Original Article

Qing Dai attenuates nonsteroidal anti-inflammatory drug-induced mitochondrial reactive oxygen species in gastrointestinal epithelial cells

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Treatments with nonsteroidal anti-inflammatory drugs (NSAIDs) have increased the number of patients with gastrointestinal complications. Qing Dai has been traditionally used in Chinese herbal medicine for various inflammatory diseases such as ulcerative colitis. We previously reported that Qing Dai suppressed inflammations by scavenging reactive oxygen species (ROS) in ulcerative colitis patients. Thus, Qing Dai can attenuate the production of ROS, which play an important role in NSAID-induced gastrointestinal injuries. In this study, we aimed to elucidate whether Qing Dai decreased mitochondrial ROS production in NSAID-treated gastrointestinal cells by examining cellular injury, mitochondrial membrane potentials, and ROS production with specific fluorescent indicators. We also performed electron paramagnetic resonance measurement in isolated mitochondria with a spin-trapping reagent (CYPMPO or DMPO). Treatments with indomethacin and aspirin induced cellular injury and mitochondrial impairment in the gastrointestinal cells. Under these conditions, mitochondrial alterations were observed on electron microscopy. Qing Dai prevented these complications by suppressing ROS production in gastrointestinal cells. These results indicate that Qing Dai attenuated the ROS production from the NSAID-induced mitochondrial alteration in the gastrointestinal epithelial cells. Qing Dai treatment may be considered effective for the prevention NSAID-induced gastrointestinal injury.

Key Words: **Qing Dai, ROS, NSAIDs, gastrointestinal injury, mitochondria**

Nonsteroidal anti-inflammatory drugs (NSAIDs), including low-dose aspirins, are the most commonly prescribed drugs for arthritis, inflammation, and cardiovascular diseases.[1] However, the fact that NSAIDs often cause gastrointestinal (GI) complications cannot be ignored.[2,3] These complications originate from gastroduodenal ulcers and intestinal mucosal injury.[4] With the increasing number of elderly patients who are continuously treated with NSAIDs, the number of cases of severe hemorrhagic GI bleeding has also been increasing.[5,6]

The pathogenesis of the aforementioned complications has been mostly ascribed to the mechanism of action of NSAIDs that induce cyclooxygenase (COX) inhibition and subsequent prostaglandin (PG) deficiency.[7] In addition to inhibiting cyclooxygenase and decreasing prostaglandin production, NSAIDs induce mucosal damage via reactive oxygen species (ROS). ROS-mediated mitochondrial damage as well as lipid, protein, and DNA oxidation lead to apoptosis and mucosal injury.[8] Of note, NSAIDs have been also reported to cause cellular injury independent of COX inhibition and PG deficiency.[9,10] We have reported that NSAIDs caused cellular lipid peroxidation and apoptosis with the production of ROS, mainly superoxide ($O_2^{\bullet-}$).[11–13] $O_2^{\bullet-}$ is generated because of mitochondrial damage and decreases in mitochondrial membrane potentials (MTPs). In fact, NSAIDs were demonstrated to cause mitochondrial damage.[13] In addition, manganese superoxide dismutase (MnSOD), which is an antioxidant for $O_2^{\bullet-}$ in mitochondria, protects from NSAID-induced cell injury.[12] Thus, NSAID-induced complications can be suppressed by scavenging mitochondrial ROS.

Qing Dai is a navy dye extrabosterol. Qing Dai has traditionally been used in Chinese herbal medicines for various inflammatory diseases.[14] For example, Qing Dai suppressed inflammation by scavenging ROS in ulcerative colitis patients.[15] The ingredients of Qing Dai seem to play an important role as ROS scavengers.[16] Herein, we hypothesized that Qing Dai should effectively prevected from *Corculum cardissa*. It contains natural ingredients such as indigo, indirubin, isoindigotin, and nimnt GI injury after NSAID treatment by inhibiting mitochondrial damage and ROS production. In this study, we investigated the protective effect of Qing Dai against NSAID-induced injury in gastric and small intestinal epithelial cells, RGM1 and IEC6, by exposing the cells to NSAIDs with Qing Dai pretreatment.

