ROS mediated cytotoxicity of porcine adrenocortical cells induced by QdNOs derivatives in vitro

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A B S T R A C T

Quinoxaline 1,4-dioxides (QdNOs) derivatives, the potent synthetic antibacterial group used in food-producing animals, are assumed to have pro-oxidant properties. However, how oxidative stress mediated their adrenal toxicity is far from clear. The aim of this study was to assess the ability of three QdNOs, i.e. olaquindox (OLA), mequindox (MEQ), and cyadox (CYA), to produce reactive oxygen species (ROS) and oxidative cell damage in porcine adrenocortical cells. Multiple approaches such as cell activity assay, biochemical detection, flow cytometry and fluorescent were used to study the integrated role of ROS homeostasis, mitochondrial redox metabolism and cell apoptosis as well as chemical stability of these drugs. The results showed that OLA and MEQ treatment evoked a significant dose and time-dependent cell damage in adrenocortical cells, well CYA displayed much less toxicity. As for the intracellular ROS production, OLA irritated a persistent and utmost release of ROS while MEQ made a similar but weaker reaction. CYA, however, had a short and unstable release of intracellular ROS. On the other hand, quinoxaline-2-carboxylic acid (QCA), one of the metabolites of OLA and MEQ, did not cause any significant production of ROS and showed relatively lower toxicity than its parents. Moreover, an imbalance in the redox metabolism and mitochondrial membrane damage has been implicated in adrenal toxicity of QdNOs. ROS scavengers partially reversed QdNOs-induced mitochondrial damage, indicating that mitochondria may be a major target and critical for ROS-mediated cell death. In a word, these results suggested that ROS is a key mediator of QdNOs-induced cell death via mitochondria-dependent pathway in adrenocortical cells. The results provide a mechanism approach in understanding the characterize of adrenal damage caused by QdNOs in vitro, which would in turn, help in designing the appropriate therapeutic strategies of these kind of feed additives.

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1. Introduction

Quinoxaline 1,4-dioxides (QdNOs) derivatives, consisting of one or two acyclic chains moiety combined with quinoxaline ring [1], are the potent synthetic antibacterial group (Fig. 1). They are widely used at subtherapeutic levels to promote growth and improve efficiency of feed conversion in animal feeding [2]. Because of different side chains in their structures, which may be relevant to specific chemical properties, the toxicity of QdNOs was not the same. For example, carbadox (CBX) and olaquindox (OLA) are well known members of QdNOs, but their toxicity and potential adverse effects have been largely reported [2–4]. In contrast, some QdNOs such as cyadox (CYA) are marked as less toxic [5]. Therefore, it remains to be defined that what exact nature of the chemical species that may be responsible for their toxicities.

Several previous studies have revealed that some QdNOs derivatives are assumed to have adrenal toxicity in vivo [6–8] and in vitro [9,10]. But how QdNOs mediates the toxicity is not fully understood. Since the N → O group reduction is the main metabolic pathway of QdNOs metabolism [11,12], the reduction of QdNOs may lead to the formation of reactive oxygen species (ROS). Much previous work supports the thesis of involvement for ROS in pathological processes induced by QdNOs [13–16]. Recent investigation from our laboratory has confirmed that the adrenal toxicity of mequindox (MEQ) is probably related to oxidative stress [17].

Mitochondrial ROS are known to be important determinants in cell function, participating in many signaling networks and also in a variety of degenerative processes [18]. The alterations in

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mitochondrial energy metabolism and redox functions may therefore be critical in determining the difference between cell death and survival. Generation of ROS associated with mitochondrial disruption and subsequent oxidative stress has been suggested as components of a final common pathway during execution of apoptotic program [19,20]. Mitochondria-dependent pathway for recognition of apoptotic cells may be a candidate target for the involvement of oxidative stress in signaling.

