Research Article

Protective Effects of Crocetin against Radiation-Induced Injury in Intestinal Epithelial Cells

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Background and Aims. Treatment options for radiation-induced intestinal injury (RIII) are limited. Crocetin has been demonstrated to exert antioxidant, antiapoptotic, and anti-inflammatory effects on various diseases. Here, we investigate the effects of crocetin on RIII in vitro. Materials and Method. IEC-6 cells exposed to 10 Gy of radiation were treated with different doses of crocetin (0, 0.1, 1, 10, and 100 μM), and cell viability was assessed by CCK-8. The levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA), myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) in culture supernatants were measured using colorimetric and ELISA kits, respectively. Cellular apoptosis was evaluated by Annexin V/PI double staining. Results. Crocetin dose-dependently improved the survival of irradiated IEC-6 cells with the optimal dose of 10 μM, as indicated by the reduction of cellular apoptosis, decreased levels of MDA, MPO, and proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ), and increased activities of antioxidative enzymes (SOD, CAT, and GPx). Conclusion. Our findings demonstrated that crocetin alleviated radiation-induced injury in intestinal epithelial cells, offering a promising agent for radioprotection.

1. Introduction

Radiation-induced intestinal injury (RIII) is a common complication of radiation therapy in patients with abdominal or pelvic malignancies, which seriously affects the quality of life and even leads to substantial mortality [1, 2]. Exposure of the small intestine to radiation may produce a large amount of free radicals and epithelial cell apoptosis, which cause impaired barrier function, followed by inflammatory response and even septicemia [3, 4]. Although RIII seriously affects the efficacy of abdominopelvic radiotherapy, there are no therapeu tic agents available to attenuate the intestinal toxicity of radiation [5].

Radioprotectors targeting oxidative damage and inflammatory reaction have been studied for decades with limited success, because of either the limited protective effect or inevitable toxicity [6]. In addition, previous studies have showed that some radioprotective agents had the risk of tumorigenesis, hampering their clinical application [6, 7].

Crocetin, an active constituent of saffron (Crocus sativus L) stigma, belongs to the large family of carotenoids [8]. Accumulated evidences have demonstrated that crocetin exerted...
beneficial effects on injured tissue [9, 10] and tumor cells
[11, 12]. It has been reported that crocetin attenuated
TNBS-induced colitis in mice by reducing inflammatory cyto-
kins and lipid peroxidation [13]. A previous study has also
proved that crocetin treatment protected against burn-
induced intestinal injury via inhibiting oxidative stress and
inflammatory response [14]. Additionally, crocetin could
inhibit the growth and metastasis of tumor cells both in vitro
and in vivo [15–17]. However, the potential role of crocetin
on RIII has not been reported. In this study, we aimed to
investigate whether and how crocetin protected against RIII.

2. Materials and Methods

2.1. Cell Culture. Rat intestinal epithelial IEC-6 cells were
obtained from the American Type Culture Collection (ATCC,
Manassas, VA, USA). Cells were maintained in high-glucose
Dulbecco’s modified Eagle’s medium (Sigma, St Louis, MO,
USA; no. D5796) with 10% fetal bovine serum, 1% penicillin/-
streptomycin, and 0.1 U/mL recombinant human insulin at
37 °C in a humidified atmosphere containing 5% CO₂. The
culture medium was changed every 2 or 3 d. The cells were
passaged as they grew to 70–80% confluence, and cells before
20th passages were used for the experiments.

2.2. Irradiation and Crocetin Treatment. The radiation pro-
cedure was performed according to our previously described
protocols [4]. Briefly, IEC-6 cells were exposed to 10 Gy doses of
radiation using a linear accelerator (Siemens PRIMUS) at a
dose-rate of 300 cGy/min. IEC-6 cells were seeded into 96-
well plates at a density of 1 × 10⁴ cells/well and grown to
70%–80% confluence prior to experiment. After 10 Gy radia-
tion, IEC-6 cells were replaced with serum-free DMEM-F12
medium and subsequently treated with different doses of croce-
tin (0, 0.1, 1, 10, and 100 μM, MP Biomedicals, Santa Ana,
CA, USA; CAS no.: 27876-94-4), then incubated for 24 h at
37 °C. After 24 h incubation, the culture medium was col-
lected for biochemical assay and ELISA and then replaced
with new fresh serum-free medium for subsequent condition
of IEC-6 cells. To determine the most effective concentration
of crocetin in the following experiments, cell viability was
assessed daily for the next 7 days after radiation. Further
studies were performed at the most effective concentration
to improve cell viability.