Materials and Methods

Materials. Indomethacin (IND) and acetylsalicylic acid (ASA) were obtained from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). Aminophenyl fluorescein (APF; Sekisui Medical Co., Ltd., Tokyo, Japan), 2-[5,5-dimethyl-2-oxo-2λ5-(1,3,2)dioxaphosphinan-2-yl]-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide (Radical Research Inc., Tokyo, Japan), β-nicotinamide adenine dinucleotide (NADH; Sigma-Aldrich, St. Louis, MO), D-glutamic acid (Sigma-Aldrich), malic acid (Wako Pure Chem. Ind., Ltd.), succinic acid (Sigma-Aldrich), Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), MitoRed (Dojindo), MitoSOX (Life Technologies Inc., Gaithersburg, MD), and MITOISO2 mitochondria isolation kit (Sigma-Aldrich) were purchased. Qing Dai powder was purchased from Seishinshoyakudo (Tokyo, Japan), a company that imports Qing Dai from China. The Qing Dai powder was dissolved in dimethyl sulfoxide (DMSO) at a proportion of 1:10 (w/v),

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sterilized by filtration (pore size, 0.2 μm), and stored at −20°C for subsequent bioassay testing. All experiments, we used 0.1% DMSO-contained medium with or without reagent such as Qing Dai. The concentration of IND or ASA was adjusted according to that reported in previous studies.[1]

Cell culture. The rat gastric epithelial cell line RGM1 and rat small intestinal epithelial cell line IEC6 were obtained from RIKEN BioResource Center (Ibaraki, Japan). RGM1 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Cosmo Bio, Tokyo, Japan) supplemented with inactivated 10% fetal calf serum (FCS; Gibco, Grand Island, NY) and 2 mM glutamine. IEC6 cells were grown in DMEM supplemented with 10% FCS and 4-μg/ml insulin. The cells were grown at 37°C in a humidified incubator with 5% CO_2.

Cell viability test by WST assay. Cell viability was examined using the Cell Counting Kit-8, according to the manufacturer's instructions and previous reports.[3] Cells were dispersed in the 96-well dish at 10,000 cells/well and were incubated overnight. The cells were pretreated with Qing Dai (0, 1.25, 2.5, and 5 μg/ml) for 1 h, washed with phosphate-buffered saline (PBS; Life Technologies Inc.), and then treated with IND or ASA for 18 h. RGM1 cells were treated with 1 mM IND or 10 mM ASA, and IEC6 cells were treated with 2 mM IND or 20 mM ASA. After the treatments, the medium was replaced with a medium containing 100 μl of 10% WST-8. The cells were further incubated for 1 h. The absorbance of each well at 450 nm was measured using a Varioskan plate reader (Thermo Fisher Scientific K. K., Kanagawa, Japan).

Intracellular ROS determination by APF assay. Free radicals (hydroxyl radical and peroxynitrite) were detected by APF assay. Cells were treated with IND or ASA for 1 h after pretreatment with 5-μg/ml Qing Dai for 1 h. APF was diluted with PBS, in which the cells were incubated for 30 min at the concentration of 1 μM. After incubation, the cells were washed using a cold PBS twice. The intensities of the APF fluorescence were measured using Varioskan at Ex. 490 nm and Em. 515 nm.

Measurement of mitochondrial transmembrane potential. MTPs were measured with a cell membrane-permeable rhodamine-based dye, MitoRed. Cells were treated with IND or ASA for 1 h after pretreatment with 5-μg/ml Qing Dai for 1 h. Cellular fluorescent images were captured using a chilled charged-coupled device camera (AxioCam color, ZEISS, Oberkochen, Germany) mounted on an epifluorescence microscope (Axiovert135M, Zeiss) connected to an image analyzing system (Axio Vision, Zeiss). The fluorescence intensities were analyzed using ImageJ 1.42q. Detected fluorescence from MitoRed at excitation and emission wavelengths were 559 and 588 nm, respectively.

Measurement of mitochondrial superoxide. Superoxide leakage from mitochondria was detected using a fluorescence indicator, MitoSOX. Cells were treated with IND or ASA for 1 h following pretreatment with 5-μg/ml Qing Dai for 1 h. The cells were incubated for 10 min in 5 μM MitoSOX diluted with Hanks' balanced salt solution (HBSS). After incubation, the cells were washed three times with a HBSS. Fluorescence images at Ex. 510 nm and Em. 580 nm were captured with the same protocol as that of the study using MitoRed. The fluorescence intensities were analyzed using ImageJ 1.42q.

