferro, A.; Latimer, K. S. First report of post-weaning multisystemic wasting syndrome in pigs in Spain. *Vet. Rec.* **141**:600–601; 1997.

[25] Gillespie, J.; Opriessnig, T.; Meng, X.; Pelzer, K.; Buechner-Maxwell, V. Porcine circovirus type 2 and porcine circovirus-associated disease. *J. Vet. Intern. Med.* **23**:1151–1163; 2009.

[26] Darwich, L.; Segales, J.; Mateu, E. Pathogenesis of postweaning multisystemic wasting syndrome caused by porcine circovirus 2: an immune riddle. *Arch. Virol.* **149**:857–874; 2004.

[27] Harding, J. C. S.; Clark, E. G.; Strokappe, J. H.; Willson, P. I.; Ellis, J. A. Postweaning multisystemic wasting syndrome: epidemiology and clinical presentation. *Swine Health Prod.* **6**:249–254; 1998.

[28] Grau-Roma, L.; Fraile, L.; Segales, J. Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. *Vet. J.* **187**:23–32; 2011.

[29] Patterson, A. R.; Opriessnig, T. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). *Anim. Health Res. Rev* **11**:217–234; 2010.

[30] Chen, X.; Ren, F.; Hesketh, J.; Shi, X.; Li, J.; Gan, F.; Huang, K. Reactive oxygen species regulate the replication of porcine circovirus type 2 via NF-kappa B pathway. *Virology* **426**:66–72; 2012.

[31] Palabiyik, S. S.; Erkekoglu, P.; Zeybek, N. D.; Kizilgun, M.; Baydar, D. E.; Sahin, G.; Giray, B. K. Protective effect of lycopene against ochratoxin A induced renal oxidative stress and apoptosis in rats. *Exp. Toxicol. Pathol.* **65**:853–861; 2013.

[32] Nannelli, A.; Rossignolo, F.; Tolando, R.; Rossato, P.; Longo, V.; Gervasi, P. G. Effect of beta-naphthoflavone on AhR-regulated genes (CYP1A1, 1A2, 1B1, 2S1, Nrf2, and GST) and antioxidant enzymes in various brain regions of pig. *Toxicology* **265**:69–79; 2009.

[33] Catherwood, M. A.; Powell, L. A.; Anderson, P.; McMaster, D.; Sharpe, P. C.; Trimble, E. R. Glucose-induced oxidative stress in mesangial cells. *Kidney Int.* **61**:599–608; 2002.

[34] Gan, F.; Chen, X.; Liao, S. F.; Lv, C.; Ren, F.; Ye, G.; Pan, C.; Huang, D.; Shi, J.; Shi, X.; Zhou, H.; Huang, K. Selenium-enriched probiotics improve antioxidant status, immune function, and selenoprotein gene expression of piglets raised under high ambient temperature. *J. Agric. Food Chem.* **62**:4502–4508; 2014.

[35] Chen, X.; Ren, F.; Hesketh, J.; Shi, X.; Li, J.; Gan, F.; Huang, K. Selenium blocks porcine circovirus type 2 replication promotion induced by oxidative stress by improving GPx1 expression. *Free Radic. Biol. Med.* **53**:395–405; 2012.

[36] Chen, X.; Ren, F.; Hesketh, J.; Shi, X.; Li, J.; Gan, F.; Hu, Z.; Huang, K. Interaction of porcine circovirus type 2 replication with intracellular redox status in vitro. *Redox Rep.* **18**:186–192; 2013.

[37] Hernández-García, D.; Wood, C. D.; Castro-Obregón, S.; Covarrubias, L. Reactive oxygen species: a radical role in development? *Free Radic. Biol. Med.* **49**:130–143; 2010.

[38] Zhu, B.; Zhou, Y.; Xu, F.; Shuai, J.; Li, X.; Fang, W. Porcine circovirus type 2 induces autophagy via the AMPK/ERK/TSC2/mTOR signaling pathway in PK-15 cells. *J. Virol.* **86**:12003–12012; 2012.

