Original Article

Ruscogenin Protects against Deoxynivalenol-Induced Hepatic Injury by Inhibiting Oxidative Stress, Inflammation, and Apoptosis through the Nrf2 Signaling Pathway: An *In vitro* Study

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Abstract

Background: Deoxynivalenol (DON) is a trichothecene mycotoxin with demonstrated cytotoxicity in several cell lines and animals, primarily owing to inflammation and reactive oxygen species accumulation. Ruscogenin (RGN), a steroidal sapogenin of Radix *Ophiopogon japonicus*, has significant anti-thrombotic/anti-inflammatory effects.

Objective: The aim of this study was to assess the protective role of RGN against DON-induced oxidative stress, which occurs through the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and is regulated by phosphoinositide 3-kinases/protein kinase B (PI3K/AKT).

Methods: The effects were examined using the HepG2 cell line. RGN and DON were suspended in serum-free medium. Cells were seeded onto plates, and then RGN, DON, or both were added over 24 h in triplicates for each group.

Results: RGN conferred protection against DON-exhibited cytotoxicity against HepG2 cells. RGN pretreatment downregulated the expression of DON-induced TNF-α and COX-2 and the formation of reactive oxygen species in a dose-dependent manner. RGN upregulated the expression of Nrf2 and its antioxidant proteins as well as mRNA levels of HO-1/NQO-1/HO-1/Nrf2. Similarly, treatment with DON + RGN resulted in upregulation of the pI3K/pAKT signaling pathway in a dose-dependent manner. Finally, RGN was also found to inhibit the DON-induced apoptosis by upregulating the levels of cleaved proteins and downregulating the expression of Bcl2.

Conclusion: The study demonstrates that RGN suppresses hepatic cell injury induced by oxidative stress through Nrf2 via activation of the pI3K/AKT signaling pathway.

Keywords: Apoptosis, deoxynivalenol, hepatotoxicity, Nrf2, oxidative stress, ruscogenin

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INTRODUCTION

Deoxynivalenol (DON) is a natural contaminant of feed and food that causes widespread economic challenges for the food/feed industry across the globe.[1-3] Furthermore, it jeopardizes the health of both animals and humans due to its toxicity, which leads to physical ailments such as anorexia and nausea,[4] toxicity to the immune system,[5] teratogenicity,[6] and carcinogenicity.[7] DON toxicity has been studied extensively for animal health,[8,9] and swine has been found to be particularly susceptible.[10]

DON toxicity-induced organ failure has been examined in various *in vivo* models.[11,12] Wheat contaminated by DON has been associated with histopathological alterations in porcine liver.[13] Furthermore, DON is also known to affect primary hepatocytes in humans and the liver cells of rats.[14] DON-induced oxidative stress could be one of the molecular mechanisms of its hepatotoxicity.[2] After being released from Kelch-like ECH-associated protein 1 (Keap1), activated nuclear factor erythroid 2-related factor 2 (Nrf2) translocates to the nucleus and forms a heterodimer with a small Maf protein that results in the activation of a series of detoxifying (phase II) antioxidant genes, such as NAD (P) H, quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase catalytic subunit (GCLC). These findings suggest the importance of the Nrf2 pathway for enhancing antioxidative defenses and survival of cells against oxidative damage.[15,16] PI3K/AKT and mitogen-activated protein kinase (MAPKs) significantly regulate the survival, growth, and apoptosis of cells linked to the activation of Nrf2.[17]

As reactive oxygen species (ROS) may play a role in hepatic injury, it is important for cells to effectively upregulate antioxidants, decrease ROS production, and scavenge free radicals, which may cause intestinal permeability and cell death. Ruscogenin (RGN) is an important steroidal sapogenin of Radix *Ophiopogon japonicus*, a Chinese herb that has long been used for the treatment of both chronic and acute diseases related to the heart.[18-21] RGN has been shown to cause significant anti-thrombotic/anti-inflammatory activities, possibly due to its ability to inhibit the upregulation of intercellular adhesion molecule-1 (ICAM-1) through tumor necrosis factor α (TNF-α)-induced nuclear factor-κB (NF-κB) p65 translocation. However, its intracellular signal transduction pathways need to be elucidated.[21,22] Therefore, we used a DON-induced oxidative stress model to determine if RGN could prevent liver damage.