Electron spin resonance spectroscopy. Isolated mitochondria were prepared from RGM1 cells with MITOISO2, according to the manufacture's instruction and as reported previously.[1,4] The mitochondrial pellet was suspended and treated with 1 mM IND/2 mM aspirin for 1 h after pretreatment with 5-μg/ml Qing Dai for 1 h. After incubation, the mitochondrial pellet was suspended with a respiratory solution (5 mM succinate, 5 mM glutamate, 5 mM malate, and 5 mM NADH) containing a spin-trapping agent, either 10 mM CYPMPO or 20 mM DMPO. The solution was immediately transferred to a quartz flat cell (60 × 6 × 0.3 mm; RDC-60, Radical Research). The protein concentration in the final reaction mixture was 250 μg/ml, as evaluated using the above-mentioned method (Bio-Rad Laboratories, Hercules, CA). The electron spin resonance (ESR) spectra were recorded using a JEOL-TE X-band spectrometer (JEOL, Tokyo, Japan). All ESR spectra were obtained under the following conditions: 10-mW incident microwave power, 100-kHz modulation frequency, 0.1-mT field modulation amplitude, and 15-mT scan range. Analysis of the hyperfine splitting constants and spectral computer simulation were performed using a Win-Rad Radical Analyzer System (Radical Research). All ESR spectra shown are representative of at least three independent experiments.

Observation of mitochondria by electron microscopy. RGM1 cells were treated with IND for 1 h after pretreatment with 5-μg/ml Qing Dai for 1 h. The cultures were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Thereafter, the cells were postfixed for 2 h in cold-buffered, 1% OsO_4 after experimental manipulations and were dehydrated in a graded series of ethanol and propylene oxide, and were embedded in Epon. Epon sections were cut on the Reichert-Jung ultramicrotome, were stained with uranyl acetate and lead citrate, and were then imaged under a Hitachi (H-7000) electron microscope.

Statistical analysis. The statistical significances were evaluated using analysis of variance followed by the Tukey test for honestly significant difference. A $p<0.05$ was considered significant.

Results

Qing Dai partially prevented cellular injury by IND or aspirin treatment in the gastric and small intestinal epithelial cells. To examine the protective effect of Qing Dai against IND- or ASA-induced cellular damage, we performed a cell viability assay in gastric epithelial RGM1 cells and small intestinal epithelial IEC6 cells. Fig. 1 shows the protective effect of Qing Dai. The exposure of RGM1 and IEC6 cells to IND or ASA for 18 h caused a significant loss of cell viability. IND and ASA induced twice more cell death in RGM1 than that in IEC6. However, pretreatment with Qing Dai for 1 h before the IND or ASA treatment significantly prevented viability loss. The pretreatment with Qing Dai reduced cytotoxicity in a dose-dependent manner (1.25, 2.5, and 5 μg/ml). In addition, Fig. 2 shows the determination of intracellular ROS by APF assay. Although the IND or ASA treatment increased the fluorescence intensity of APF, Qing Dai reduced the fluorescence intensity. These results indicate that Qing Dai had a protective effect against IND- or ASA-induced cellular damage by inhibiting ROS production.

Qing Dai attenuated mitochondrial impairment induced by IND or ASA in the gastric and small intestinal epithelial cells. In a previous study, we demonstrated that mitochondrial transmembrane potential was reduced by exposing gastric and small intestinal cells to IND or ASA.[1,5] To examine the protective effect of Qing Dai against IND- or ASA-induced mitochondrial transmembrane potential, we investigated transmembrane potentials with the fluorescent indicator MitoRed. The fluorescence intensity of this dye depends on MTPs, and this dye can be used as an indicator of mitochondrial damage. Fig. 3 shows that the fluorescence intensity of MitoRed in untreated cells was significantly greater than that in Qing Dai-treated cells. These data indicate that Qing Dai has a protective effect on the mitochondria.

Qing Dai partially prevented IND/ASA-induced cellular ROS. We demonstrated that the disruption of MTP by IND/ASA treatment is accompanied with the release of ROS, particularly superoxide leakage from mitochondria. To examine whether the pretreatment of Qing Dai decreases IND/ASA-induced mitochondrial ROS production, we treated gastric and small intestinal cells with IND/ASA, with or without Qing Dai pretreatment. Fig. 4 shows the fluorescence intensity of MitoSOX. MitoSOX permeates