[39] Soleimany, F.; Jinap, S.; Abas, F. Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. *Food Chem.* **130**:1055–1060; 2012.

[40] Han, Z.; Zhao, Z.; Shi, J.; Liao, Y.; Zhao, Z.; Zhang, D.; Wu, Y.; De Saeger, S.; Wu, A. Combinatorial approach of LC–MS/MS and LC–TOF–MS for uncovering in vivo kinetics and biotransformation of ochratoxin A in rat. *J. Chromatogr. B* **925**:46–53; 2013.

[41] Guo, L. J.; Lu, Y. H.; Wei, Y. W.; Huang, L. P.; Liu, C. M. Porcine circovirus type 2 (PCV2): genetic variation and newly emerging genotypes in China. *Virology J* 7; 2010.

[42] Li, X.; Wang, X.; Han, C.; Wang, X.; Xing, G.; Zhou, L.; Li, G.; Niu, Y. Astragaloside IV suppresses collagen production of activated hepatic stellate cells via oxidative stress-mediated p38 MAPK pathway. *Free Radic. Biol. Med.* **60**:168–176; 2013.

[43] Lu, T. H.; Hsieh, S. Y.; Yen, C. C.; Wu, H. C.; Chen, K. L.; Hung, D. Z.; Chen, C. -H.; Wu, C. C.; Su, Y. C.; Chen, Y. W. Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury. *Toxicol. Lett.* **204**:71–80; 2011.

[44] Pozzo, L.; Cavallarin, L.; Nucera, D.; Antoniazzi, S.; Schiavone, A. A survey of ochratoxin A contamination in feeds and sera from organic and standard swine farms in northwest Italy. *J. Sci. Food Agric* **90**:1467–1472; 2010.

[45] Klarić, M.Š.; Rumora, L.; Ljubanović, D.; Pepeljnjak, S. Cytotoxicity and apoptosis induced by fumonisin B1, beauvericin and ochratoxin A in porcine kidney PK15 cells: effects of individual and combined treatment. *Arch. Toxicol.* **82**:247–255; 2008.

[46] Klarić, M.Š.; Želježić, D.; Rumora, L.; Peraica, M.; Pepeljnjak, S.; Domijan, A. -M. A potential role of calcium in apoptosis and aberrant chromatin forms in

[47] Commission, E. Commission recommendation 2006/576/EC August 17th 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. *Off. J. Eur. Union* **49 (L229)**:7–9; 2006.

[48] Hygienical standard for feeds—toleration of ochratoxin A and zearalenone in feeds. *GB 13078.2-2006;* Beijing: Standardization Administration of China; 2006.

[49] Stoev, S.; Vitanov, S.; Anguelov, G.; Petkova-Bocharova, T.; Creppy, E. Experimental mycotoxic nephropathy in pigs provoked by a diet containing ochratoxin A and penicillic acid. *Vet. Res. Commun.* **25**:205–223; 2001.

[50] Cabassi, E.; Miduri, F.; Corradi, A.; Cantoni, A.; Fusari, A.; Gregori, A.; Valente, L. Experimental direct and indirect prolonged ochratoxin A intoxication in swine. *Ann. Fac. Med. Vet. Univ. Parma* 25; 2005.

[51] Stoev, S.; Paskalev, M.; MacDonald, S.; Mantle, P. Experimental one year ochratoxin A toxicosis in pigs. *Exp. Toxicol. Pathol.* **53**:481–487; 2002.

[52] Ringot, D.; Chango, A.; Schneider, Y. -J.; Larondelle, Y. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chem.-Biol. Interact* **159**:18–46; 2006.

[53] Wen, L. B.; Guo, X.; Yang, H. C. Genotyping of porcine circovirus type 2 from a variety of clinical conditions in China. *Vet. Microbiol.* **110**:141–146; 2005.