MATERIALS AND METHODS

Drugs and chemicals

A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining kit along with DON and RGN were obtained from Sigma–Aldrich, St. Louis, MO, USA. Antibodies against pAKT (#44-621G), AKT (#44-609G), pPI3K (#PA5-104853), PI3K (#PA5-29220), BCL2 (#PA5-27094), Poly (ADP-ribose) polymerase (PARP) (PA5-16452), Heme oxygenase-1 (HO-1) (#PA5-77833), *NQO1* (#PA5-82294), pNrf2 (#PA5-105664), and β-actin (#PA5-78716) were obtained from Invitrogen (Waltham, MA, USA). Anti-cleaved caspase-3 (ab32042) antibody was obtained from Abcam (Branford, CT, USA). Lipofectamine 2000 (11668027) and Nrf2 Small interfering RNA (siRNA) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture

HepG2 was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with heat-inactivated fetal bovine serum (FBS), 100 U/ml streptomycin, and 100 U/ml penicillin. Cells were maintained in a humidified CO_2 atmosphere in 100-mm dishes at an incubation temperature of 37°C.

Cell culture treatment

RGN and DON were suspended in serum-free medium. Cells were seeded onto plates, and then RGN, DON, or a mixture of them was added over 24 h in triplicate for each group. Cells were added to a solution of 0.25% trypsin (w/v) and 0.52 mM EDTA (Cat. no: R001100, Thermo Fisher Scientific Inc, Waltham, MA, USA).

Determination of TNF-α and COX-2 Levels

The effects of DON were used to evaluate the viability of HepG2 cells and to determine the treating potentiality of RGN. In addition, this study examined the activity and expression patterns of cytokine TNF-α and cyclooxygenase-2 (COX-2) after RGN pretreatment in DON-treated HepG2 cells.

Intracellular reactive oxygen species assay

Seeding of HepG2s was done in an 8-well chamber containing 10% DMEM with growth at 80% confluence. Following this, dichlorofluorescin diacetate (DCFH2-DA) was used to wash cells using phosphate buffered saline (PBS). A fluorescence microscope (Leica D6000) was used to measure intracellular ROS.[23] For measuring ROS generation, the percentage of fluorescence intensity was compared with untreated control cells, which were arbitrarily assigned a value of 100%.