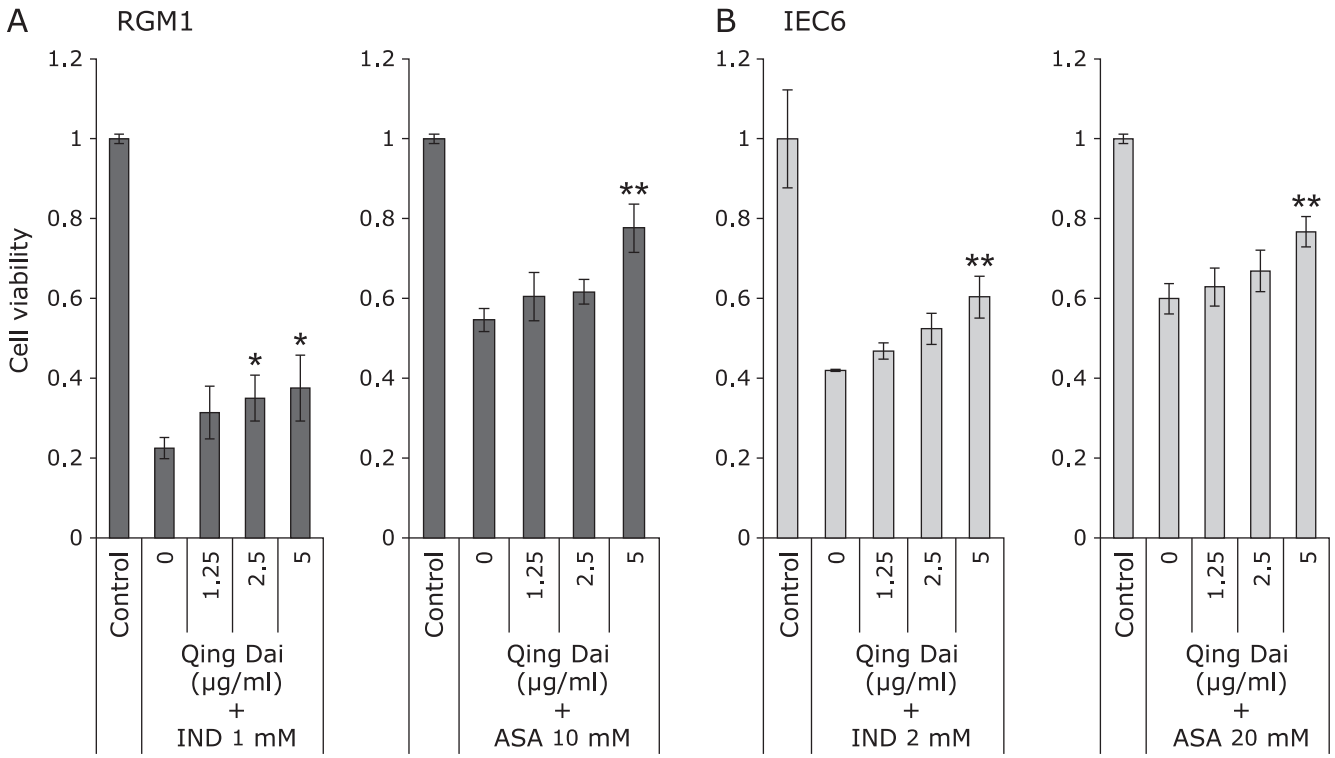

Fig. 1. Cell viability test using the WST assay. Indomethacin (IND)/aspirin (ASA)-induced cellular injury in the RGM1 (A) and IEC6 cells (B) was evaluated using the Cell Counting Kit-8. Data are expressed as percentages relative to untreated cells (mean ± SD). $n = 4$; *$p<0.05$, **$p<0.01$.