Base on the above information, to further elucidate the mechanism of QdNOs, it is necessary to delineate the link among chemical property, ROS homeostasis, mitochondrial redox metabolism and apoptosis to compare and characterize their adrenal cytotoxicities. In this study, we have further explored the deleterious effects on porcine adrenocortical cells induced by QdNOs. Three QdNOs derivatives, i.e. OLA, mequindox (MEQ) and CYA were selected because they are illustrative of the clinical behavior of this class of compounds. The mode of action and mechanism were investigated in more details. Numerous methods have been designed for the purpose of the investigation. QdNOs-induced cell injury and apoptosis were determined by methylthiazol tetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release, flow cytometry and fluorescent double staining, respectively. The mitochondrial membrane potential was measured to evaluate the mitochondrial redox metabolism and respiratory function. The mediation of superoxide anion radicals was testified by dynamic detection of intracellular metabolism and respiratory function. With each preparation, there were six replicates per treatment. Data were obtained from different cell preparations, each being prepared from 4 to 8 adrenal glands.

2. Materials and methods

2.1. Reagents and materials

Olaquindox (OLA, C₁₂H₁₃N₃O₄, FW 263.25, 99.8%) was obtained from the China Institute of Veterinary Drug Control (Beijing, China); mequindox (MEQ, C₁₁H₁₀N₂O₃, FW 218.21, 98%) was purchased from Beijing Zhongnongfa Pharmaceutical Co. Ltd. (Huanggang, PR China); cyadox (CYA, C₁₂H₉N₅O₃, FW 271.23, purity 98%) was obtained from the Institute of Veterinary Pharmaceuticals (Wuhan, PR China); quinoxalinine-2-carboxylic acid (QCA, C₉H₆N₂O₂, FW 228, 99%) was purchased from Sigma. All four compounds were dissolved in dimethyl sulfoxide (DMSO, Amresco, USA) and then diluted in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, USA) at desired concentration.

Collagenase (type 1; 268 U/mg) was obtained from Invitrogen (USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Biotech (USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), Rhodamine 123 (Rh 123), N-acetylcysteine, and Hoechst 33258 were purchased from Sigma Chemicals Co. (St Louis, Missouri, USA). Acridine orange (AO) and ethidium bromide (EB) were purchased from Amerisco (USA). Lactate dehydrogenase (LDH), SOD, reduced glutathione (GSH), malondialdehyde (MDA) assay reagents were from Jiancheng-Bioeng Institute (Nanjing, China). Fetal bovine serum (FBS) was from GIBCO Life Technologies (Logan, UT).

2.2. Cell culture and drug treatment

Adrenals of pigs (live-weight below 30 kg) were removed at a slaughterhouse. Immediately after the abdomens were opened, the adrenals were removed and stored in ice-cold D-hanks solution (NaCl 8.0 g, KCl 0.4 g, Na₂HPO₄·12H₂O 0.134, KH₂PO₄ 0.06 g, NaHCO₃ 0.35 g l⁻¹, pH 7.2–7.4). The surrounding tissue, capsule and medulla of the adrenals were removed and the remaining tissue was minced. Minced cortical tissue suspended in D-hanks solution (0.2 g/ml) was treated with collagenase (2.5 mg/ml) at 37°C for 45 min. After being passed through a 100 μm sieve, the cells were washed twice with D-hanks and DMEM, as follows. The cell suspension was centrifuged (100 × g) for 10 min, the supernatant was decanted and the cells in the pellet were resuspended in fresh medium. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). Cells were seeded at an appropriate density according to each experimental scale and cultured in a water-saturated atmosphere of 5% CO₂ at 37°C.

All experiments were carried out 12 h after cells were seeded and the culture medium was refreshed with a new medium. The porcine adrenocortical cells were exposed to various concentrations (1–100 μM) of OLA, MEQ, CYA and QCA for indicated time. Control cells were treated with vehicle alone (final DMSO concentration not more than 0.5%). Selection of the doses and time points for the test compounds were based on the literatures, MTT cell survival tests and our practice study. Data were obtained from different cell preparations, each being prepared from 4 to 8 adrenal glands. With each preparation, there were six replicates per treatment.