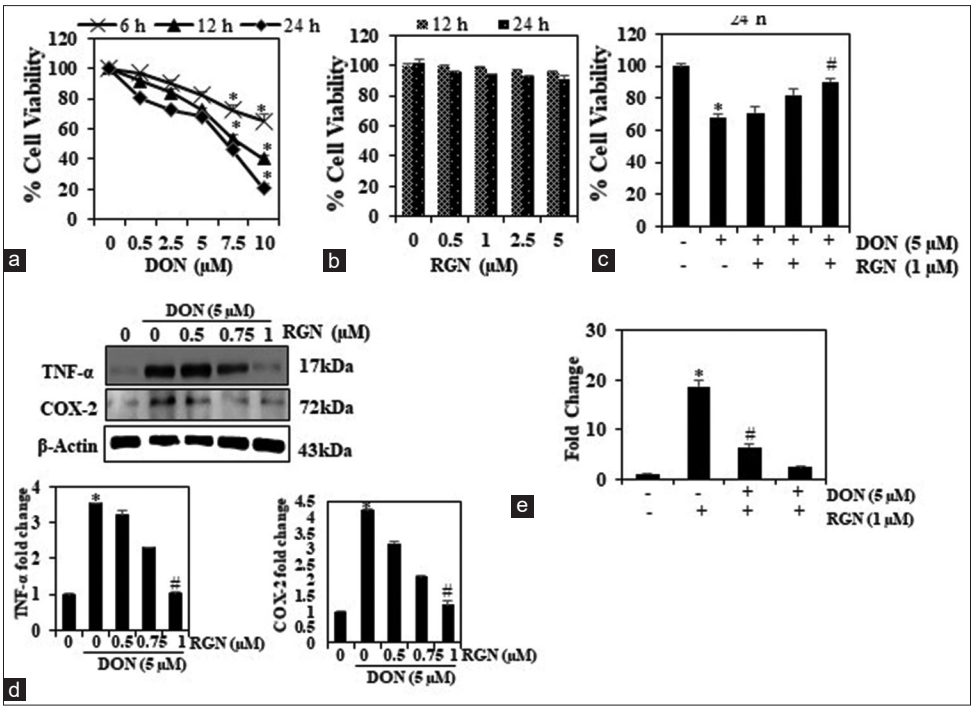

Figure 1: The effects of DON (5 μm), RGN (0-5 μm), or a combination thereof on the proliferation of HepG2 cells. (a) DON on HepG2 viability (0–10 μm for 6, 12, or 24 h, respectively). (b) Cell viability effect of RGN on HepG2 (0–5 μm for 24 h). (c) RGN protects against the cytotoxic effect of DON. (d) SDS-PAGE was done after different concentrations of DON (5 μm) or RGN (0-1) were added to cells for 24 hours. Expressions of TNFα and COX-2 were analyzed. (e) DCFH2-DA was used to measure intracellular ROS generation. Data are mean ± SD of values (n = 3), *P < 0.05 represents significant difference compared to DON alone. #P < 0.05 for RGN compared with the DON treatment groups

Nrf2-siRNA transient transfection

To ascertain the significance of Nrf2 regulation, a Nrf2 knockdown model was developed using si-RNA transfection. Cell plating was carried out on 6-well plates to attain 40–60% confluency. Opti-MEM (500 μl) was used as a culture medium, while lipofectamine RNAiMAX reagent was used to transfected the cells. In another tube, RNAiMAX reagent, Opti-MEM, and siRNA100 pM were combined. After obtaining siRNA transfected with lipofectamine RNAiMax, the RGN manufacturers' instructions were followed. The aforementioned combination was incubated for almost half an hour. After adding the solution to the plates, the solution was incubated for 6 hours. This was followed by culturing of cells at normal temperature after replacing the transfection medium with the standardized growth medium.[16]

Western blotting

Cells were scraped and then rinsed in PBS, and cytoplasmic extract was prepared. A Bio-Rad protein assay helped to determine albumin/protein concentration of bovine serum as a reference standard. Meanwhile, sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve equal protein amounts (50 μ*g*) and move them across membranes of nitrocellulose. The membranes were blocked with 5% skimmed milk for 30 minutes at room temperature and incubated for 120 minutes.

Next, the membranes were incubated with goat anti-mouse/anti-rabbit (secondary) antibody. A chemiluminescence substrate that was significantly enhanced was used to develop the membranes. An imaging system (LI-COR chemiluminescence, Lincoln, NE, USA) was used to examine the samples. Graphs of the densitometric band's intensities were developed using the Image Studio™ Lite software version 5 (LI-COR Biosciences, Lincoln, NE, USA).[24]

TUNEL assay

When the concentration of DON are high, it can lead to hepatic necrosis and apoptosis.[25] Accordingly, this study assessed also the ability of RGN to curtail apoptosis induced by DON in hepatic cells using the TUNEL assay.

HepG2 cells at the logarithmic growth stage were loaded in a 6-well plate and supplemented with DON or RGN. The medium was then removed, and the cells were cleaned with PBS and processed with 4% paraformaldehyde for 20 min. The paraformaldehyde was removed, the cells were re-washed with PBS, and they were incubated with TUNEL reagent (11684817910, Roche, Mannheim, Germany). After washing with PBS, cells were counterstained with 0.1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Finally, the cells were examined under a Leica D6000 fluorescence microscope (Leica, Wetzlar, Germany). All morphometric