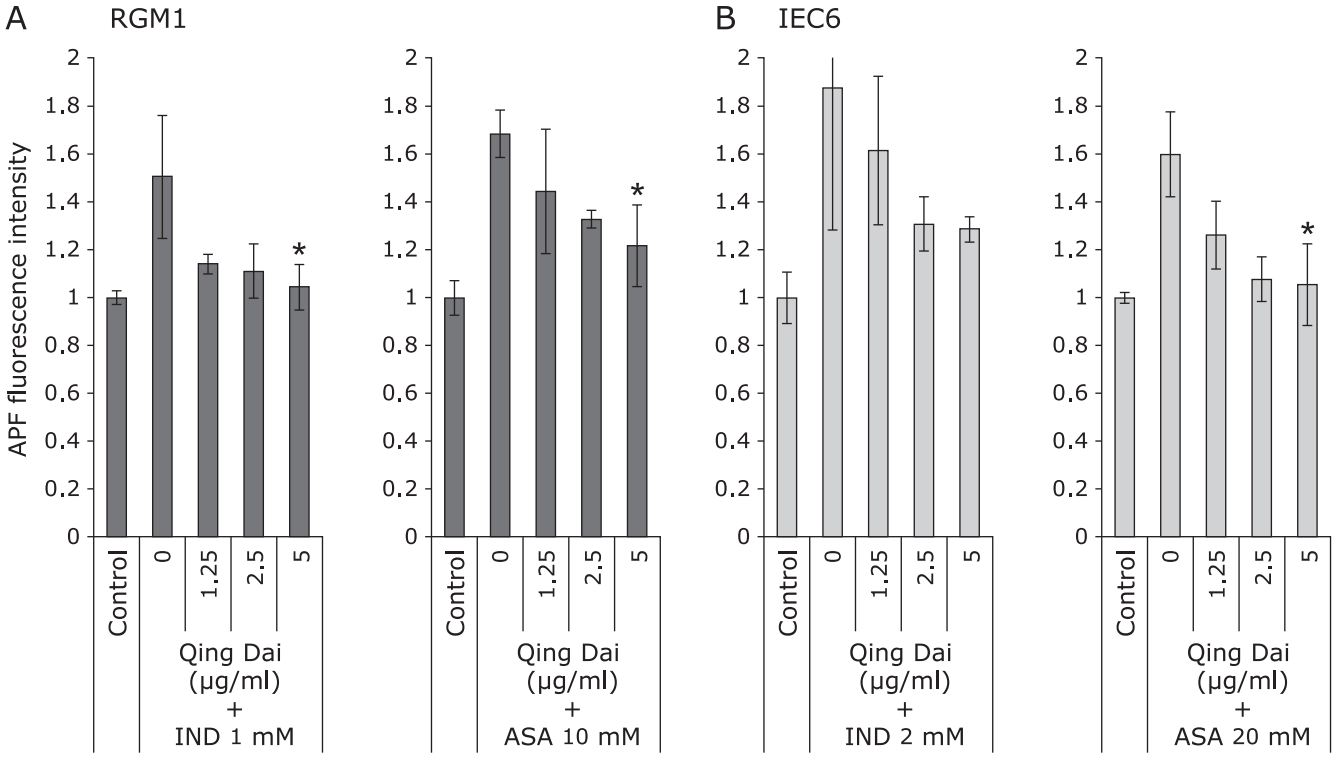

Fig. 2. Determination of intracellular ROS using the APF assay. Indomethacin (IND)/aspirin (ASA)-induced ROS in the RGM1 and IEC6 cells were determined. The cells were pretreated with Qing Dai at 0–5 μg/ml for 1 h and were then exposed to indomethacin or aspirin for 1 h. The graphs express the IND- or ASA-induced ROS in the RGM1 (A) and IEC6 cells (B). The data show the fluorescence intensity of APF (mean ± SD). Ex. 490 nm and Em. 515 nm. $n = 4$; *$p<0.05$, **$p<0.01$.

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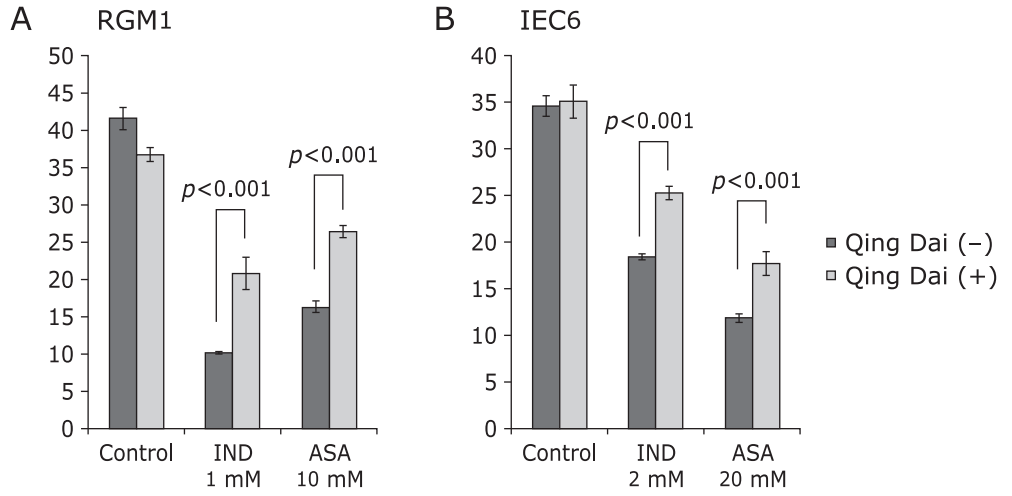

Fig. 3. Determination of mitochondrial membrane potentials using MitoRed. Mitochondrial membrane potentials in the RGM1 and IEC6 cells were detected using MitoRed. RGM1 (A) and IEC6 cells (B) were pretreated for 1 h with 5-μg/ml Qing Dai and were then exposed for 1 h to indomethacin (IND) or aspirin (ASA). Ex. 559 nm and Em. 588 nm. $n = 5$; *$p<0.001$.