2.3. Analysis of cell viability

Cell survival was observed with phase-contrast microscope (OLYMPUS, Japan). At the same time, cell viability was evaluated by the reduction of MTT [21] and LDH release. Briefly, adrenocortical cells (1 × 10⁵ cells/ml) were treated with suggested concentrations of OLA, MEQ, CYA or QCA for 0.5–24 h at 37°C. After 3 h of incubation with MTT (0.5 mg/ml), cells were lysed in DMSO and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (TECAN A-5082, meglan, Austria). Cell viability was expressed as a percent of the control culture value.

LDH was determined as previously described [22]. The absorbance was measured at 440 nm using a microplate reader as described above. LDH release (% of total) was calculated as the percentage of LDH in the medium versus total LDH activity in the cells.

2.4. Flow cytometry for apoptosis

The apoptosis assay using flow cytometry was performed according to the vendor’s protocol (BD Pharmingen, BD BioSciences,
San Jose, USA). Briefly, cells from 60% to 70% confluent plates were washed in D-hanks and resuspended (1 × 10^6 cells/ml) in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl\(_2\)). A fraction (100 μl/1 × 10^5 cells) of the cell suspension was incubated with 5 μl Annexin V conjugated to FITC and 5 μl propidium iodide (PI) for 15 min at 25 °C in the dark. 500 μl of binding buffer was added to the suspension and apoptosis was measured immediately using a CyAn ADP\(^{TM}\) with Summit\(^{TM}\) software (Beckman, USA). The apoptotic cells were estimated by the percentage of cells that stained positive for Annexin V–FITC while remaining impermeable to PI (AV+/PI–). This method was also able to distinguish viable cells (AV–/PI–) and cells undergoing necrosis (AV+/PI+).

### 2.5. Measurement of apoptosis by AO/EB double staining

Evaluation of apoptosis was also done by examining the differential uptake of two fluorescent DNA-binding dyes, AO and EB. AO is taken up by all cells and stains the nuclei bright green, whereas EB is taken up only by cells that have lost membrane integrity and stains the nuclei bright orange [23]. Four types of cells can be distinguished according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. (1) Normal cells have uniform bright green nuclei with organized structure. (2) Early apoptotic cells (which still have intact membranes but have started to undergo DNA cleavage) have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments. (3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. (4) Necrotic cells have a uniformly orange to red nuclei with organized structure. 1 μl of AO (1 mg/ml) and EB (1 mg/ml) were added to 1 ml of cell suspension and incubated for 30 min at 37 °C in a well of 6-well plates. After the cells were washed with D-hanks, AO/EB fluorescence was examined with fluorescence microscope fitted with a camera (Olympus made in Japan.) at 400× magnification.

### 2.6. Measurement of intracellular ROS

Determination of intracellular oxidant production in porcine adrenocortical cells was based on the oxidation of DCFH-DA by intracellular ROS, resulting in the formation of the fluorescent compound 2′,7′-dichlorodihydrofluorescein [24]. 200 μl of cells were seeded in black 96-well plates at density of 2 × 10^5/well. ROS probe dye 2′,7′-DCFH-DA (10 μM) was added and incubated for 30 min at 37 °C. After the cells were washed with D-hanks, cells were exposed to different concentrations of chemicals in D-hanks. After the administration of chemicals, the fluorescence intensity (relative fluorescence units) was measured within 4 h at 485 nm excitation and 530 nm emission in a fluorescence microplate reader (TECAN, Austria). ROS level (% of control) was expressed as the fluorescence of the treated samples compared to the fluorescence of the control samples.

### 2.7. Measurement of oxidative stress indices

After the desired time of treatment, cells were harvested, washed with D-hanks and homogenized in 10 mM phosphate buffer containing 0.15 M KCl, 0.1 mM EDTA, 1 mM DTT and 0.1 mM phenylmethylsulfonylfluoride (pH 7.4) at 4 °C. Mitochondria and postmitochondrial fractions were prepared by centrifugation using the Mitochondria/cytosol Fractionation Kit (Beyotime Inst. Biotech, Peking, PR China).