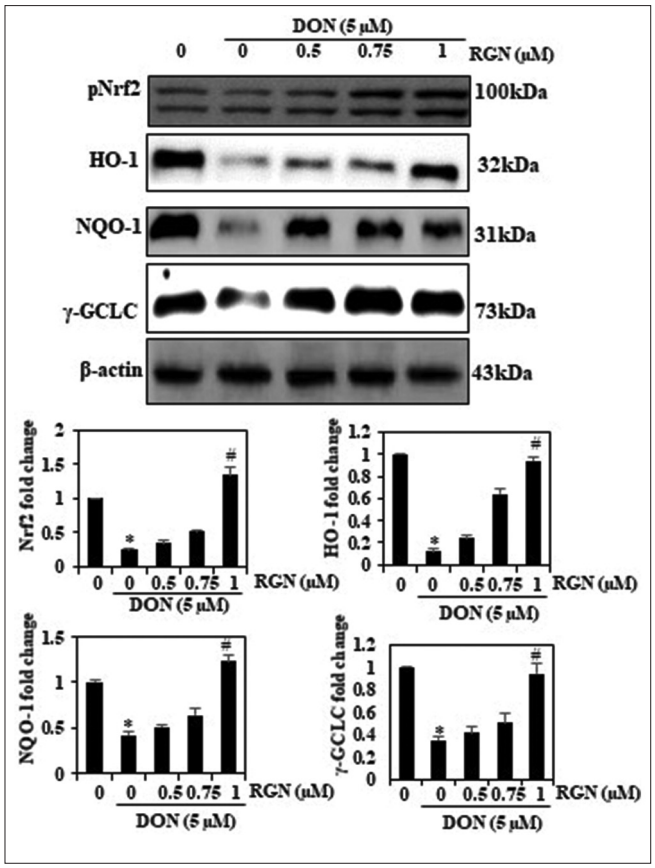

Figure 2: RGN induces Nrf2 expression in DON-treated HepG2 cells. Membranes were investigated using anti-Nrf2, anti-γ*GCLC*, and anti-HO-1 antibodies. Data are mean ± SD of values (n = 3), and **P* < 0.05 represents key differences compared to DON alone. #*P* < 0.05 for RGN compared with the DON treatment groups

studies were executed three times. TUNEL-positive cells were detected as brilliant green, and the cell nuclei were detected through UV light microscopy at 454 nm. Images were obtained through microscopy (200× magnification), and a Leica D6000 fluorescence microscope was used for measurement (Leica, Wetzlar, Germany).

RT-PCR

HepG2s cells were treated using DON and/or RGN for 24 hours. The conversion of RNA into cDNA was done using Taq polymerase and superscript reverse transcriptase through the reverse chain reaction of transcription polymerase. RT-PCR was used to analyze the relative expression of Nrf2. A ViiA-7 system (Applied Biosystems, Foster City, CA, USA) and an SYBR Green system were used in conjunction to carry out RT-qPCR. The mRNA expression of all genes was converted to β-actin expressions. The cycle threshold (Ct) value was used to calculate the fold change in several groups.

Statistical analysis

One-way analysis of variance followed by Tukey's post hoc test analysis was used for inter-group comparison.

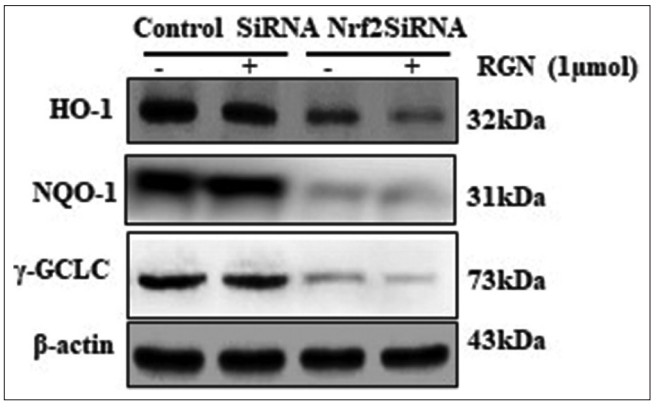

Figure 3: Effect of Nrf2 knockdown on RGN-mediated HO-1, NQO-1, and γ-GCLC expression in HepG2 cells. (a) HO-1, NQO-1, and γ-GCLC protein after carrying out western blot analysis. Data are represented as the mean ± SD of values (*n* = 3), and *P* < 0.05 represents key differences compared to DON alone. #*P* < 0.05 for RGN compared with the DON treatment groups

Results were expressed as mean ± SD, and $P < 0.05$ was considered significant.

RESULTS

Ruscogenin inhibits DON-induced cell death

The DON-exhibited cytotoxicity against HepG2 cells at varying times and doses are shown in Figure 1a. The toxicity of RGN to HepG2 cells was not significant even at a dose of 5 μmol [Figure 1b]. Significant protection against DON-induced cell death was noted in cells preincubated with DON (5 μmol) and RGN (1 μmol) [Figure 1c].

Ruscogenin inhibits deoxynivalenol-induced TNF-α and COX-2 expression and reactive oxygen species production

DON stimulation resulted in overexpression of both TNF-α and COX-2, but the use of RGN suppressed their expression in a dose-dependent manner [Figure 1d], indicative of inhibitory actions by RGN against pro-inflammatory cytokines induced by DON. In addition, RGN pretreatment significantly reduced DON-induced ROS formation in a dose-dependent manner [Figure 1e].