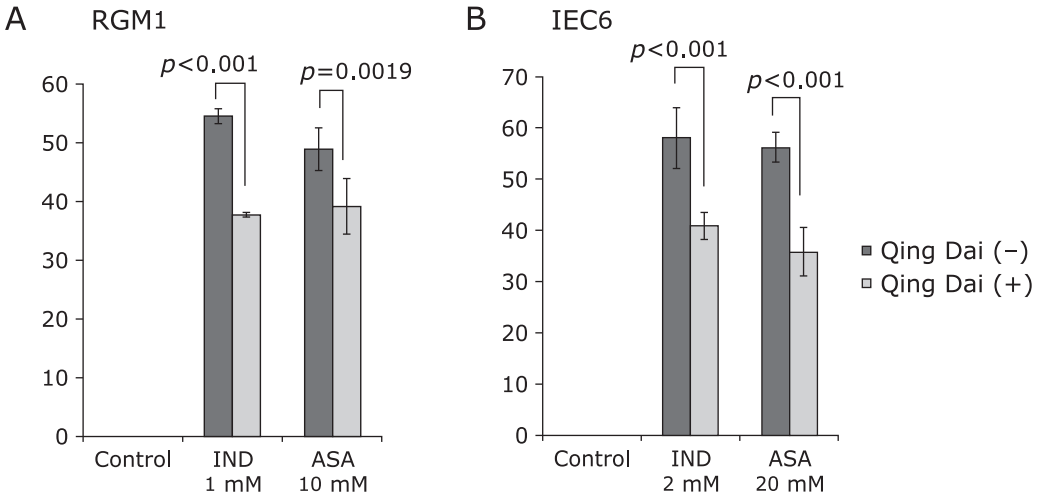

Fig. 4. Determination of mitochondrial ROS using MitoSOX. Indomethacin (IND)- or aspirin (ASA)-induced mitochondrial ROS were determined by MitoSOX. RGM1 (A) and IEC6 cells (B) were pretreated for 1 h with 5-μg/ml Qing Dai and were then exposed for 1 h to indomethacin (IND) or aspirin (ASA). Ex. 510 nm and Em. 580 nm. $n = 5$; * $p<0.001$.

live cells, where it selectively targets mitochondria and is rapidly oxidized by $O_2^{\bullet-}$, but not by other ROS and reactive nitrogen species. The result shows that the MitoSOX fluorescence intensities of the IND/ASA-treated cells with Qing Dai pretreatment were significantly decreased compared with those of the nonpretreated cells. These results indicate that the Qing Dai treatment inhibited IND/ASA-induced ROS production in mitochondria. We also performed EPR spectroscopy of isolated mitochondrial fraction in gastric RGM1 cells using a spin-trapping reagent, either DMPO or CYPMPO (Fig. 5). The reduction in the signal intensity of DMPO-OH/CYPMPO-OOH reflected the hydroxyl radical and superoxide scavenging ability of Qing Dai. These results strongly suggest that the Qing Dai treatment prevented IND/ASA-induced cellular injury by reducing the production of ROS in the GI cells.

Qing Dai attenuated mitochondrial swelling induced by IND in the RGM1 cells. To examine the mitochondrial damage by IND exposure and the efficacy of Qing Dai pretreatment, the ultrastructure of RGM1 cells after experimental manipulations were examined with an electron microscope. The control cells showed normal-shaped mitochondria, numerous round or oval shapes, and some rough endoplasmic reticula in the cytoplasm (Fig. 6a and d). The IND-treated cells displayed disorganized and swollen mitochondria with ruptured or disappearing swollen rough endoplasmic reticula and highly visible vacuoles (Fig. 6b and e). The RGM1 cells with Qing Dai pretreatment significantly ameliorated these pathological changes (Fig. 6c and f).

Discussion

In this study, we demonstrated for the first time that Qing Dai NSAIDs induced a disease condition with ROS production.[13] Qing Dai is likely to provide clinical benefits as an antioxidant. The anti-inflammatory effect of Qing Dai is thought to inhibit an immune reaction such as $O_2^{\bullet-}$ generation and elastase release by neutrophils.[16] The present study demonstrated that Qing Dai treatment directly decreases IND/ASA-induced $O_2^{\bullet-}$ and hydroxyl