The oxidative stress parameters were measured in both the mitochondria and postmitochondrial supernatant (PMS). Lipid peroxidation was measured as the malondialdehyde (MDA) formed using the kit for MDA assay. SOD and GSH were measured by using commercial kits from Jiancheng-Bioeng Institute (Nanjing, China).

### 2.8. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was measured by using rhodamine123 fluorescent dye. Rhodamine123 could enter the mitochondrial matrix and cause photoluminescent quenching dependent on mitochondrial transmembrane potential (∆Ψm). Adrenal cells were incubated with rhodamine123 (10 μg/ml) for 30 min at 37 °C. After incubation, cells were rinsed with D-hanks. The fluorescence was measured at an excitation wave length of 488 nm and an emission wave length of 510 nm using a CyAn ADP\(^{TM}\) with Summit\(^{TM}\) software (Beckman, USA). In order to demonstrate if ROS are involved in QdNOs-induced apoptosis, free radical-scavenging agents, N-acetylcysteine (1 mM), SOD (100 U/ml) and β-mannitol (10 mM) were used in this study. They were pre-incubated with the cells respectively 30 min before the chemical treatment without rinsing the cell cultures prior to administering the QdNOs.

### 2.9. Statistical analysis

Statistical analysis was performed using SPSS 11.5 for windows. All results were presented as mean ± S.E.M. Group differences were analyzed using one-way analysis of variance (ANOVA) followed by LSD's post hoc tests. A probability of P < 0.05 was considered significant.

### 3. Result

#### 3.1. The dose- and time-response cytotoxic effect of QdNOs on porcine adrenocortical cells

The results of MTT assay demonstrated a dose- and time-dependent decrease in porcine adrenocortical cells activity induced by OLA, MEQ or CYA, respectively (Fig. 2). OLA displayed the most potent cytotoxicity among the three compounds under the same experimental conditions. It inhibited cell viability to 15.79 ± 2.05% at 100 μM after 24 h of incubation. CYA, however, showed only the lowest toxicity on cells at a high dose after 24 h of incubation. As one of the main metabolites of OLA and MEQ, QCA showed relatively lower toxicity than three QdNOs. To standardize the assays, concentration of 100 μM and 4 h of exposure time were chosen for subsequent studies to assess apoptosis or intracellular ROS and oxidative stress.

The LDH leakage assay has been employed as an indicator of cytotoxicity in cells. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage [25]. As shown in Fig. 3, after 4 h of incubation, the release of LDH (% of total) increased in OLA, MEQ and CYA groups compared to control group (P < 0.05), but was not affected in QCA group (Fig. 3). The result was coincided with the cell survival rate as determined by MTT method, which further confirmed the cell toxicity of QdNOs.

#### 3.2. The effect of QdNOs on the apoptosis of porcine adrenocortical cells

As shown in Fig. 4A, an increased rate of apoptosis induced by QdNOs was determined by FACS analysis using Annexin V–FITC/PI assay (Fig. 4A). More apoptotic cells were identified after OLA treatment than those seen with CYA or MEQ. Also, OLA treatment induced significant late apoptosis/necrosis in porcine adrenocortical cells (∼38%) while MEQ (∼20%) and CYA (∼12%) induced less cell death. However, in the QCA treated groups a slightly apoptotic alteration (∼8%) of late apoptosis could be measured.
Similarly, the result of AO/EB double staining showed that significant increase in the apoptotic/necrosis cells were seen after the cells were treated with 100 μM QdNOs. OLA displayed the most potent cytotoxicity among the three compounds. Compared to the parental drugs, QCA showed relatively lower toxicity than three kinds of QdNOs (Fig. 4B).

3.3. Time-response effect of QdNOs on generation of intracellular reactive oxygen species (ROS)

As shown in Fig. 5, three QdNOs could all increase ROS production in adrenocortical cells in a time-dependent manner, which suggested that ROS is a key mediator of QdNOs-induced cell death in adrenocortical cells. OLA, on the other hand, did not cause any significant production of ROS.