[54] Guo, L.; Fu, Y.; Wang, Y.; Lu, Y.; Wei, Y.; Tang, Q.; Fan, P.; Liu, J.; Zhang, L.; Zhang, F.; Huang, L.; Liu, D.; Li, S.; Wu, H.; Liu, C. A porcine circovirus type 2 (PCV2) mutant with 234 amino acids in capsid protein showed more virulence in vivo, compared with classical PCV2a/b strain. *PLoS One* **7**:e41463; 2012.

[55] Schaaf, G. J.; Nijmeijer, S. M.; Maas, R. F.; Roestenberg, P.; de Groene, E. M.; Fink-Gremmels, J. The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. *Biochim. Biophys. Acta* **1588**:149–158; 2002.

[56] Nencioni, L.; Iuvara, A.; Aquilano, K.; Ciriolo, M. R.; Cozzolino, F.; Rotilio, G.; Garaci, E.; Palamara, A. T. Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. *FASEB J* **17**:758; 2003.

[57] Jin, X.; Song, L.; Li, Z.; Newton, I. P.; Zhao, M.; Liu, W. Dichlorodiphenyldichloroethylene exposure reduces r-GCS via suppressed Nrf2 in HepG2 cells. (in press). *Environ. Toxicol.* ; 2014.

[58] Meister, A. Glutathione metabolism and its selective modification. *J. Biol. Chem.* **263**:17205–17208; 1988.

[59] Žegura, B.; Lah, T. T.; Filipič, M. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. *Mutat. Res. Genet. Toxicol. Environ. Mutagen* **611**:25–33; 2006.

[60] Bailey, H. H.; Mulcahy, R. T.; Tutsch, K. D.; Arzoomanian, R. Z.; Alberti, D.; Tombes, M. B.; Wilding, G.; Pomplun, M.; Spriggs, D. R. Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione. *J. Clin. Oncol.* **12**:194–205; 1994.

[61] Patten, A. R.; Brocardo, P. S.; Sakiyama, C.; Wortman, R. C.; Noonan, A.; Gil-Mohapel, J.; Christie, B. R. Impairments in hippocampal synaptic plasticity following prenatal ethanol exposure are dependent on glutathione levels. *Hippocampus* **23**:1463–1475; 2013.

[62] Garaci, E.; Palamara, A. T.; Ciriolo, M. R.; D'Agostini, C.; Abdel-Latif, M. S.; Aquaro, S.; Lafavia, E.; Rotilio, G. Intracellular GSH content and HIV replication in human macrophages. *J. Leukocyte Biol.* **62**:54–59; 1997. ([Erratum in *J. Leukocyte Biol.* 70(2):340; 2001]).

[63] Planz, O.; Pleschka, S.; Ludwig, S. MEK-specific inhibitor U0126 blocks spread of Borna disease virus in cultured cells. *J. Virol.* **75**:4871–4877; 2001.

[64] Cai, Y.; Liu, Y.; Zhang, X. Suppression of coronavirus replication by inhibition of the MEK signaling pathway. *J. Virol.* **81**:446–456; 2007.

[65] Wei, L.; Zhu, Z.; Wang, J.; Liu, J. JNK and p38 mitogen-activated protein kinase pathways contribute to porcine circovirus type 2 infection. *J. Virol.* **83**:6039–6047; 2009.

[66] Wei, L.; Liu, J. Porcine circovirus type 2 replication is impaired by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. *Virology* **386**:203–209; 2009.

[67] Wang, Y.; Liu, J.; Cui, J.; Xing, L.; Wang, J.; Yan, X.; Zhang, X. ERK and p38 MAPK signaling pathways are involved in ochratoxin A-induced G2 phase arrest in human gastric epithelium cells. *Toxicol. Lett.* **209**:186–192; 2012.