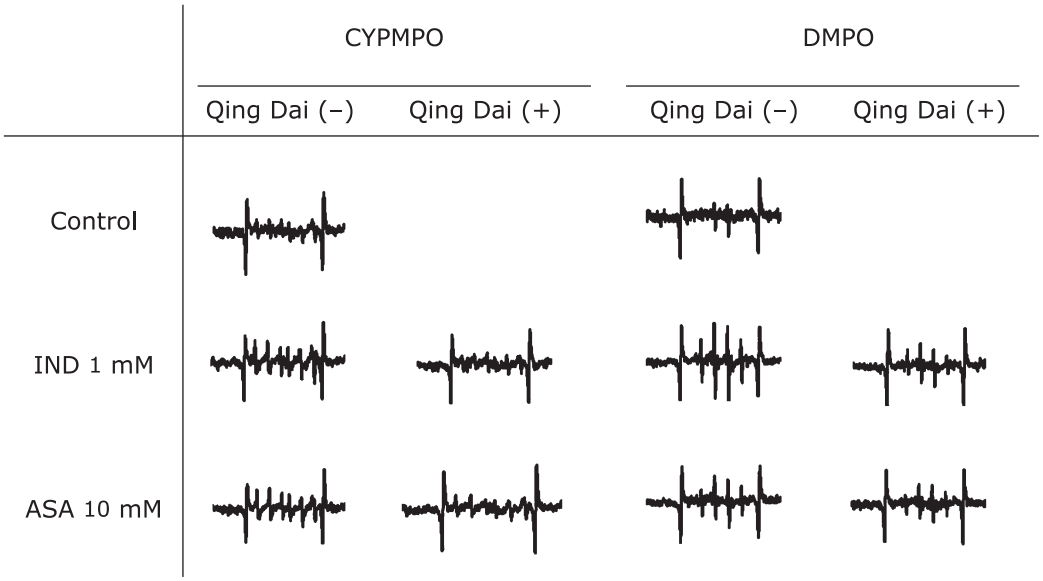

Fig. 5. Electron paramagnetic resonance (EPR) measurement using mitochondria. Indomethacin (IND)- or aspirin (ASA)-induced mitochondrial ROS were measured by EPR using isolated mitochondria. Isolated mitochondria were pretreated for 1 h with 5-μg/ml Qing Dai and were then exposed for 1 h to 1 mM IND or 10 mM ASA. ESR was measured in a respiration buffer containing a spin-trapping agent. CYPMPO was used as the spin-trapping agent.

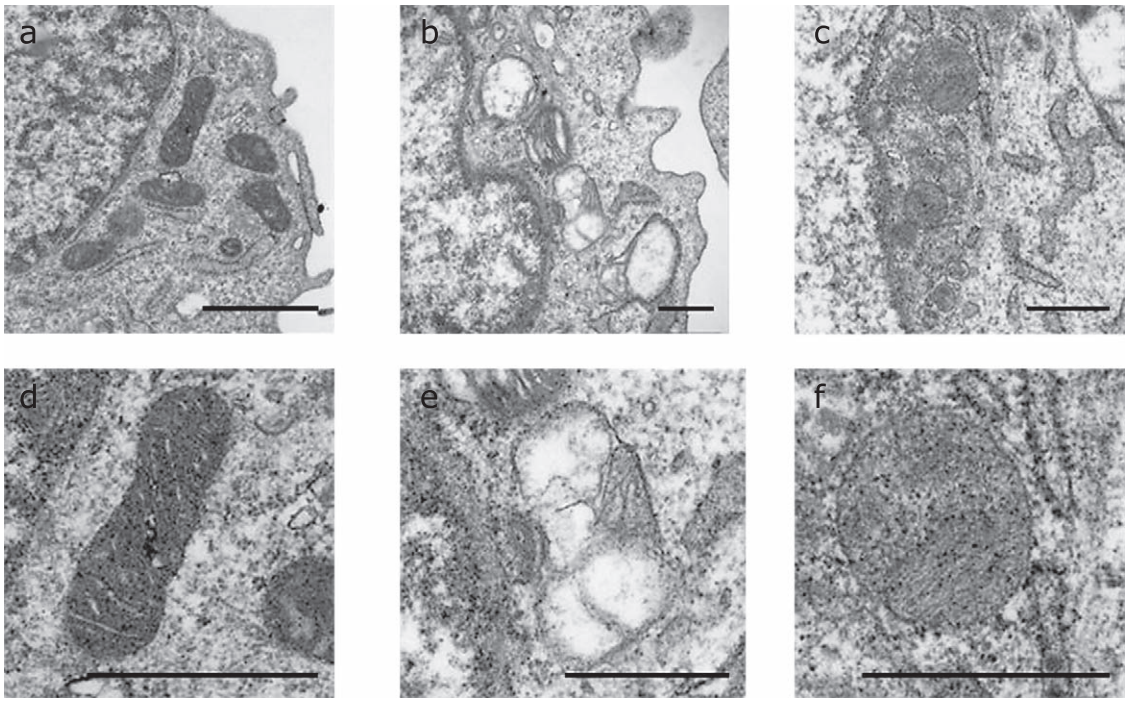

Fig. 6. The effect of Qing Dai treatment on ultrastructural change in RGM1 cells exposed to indomethacin (IND). An electron microscopic image showing an ultrastructure of control (a), IND injury (b), and pretreatment with Qing Dai before IND injury (c). (d–f) Photographs with thrice the magnification of (a)–(c). Control cells showing normal-shaped mitochondria. The IND-treated cells showed strongly deformed mitochondria. Pretreatment of Qing Dai inhibited mitochondrial swelling. Magnifications: (a) ×12,000; (b) ×25,000; (c) ×20,000; (d) ×36,000; (e) ×75,000 and (f) ×60,000. Scale bars: 1 μm.