Three QdNOs all showed their special characteristics in intracellular ROS release in adrenocortical cells. The rank order was OLA > MEQ > CYA, which were coincided with the regular pattern in cytotoxicity. We observed that the ROS production increased (1.5–2.6-fold) and lasted as long as 4 h after OLA treatment. MEQ had a similar but weaker effect compared to OLA. CYA induced a weak and unstable release in ROS production reaching a dominant peak (~2-fold) in about 30 min, and then decreased dramatically to less than 1.5-fold and remained this level to the end of the experiment.

3.4. The effect of QdNOs on oxidative stress indices

Mitochondria are the major source and a susceptible target of ROS. Increased production of ROS in cells may lead to alterations in mitochondrial redox metabolism and respiratory functions. Fig. 6A–C showed that after QdNOs treatment, there were significant increases in MDA formation, SOD activity as well as decrease in GSH levels both in the mitochondria and PMS, indicating alterations of the mitochondrial redox metabolism in porcine adrenocortical cells. The maximum induction was seen after OLA treatment. In contrast, no significant changes in oxidative stress parameters were observed after QCA treatment.

3.5. The effect of QdNOs on mitochondrial membrane potential (Δψ)

As shown in Fig. 7, mitochondrial membrane potential (Δψ) decreased to 44–73% after 4 h of QdNOs treatment, where more mitochondrial toxicity were identified after OLA than that seen with MEQ or CYA. In contrast, treatment of three free radical-scavenging agents, i.e. N-acetylcysteine, d-mannitol and SOD have all partially reversed the decrease of Δψ induced by QdNOs, suggesting a participation of ROS and oxidative stress in mitochondrial damage after QdNOs treatment. However, QCA showed no significant effect on Δψ. These results indicated the implication of
mitochondrial dysfunction in the pathogenesis of cell apoptosis and the sensitivity of $\Delta \psi$ towards mitochondrial ROS generated by QdNOs.

4. Discussion

The present study strongly demonstrated the pro-oxidant effects of QdNOs and suggested that increased intracellular ROS has mediated a significant time and dose-dependent cytotoxicity in porcine adrenocortical cells exposed to QdNOs via a mitochondrial-dependent pathway. The marked quantitative differences of QdNOs observed in the adrenal toxicities as well as the production of intercellular ROS have been investigated in the present study. OLA, which showed the most toxicity among three QdNOs derivatives, irritating a persistent and utmost release of intracellular ROS, thus triggered a cascade of cytotoxicity reaction. CYA, however, had a characterization of short and unstable release of intracellular ROS, and displayed much lower toxicity than that of OLA and MEQ. N-acetylcysteine, d-mannitol and SOD, which scavenges reactive oxygen [26,27], can promote the removal of oxidative free radicals and reduce QdNOs-induced mitochondrial damage. QCA treatment,
Fig. 6. (A–C) Effect of cyadox, olaquindox and mequindox on MDA, SOD and GSH. Mitochondrial and postmitochondrial supernatant (PMS) from control and treated porcine adrenocortical cells were prepared. MDA, GSH levels and SOD activity were measured by commercial assays. Values are expressed as mean ± S.E.M., n = 6. Significant statistical difference was indicated by *P < 0.05; **P < 0.01 versus control.

on the other hand, maintained the mitochondrial redox and respiratory functions without a marked effect on ROS production and cell viability, suggesting the participation of free radical intermediates in QdNOs' toxicity.

The results also showed that low doses of QdNOs displayed little toxicity on porcine adrenocortical cells. However, long term of high doses (50–100 μM) exposure to OLA and MEQ could lead to significant cell damage, mainly including cell apoptosis and necrosis, increasing intracellular ROS, mitochondria dysfunction and redox imbalance. Considering the clinical doses (30 and 5 μg kg⁻¹ in pig tissue) [28] of QdNOs, these feed additives are rather safe if normally used.