Ruscogenin activates Nrf2 signaling proteins

In the Western blot analysis, Nrf2 and antioxidant proteins were found to have lower expression (γ-GCLC, NQO-1, and HO-1) after the exposure of HepG2s cells to DON. However, RGN (1 μmol) significantly (dose dependent) reversed this trend, thereby upregulating the expression of Nrf2 and its antioxidant proteins [Figure 2].

Nrf2 knockdown attenuates ruscogenin's protection of HepG2 cells under oxidative stress

There were markedly reduced protein expression levels of HO-1, NQO-1, and γ-GCLC following transfection with

si-Nrf2 in the DON/RGN treatment group compared with that in the control group [Figure 3].

Ruscogenin upregulates mRNA NQO-1, HO-1, and Nrf2

HepG2 cells pretreated with DON (5 μmol) or RGN (0-1 μmol) showed high mRNA levels of HO-1/NQO-1/HO-1/Nrf2 in the RT-PCR evaluation [Figure 4]. The expression remained considerably higher at 1 μmol RGN. These results suggest that RGN activates Nrf2 signaling with DON-induced oxidative stress in a dose-dependent manner.

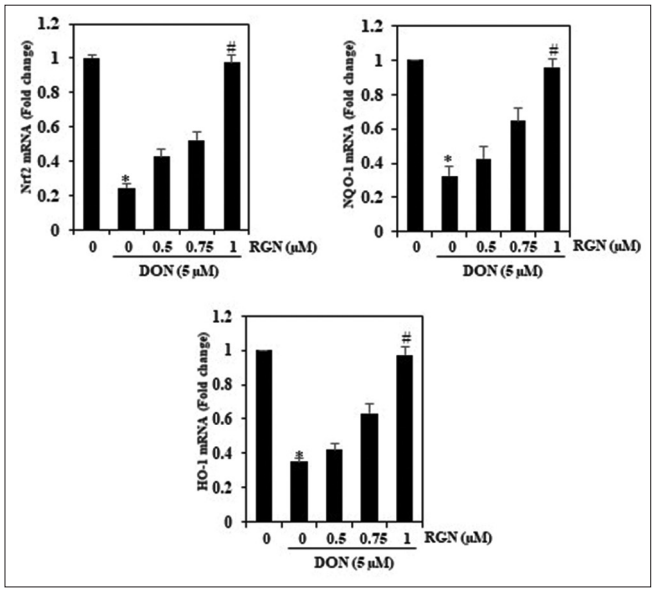

Figure 4: RT-PCR was used to assess the mRNA level of Nrf2, NQO-1, and HO-1. Data are represented as the mean ± SD of values (n = 3), and P < 0.05 represents key differences compared to DON alone. #P < 0.05 for RGN compared with the DON treatment groups

Ruscogenin activates PI3/AKT signaling

Western blotting was used to evaluate the ability of RGN to curtail oxidative stress induced by DON through the activation of pI3K/pAKT signaling pathway. The pI3K/pAKT signaling pathway was downregulated in cells treated with DON (5 μM) alone, but considerably upregulated in a dose-dependent manner when treated with DON and RGN [Figure 5a].

Nrf2 activation by ruscogenin caused by signaling cascades of PI3K/AKT in hepatic cells

To determine the important pathways of signaling concerning the activation of Nrf2 induced by RGN, PI3K/AKT inhibitors were used to treat HepG2 for half an hour, which was followed by RGN treatment (1 μM). The Nrf2 activation was found to be significantly suppressed in HepG2 cells by PI3K/AKT inhibitors [Figure 5b], indicating that the effects of RGN are dependent on the AKT-Nrf2/PI3K signaling.

Ruscogenin inhibits DON-induced apoptosis

The TUNEL assay data demonstrate that RGN has the ability to curtail hepatotoxicity induced by