2.3. Cell Viability Assay. The viability of IEC-6 cells was
assessed by CCK-8 assay, and all the steps followed the man-
facturer’s instruction (Dojindo Laboratories, Kumamoto,
Japan; no. CK04). IEC-6 cells were cultured in 96-well plates
with a density of 1 × 10⁴ cells/well for 24 h. After 10 Gy radia-
tion and treatment with different doses of crocetin for 24 h,
10 μL of CCK-8 was added to each well and for incubation for
another 1 h at 37 °C. Cell viability was measured daily for 7
consecutive days after radiation. Absorbance of each well
was determined at 450 nm using a Multiskan Spectrum
(Thermo Fisher, CA, USA). The experiment was independ-
dently repeated at least three times.

2.4. Biochemical Measurements. Malondialdehyde (MDA,
Beyotime Institute of Biotechnology, Shanghai, China; no.
S0131) levels, superoxide dismutase (SOD, Abcam, Cam-
bridge, MA, USA; no. ab65354) activities, catalase (CAT,
Sigma, St Louis, MO, USA; no. CAT100) activities, glutathi-
one peroxidase (GPx, Beyotime Institute of Biotechnology,
Shanghai, China; no. S0056) levels, and myeloperoxidase
(MPO, Abcam, Cambridge, MA, USA; no. ab105136) activi-
ties in the cell culture supernatants were measured at 1, 3, 5,
and 7 d after radiation using commercial assay kits, respec-
tively, according to the manufacturer’s protocols.

2.5. Cell Apoptosis Assay. Cell apoptosis was detected at 1, 3,
5, and 7 days after radiation using Annexin V-FITC/PI Apo-
ptosis Detection Kit (BD Biosciences, San Diego, CA, USA)
based on our previously described procedures [18]. Briefly,
IEC-6 cells were plated in 6-well plates at a concentration
of 1 × 10⁵ cells/well. The cells in all groups were incubated
and then harvested at 1, 3, 5, and 7 d after treatment of croce-
tin, washed with PBS twice, resuspended in binding buffer,
and stained with Annexin V and propidium iodide (PI) for
10 min at room temperature in the dark. Annexin V fluores-
cence was measured using a flow cytometer (BD Biosciences),
and the membrane integrity of the cells was simultaneously
assessed by the PI exclusion method.

2.6. Cytokine Assay. Proinflammatory cytokines tumor necro-
sis factor-α (TNF-α, R&D Systems, Minneapolis, MN, USA;
no. PMTA00B), interleukin-1β (IL-1β, RayBiotech, Peachtree
Corners, GA, USA; no. ELM-IL1b-1), and interferon-γ (INF-
γ, R&D Systems, Minneapolis, MN, USA; no. PMIF00) levels
were obtained from the cell culture supernatants at 1, 3, 5,
and 7 days after radiation and were measured using ELISA kits
according to the manufacturer’s instructions.

2.7. Statistical Analysis. The differences of all measured
parameters among groups were analyzed by one-way analysis
of variance followed by Student-Newman-Keuls- (SNK-) q
test and between two groups by Student t-test. All analyses
were performed with SPSS statistics package (IBM SPSS,
Chicago, IL, USA). Data were considered statistically signi-
ficant for P < 0.05.