Although some experts thought that the different toxicity of QdNOs is partly due to their metabolisms of absorption and contribution in vivo [29–31], it is insufficient to interpret their toxicity in vitro. Due to the special chemical structure of QdNOs, it is possible that free radical metabolites may be responsible for the cytotoxicity observed in porcine adrenocortical cells [13–16]. The reduction of QdNOs results in the formation of a semiquinone free radical by a one-electron reduction of the quinone. Semiquinones are readily autooxidizable, which leads to non stoichiometric oxidation of NADPH and oxygen consumption. The oxidative stress associated with autooxidation of a semiquinone free radical, which produces superoxide anion, hydrogen peroxide, and other active oxygen species, can be extremely cytotoxic, as illustrated in Fig. 8. The metabolites of these compounds, however, exerted lower cytotoxicity and pro-oxidant property than their parent drugs. It is clear that additional work is required before a comprehensive knowledge of QdNOs toxicology potential and mechanisms of action can be achieved. More full-scale investigations focused on the mechanism and toxicity of these compounds were conducted currently in our lab. Their metabolism as well as chemical properties such as stabilities, pKa or octanol:water partition coefficients should be considered and investigated deeply (currently being implemented in our laboratory) to better characterize the ability of these QdNOs to partition into the lipid phase.

Mitochondria are the major cellular source for generation of reactive oxygen species (ROS) and at the same time the main tar-

Fig. 7. The effect of QdNOs on mitochondrial membrane potential. N-acetylcysteine (1 mM), SOD (100 U/ml) and D-mannitol (10 mM) were pre-incubated with the cells 30 min before the drug treatment without rinsing the cell cultures prior to administering the QdNOs. Values are expressed as mean ± S.E.M., n = 6. Significant statistical difference were indicated by *P < 0.05; **P < 0.01 versus control and #P < 0.05; ##P < 0.01 versus the same group which not treated with pharmacological tools.

Fig. 8. Two-electron reduction of QdNOs, and production of reactive oxygen species during its one-electron reduction to a semiquinone radical.
gets of ROS-induced oxidative damage [32]. In addition to known mitochondrial functions, mitochondria can serve the critical role of a cellular “signal amplifier” when a low amount of ROS in or around mitochondria can be significantly amplified by mitochondria and converted into a pathological ROS signal. The mitochondrion-to-mitochondrion RIRR (ROS-induced ROS release) constitutes a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial and cellular injury [33]. In situ-generated ROS can open the permeability transition pore with subsequent ΔΨm and can cause cellular damage such as apoptosis, enzyme inactivation and cytochrome c release into the cytosol [34–36]. In our present study, it has been demonstrated, for the first time that QdNOs-induced alterations in mitochondrial redox functions are critical to promote ROS-mediated cell death. The decrease of ΔΨm induced by QdNOs showed a ROS-mediated mitochondrial dysfunction in the cells. Thus, the alterations in mitochondrial function in adrenocortical cells by QdNOs may be a major cause of apoptosis and cell death. GSH is one of the most prominent antioxidant defense components in the tissue. Besides serving as a substrate for glutathione-related enzymes such as GPx, GSH acts as a free radical scavenging ROS [37]. Mitochondrial GSH pool was inhibited under this condition, indicating a QdNOs-induced imbalance of redox both in PMs and mitochondria. Furthermore, the increased SOD activity also suggests that increased mitochondrial superoxides are being metabolized. The increased oxidative stress caused by the increased ROS production and Lipid peroxidation further aggravated the mitochondrial dysfunctions leading to cell death. Our results have provided clear evidence that the QdNOs-induced cell death (both apoptosis and necrosis) is a result of increased oxidative stress signaling and alterations in the mitochondrial redox and respiratory functions.

In summary, the results of this study indicate that QdNOs exposure is involved in the generation of ROS and a state of oxidative damage which in turn may elicit their cytotoxicities through mitochondria-dependent pathway. The characterization of three QdNOs differs a lot in the cytotoxicity and intracellular ROS release. The results provide a mechanism approach in understanding the characterize of adrenal damage caused by QdNOs in vitro, which would in turn, help in designing the appropriate therapeutic strategies of these kinds of feed additives.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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