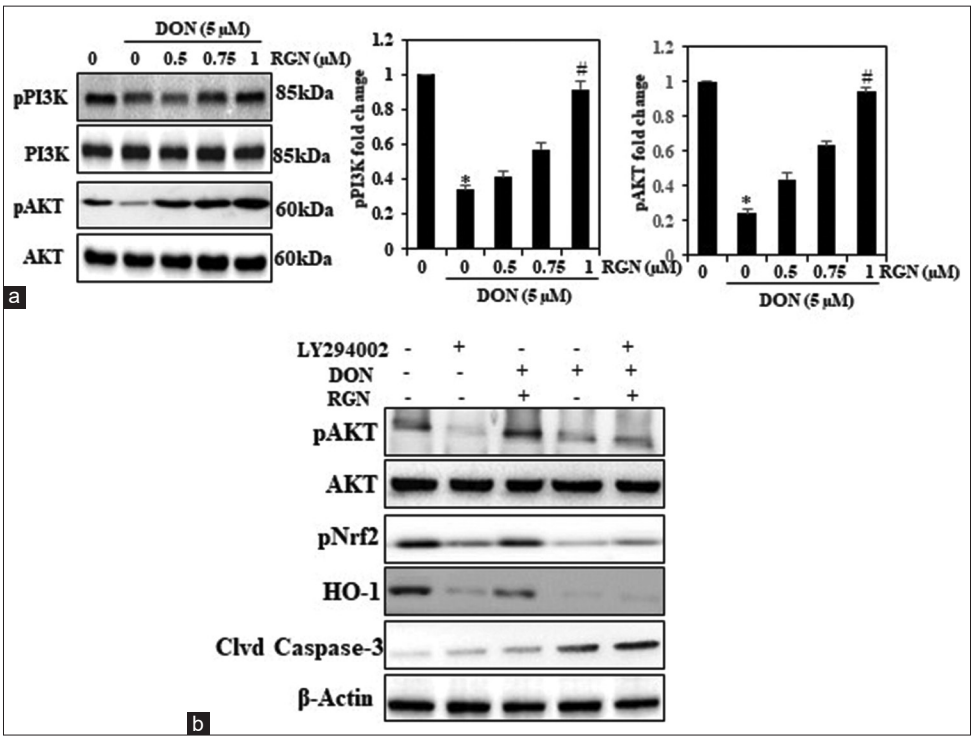

Figure 5: PI3/AKT-mediated Nrf2 signaling enhanced by RGN. (a) pAKT and pPI3K proteins after carrying out western blot analysis. (b) The pAKT, Nrf2, HO-1, and cleaved caspase3 were traced by western blotting. Data was represented as the mean ± SD of values (n = 3), and P < 0.05 represents key differences compared to DON alone. On the other hand, #P < 0.05 and RGN compared with the DON treatment groups

DON [Figure 6a]. The expression of Bcl2 was significantly reduced, whereas those of cleaved apoptosis marker protein levels were increased. After RGN treatment, there was a marked reversal in the upregulation of cleaved protein levels and Bcl2's downregulation, suggesting the ability of RGN to suppress apoptosis induced by DON in HepG2 cells [Figure 6b].

DISCUSSION

A growing body of research suggests that the study of trichothecenes is important for both public health and agriculture.[2,26] DON is a mycotoxin that is detected frequently in both cereals and the products derived from them. Over the past few decades, DON's chronic/acute toxicity has been studied extensively in many studies carried out worldwide.[27] In the current study that aimed at determining how RGN treatment alters DON-induced stress in HepG2 cells, it was found that while DON resulted in oxidative damage, RGN pretreatment caused this impact to be majorly inhibited [Figure 7]. The findings of this study are coherent with those reported previously.[28-30]

pPI3K and pAKT inhibitions have been found to exacerbate inflammation and increase the likelihood of hepatic cancers. This is attributed to the fact that AKT's hepatic elimination exacerbates damage to the liver and causes hepatocellular carcinoma and inflammation.[25] The signaling pathway of PI3K/AKT plays a key role in regulating Nrf2 signaling.[31-33] Hence, we examined the potential of RGN-induced Nrf2 signaling (mediated by PI3K/AKT). In this study, while DON alone downregulated the PI3K/pAKT signaling pathway, treatment with DON and RGN considerably upregulated their activation in a dose-dependent manner. Therefore, RGN likely confers protection against the oxidative stress induced by DON through this pathway. Furthermore, major changes were not found in AKT protein levels, which suggests that enhanced AKT protein phosphorylation contributes to RGN-induced Nrf2 signaling.