3. Results

To evaluate the therapeutic mechanisms of crocetin in
radiation-induced intestinal injury (RIII), we established
in vitro experimental systems (Figure 1). To determine the
optimal concentration of crocetin on irradiated IEC-6 cells,
the cell viability of each group was tested by CCK-8 assay.
The cell viability of IEC-6 cells was significantly decreased
after radiation (Figure 2(b)), whereas treatment with crocetin
at concentrations of 0.1 μM, 1 μM, and 10 μM improved the
survival of irradiated IEC-6 cells in a dose-dependent man-
ner with the maximal effect achieved at 10 μM (Figures 2(a)
and 2(b)). In contrast, 100 μM of crocetin showed a decrease
on the cell viability of irradiated IEC-6 cells compared to that
of the irradiated group (Figures 2(a) and 2(b)). According to
the results, 10 μM was the most effective dose of crocetin
to improve the viability of irradiated IEC-6 cells, which was
used for subsequent experiments.
3.2. Crocetin Attenuated Oxidative Stress in Irradiated IEC-6 Cells. To investigate the effect of crocetin on oxidative stress, we examined the levels of SOD, GPx, CAT, and MDA in culture supernatants by colorimetric assays. While radiation led to increased level of MDA, this increase was alleviated by crocetin ($P < 0.05$). Conversely, treatment of irradiated IEC-6 cells with crocetin significantly elevated the activities of endogenous antioxidant enzymes (SOD, GPx, and CAT), compared to the irradiated group (Figures 3(a), 3(c), and 3(d), $P < 0.05$). These data suggested that crocetin exerted an antioxidant effect in irradiated IEC-6 cells.

3.3. Crocetin Ameliorated Apoptosis in Irradiated IEC-6 Cells. We further evaluated the effect of crocetin on apoptosis of irradiated IEC-6 cells by Annexin V/PI double staining. The percentage of apoptotic cells increased after radiation compared to the control group (Figure 4(b), $P < 0.05$), whereas crocetin dramatically reduced the apoptosis of irradiated IEC-6 cells on day 3 and day 5 ($P < 0.05$), with less effects on day 7 (Figures 4(a)–4(c)). These results indicated that crocetin reduced radiation-induced intestinal epithelial apoptosis.

3.4. Crocetin Inhibited Inflammation in Irradiated IEC-6 Cells. To explore the effect of crocetin on inflammatory response in irradiated IEC-6 cells, the levels of proinflammatory cytokines in culture supernatants were assessed. Exposure to radiation remarkably increased the levels of TNF-$\alpha$, IL-1$\beta$, and IFN-$\gamma$, while administration of crocetin dramatically
decreased this effect (Figures 5(a)–5(c), \( P < 0.05 \)). Consistent with the results of proinflammatory cytokines, crocetin significantly suppressed MPO activity (Figure 5(d), \( P < 0.05 \)), suggesting crocetin attenuated radiation-induced inflammation in IEC-6 cells.

### 4. Discussion

Though agents ameliorating radiation-induced damage by reducing oxidants stress and inflammation may exert protective effects against RIII, the potential toxicity and tumorigenicity must be addressed before their clinical application [6]. In contrast, crocetin could be an alternative radioprotector for RIII with low toxicity [19, 20] and antitumor properties [11, 21]. In our study, we demonstrated the protective effects of crocetin against radiation-induced injury in intestinal epithelial cells and the underlying mechanisms could be attributed to inhibition of oxidative stress, cellular apoptosis, and inflammatory response, suggesting a safe and effective strategy for RIII.

There are some important discoveries in our work. First, the protective effects of crocetin in different concentrations on irradiated IEC-6 cells were investigated. In this study, we demonstrated that lower concentrations (0.1 \( \mu \text{M}, 1 \mu \text{M}, \) and \( 10 \mu \text{M} \)) of crocetin improved the survival of irradiated IEC-6 cells in a dose-dependent manner, showing the most pronounced effect at the dose of 10 \( \mu \text{M} \). Consistent with our findings, Yoshino et al. found that crocetin at 1–10 \( \mu \text{M} \) protected HT22 cells against \( \text{A}_\beta_{1-42} \)-induced neuronal cell death [22]. Conversely, it was reported previously that high doses of crocetin exerted cytotoxic effects on healthy monocytes and Alzheimer’s disease monocytes [23]. Our study also found that 100 \( \mu \text{M} \) of crocetin decreased cell viability whereas no cytotoxicity was observed at 0.1–10 \( \mu \text{M} \), suggesting the safe concentration of crocetin should be lower than 100 \( \mu \text{M} \). These findings suggested that 10 \( \mu \text{M} \) was relatively
a safe and effective dose of crocetin to protect irradiated IEC-6 cells.