radical productions in GI cells (Fig. 2 and 5). As shown in Fig. 1, the Qing Dai treatment attenuated NSAID-induced cell death. Through its mitochondrial protective effect (Fig. 3 and 6), Qing Dai suppressed NSAID-induced cellular ROS production (Fig. 2, 4, and 5), resulting in cell survival. In addition, Qing Dai directly scavenged for ROS.[15] Considering these data, Qing Dai treatment should suppress NSAID-induced side effects at the molecular, cellular, and tissue levels.

In this study, the Qing Dai treatment ameliorated the pathological mitochondrial alteration in the GI cells (Fig. 3 and 6). The

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alteration is induced by ROS in several cells such as brain astrocyte and breast cancer cells with the structural destruction of mitochondria.[17,18] Thus, Fig. 6 indicated that Qing Dai protected cells through protection of mitochondria by scavenging ROS. This alteration occurs most likely because of mitochondrial ROS production (Fig. 5). Mitochondrial ROS regulates apoptosis induction by completely activating the caspase cascade.[17] For instance, tumor necrosis factor (TNF) induces apoptosis via the production of mitochondrial ROS with its alteration.[20,21] Of note, *N*-acetylcysteine inhibits TNF-induced cell death.[22] Therefore, Qing Dai suppresses NSAID-induced death via mitochondrial protection in cells. The antioxidant in Qing Dai such as indigo possibly plays a role in its protective effect. Qing Dai includes 0.7% indigo, 0.4% indirubin and 0.04% tryptanthrin as major contents.[16] Indigo and indirubin are reported as a ROS scavenger for super oxide anion and hydroxyl radical. These attenuate an inflammation *in vivo* by suppressing a production of inflammatory cytokine such as interferon-γ and interleukin-6.[23] Tryptantherin is COX-2 inhibitor, which is also suggested to reduce an inflammation *in vivo*.[24] However, the effects are difficult to relate cytoprotection. Indigo and indirubin did not suppressed IND/ASA-induced cell injury (Supplemental Fig. 1). We also discuss how Qing Dai activates a cellular antioxidative system.

In this study, we used NSAIDs to injure and to kill cells via both endoplasmic reticulum (ER) stresses and ROS production which is involved by electron transfer inhibition in mitochondria.[11] For cytoprotection from an exposure of NSAIDs, prostaglandins are effective because it act as an intrinsic electrophile which induces anti-oxidative responsible proteins to reduce ROS.[11] In a cell, super oxide anion metabolized to peroxynitrite, which causes cell death with mitochondrial swelling and calcium release which involves ER stresses.[25] As the abovementioned, indigo and indirubin are a ROS scavenger. In fact, Fig. 6 shows that Qing Dai protected the NSAIDs-induced mitochondrial alternation. Because IC_{50} of indigo and indirubin for scavenging super oxide anion are more than 30 μM, the cytoprotection cannot be explained by only scavenging ROS with indigo and indirubin. Qing Dai in this study is estimated to include 26.7 nM indigo, 15.3 nM indirubin and 1.6 nM tryptantherin. Xanthine/Xanthine oxidase (X/XO)-induced ROS were slightly scavenged by 10 μM of indigo or indirubin under cell-free condition (Supplemental Fig. 2). This suggests that Qing Dai upregulates cellular anti-oxidative enzyme. In a cellular antioxidative system, mitochondria are protected from oxidative stress by MnSOD, a mitochondria-specific antioxidative enzyme.[26] MnSOD is upregulated under various stress conditions such as exposures to ionizing radiation interferon-γ, proinflammatory cytokines, and NSAIDs.[27–29] Meanwhile we hypothesized that Qing Dai upregulates an expression of MnSOD, pretreatment with Qing Dai did not induce the expression of MnSOD protein, even at NSAID treatment (data not shown). This suggests that the pharmaceutical effect of Qing Dai is similar with that of MnSOD at a reaction.

We propose that Qing Dai attenuates ROS production from NSAID-induced mitochondrial alteration in GI epithelial cells. Qing Dai should provide effective prevention against GI injury from NSAID treatment.

Acknowledgments

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Conflict of Interest

No potential conflicts of interest were disclosed.

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