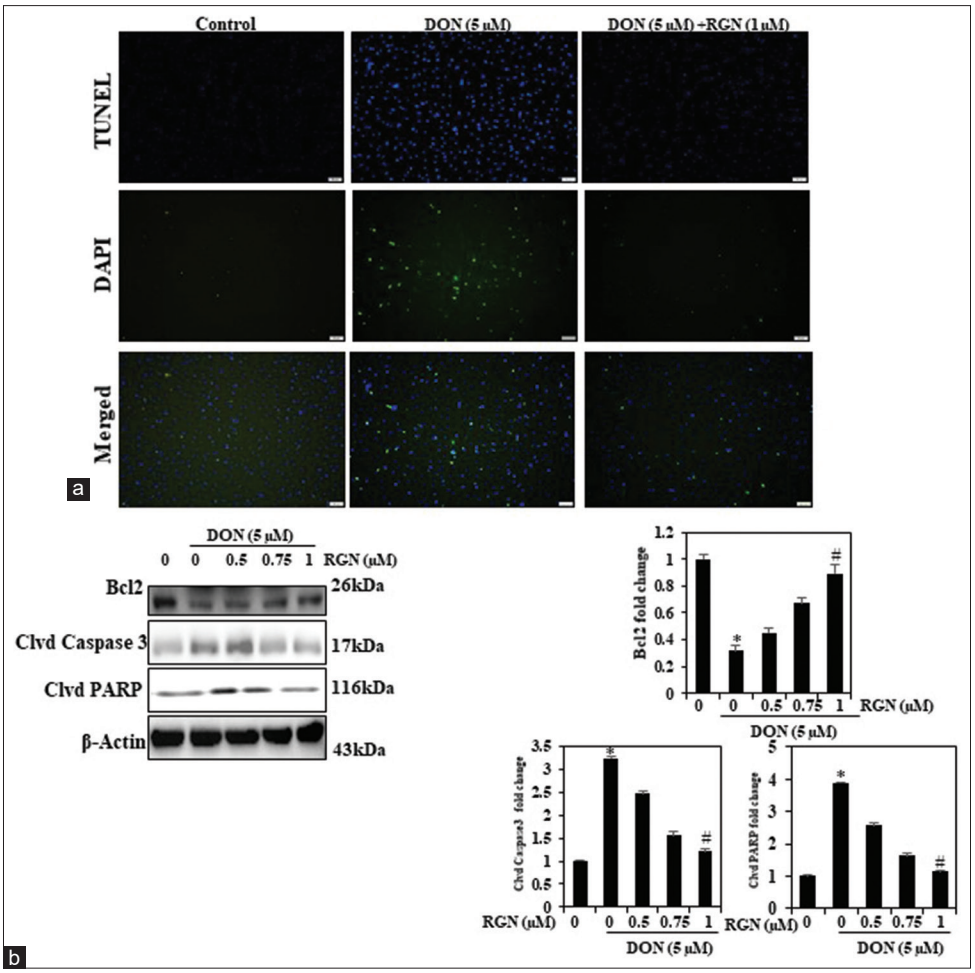

Figure 6: RGN suppresses DON-induced apoptosis in HepG2 cells. (a) TUNEL assay results. (b) Analysis of Bcl-2, cleaved caspase-3, and cleaved PARP by western blot. Data are denoted as the mean ± SD of values (n = 3), with *P < 0.05 denoting major differences compared to DON treatment alone. #P < 0.05 for RGN compared with the DON treatment groups

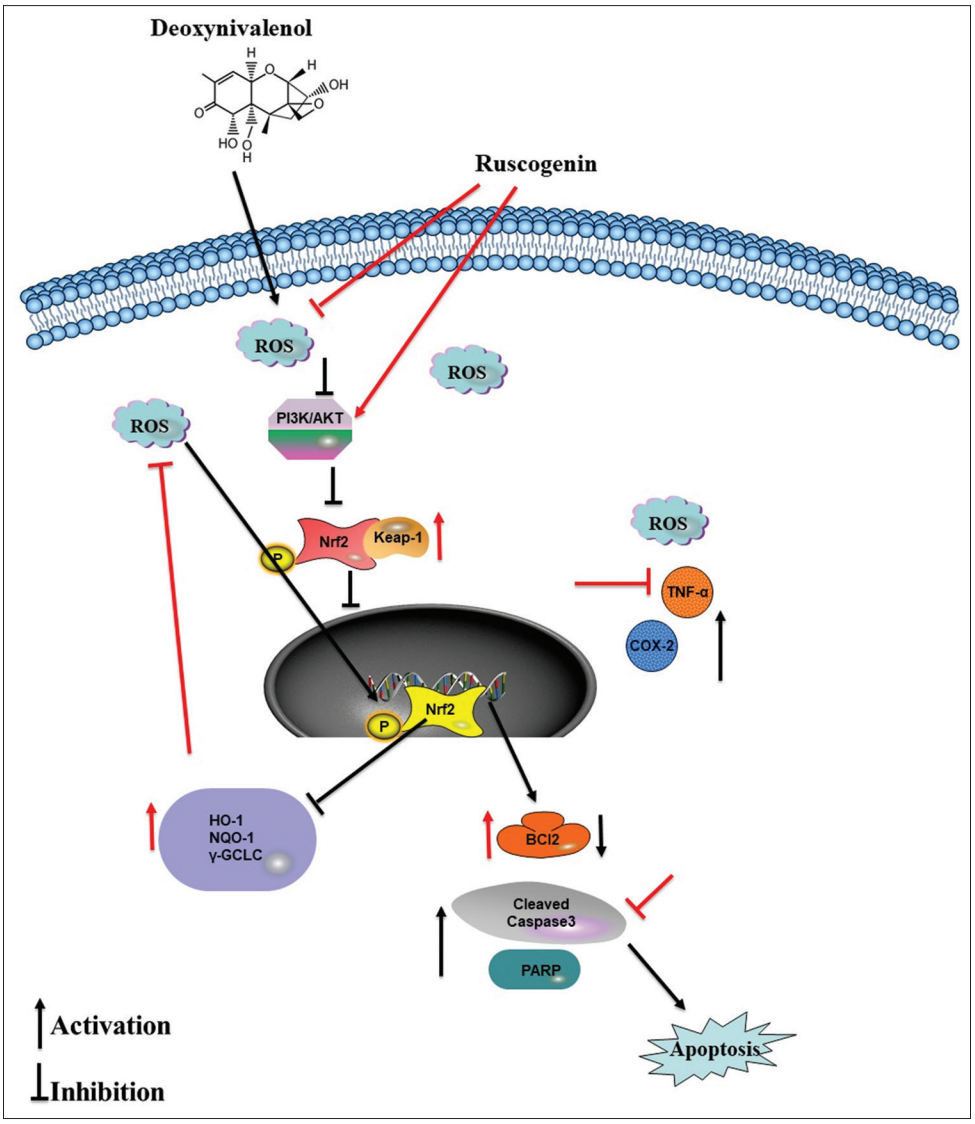

Figure 7: Schematic representation of RGN conferring protection against the DON induced hepatic injury

A PI3K/AKT inhibitor and RGN were used to treat HepG2s to determine the pathway involved. Our results show that the signaling was blocked by the PI3K/AKT signaling pathway in the presence of RGN. However, some scholars state that regardless of oxidative stress, Nrf2-ARE activation can be modulated by kinase pathways such as PI3K, which can mediate the activation.[1,30] The Nrf2 protein expression was then assessed using Western blotting to ascertain if RGN is capable of upregulating HO-1 by activating Nrf2 and isolating proteins. The findings show that RGN treatment did enhance Nrf2 protein expression significantly, which is suggestive of the potential of RGN in stimulating HO-1 expression through the Nrf2 signaling regulation.

Nrf2 knockdown cells were used to ascertain whether curtailing signaling regulated by Nrf2 reduced RGN-induced HO-1 expression. Nrf2 inhibition was found to be strongly affected by HO-1 expression triggered by RGN. Thus, RGN impacts the functions of antioxidants through the upregulation of HO-1 expression. RGN's mechanisms of action against cytotoxicity triggered by DON were also examined. According to the findings, RGN was able to mediate gene alterations by DON, which were partially reversed in cleaved PARP/cleaved caspase-3/Bcl-2. DON is also capable of inducing death of HepG2 cells through the instigation of cell apoptosis. Therefore, by blocking the apoptotic pathway, RGN potentially offers protection against the damage done by DON.

CONCLUSION

This is the first study to show that RGN pretreatment can ameliorate oxidative stress induced by DON in

human hepatic cells. The likely mechanism of action by which RGN confers protections is through enhancing the antioxidant capacity while leveraging the PI3K/AKT pathway to activate Nrf2 signaling.

Data availability statement

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Peer review

This article was peer-reviewed by two independent and anonymous reviewers.

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Conflicts of interest

There are no conflicts of interest.

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