Second, our study demonstrated the mechanisms of crocetin on RIII. Previous studies showed that crocetin exerted beneficial effects on tissue regeneration by reducing oxidative stress, inhibiting cellular apoptosis, and attenuating inflammatory response [24–26]. Recently, a study further investigated that crocetin protected ultraviolet A radiation-induced skin damage by reducing oxygen species production and cellular apoptosis [27]. Similar with these studies, we observed that crocetin inhibited oxidative stress, the occurrence of apoptosis, and inflammation in irradiated IEC-6 cells, suggesting crocetin could attenuate intestinal toxicity induced by radiation.

Though some substances have shown variable degrees of radioprotective properties, the application of most agents is hindered by toxicity and narrow therapeutic time windows [28]. Crocetin has been reported to treat a wide range of diseases with low toxicity [19, 20]. Milajerdi et al. suggested that LD_{50} values of saffron stigma extracts containing crocetin could be very higher than the therapeutic dose [29]. A clinical study also reported that no adverse changes in volunteers were observed after crocetin was administrated at the dose of 37.5 mg/d for 4 weeks [30]. Moreover, crocetin could inhibit the proliferation and invasion of various tumor cells including intestinal cancer [31]. Kim et al. have demonstrated that crocetin could increase the death of HCT-116 colorectal cancer cells [11]. Ray et al. have also demonstrated that crocetin could induce p53-mediated cell death by p73-mediated FAS-FADD-caspase-8 activation and BID cleavage in colorectal cancer cells [32]. As RIII commonly occurs in patients with abdominopelvic malignancies receiving local radiation therapy, crocetin represents a promising therapy to attenuate radiation-induced injury of intestine and, at the same time, inhibit tumor growth. However, the possible optimal doses in vivo still need to be further studied.

This study has potential limitations. First, the effect of crocetin on tumor cell lines after radiation was not studied because previous studies have reported the antitumor effects

![Figure 4: Crocetin ameliorated apoptosis in irradiated IEC-6 cells. (a) Apoptosis of IEC-6 cells was detected by flow cytometry after Annexin V/PI staining 3 days after radiation. The left upper quadrant contains necrotic cells (%); the upper right quadrant contains late apoptotic cells (%); the lower left quadrant contains live cells (%); and the lower right quadrant contains early apoptotic cells (%). (b) The percentage of total apoptotic cells and dead cells was calculated on day 3 after radiation. (c) Apoptotic ratio of IEC-6 cells was detected by Annexin V/PI double staining at 1, 3, 5, and 7 days after radiation. Data were expressed as mean ± SD of three independent experiments. *P < 0.05 compared to the control group, **P < 0.01 compared to the IR group. IR group: irradiation group, IEC-6 cells exposed to 10 Gy of radiation. Control group: IEC-6 cells without irradiation or crocetin.](image-url)
of crocetin on various tumor cells [11, 31]. Second, the solubility and the bioavailability of crocetin require optimization before being used as an effective radioprotective agent [12]. This problem may be solved with cyclodextrins or similar molecules. For example, Wong et al. suggested that crocetin-γ-cyclodextrin inclusion complex could enhance the solubility, bioavailability, and applicability of crocetin [33]. Puglia et al. showed that solid lipid nanoparticles containing crocetin improved its solubility, stability, and pharmacokinetic properties, offering an appropriated approach to resolve this issue [34]. Third, this is a study in cell model only providing preclinical clues for radioprotection of crocetin; more studies are needed to demonstrate its effects on RIII in animal models and further in clinical studies.

5. Conclusions

In conclusion, the present study suggests that crocetin could be an attractive agent for RIII not only attenuating intestinal injury induced by radiation via inhibiting oxidative stress, cellular apoptosis, and inflammatory response but also improving the efficacy of cancer cure with potential antitumor effects.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

Authors’ Contributions

Chen Zhang, Kequan Chen, Jinghua Wang, Zhongwen Zheng, and Yujun Luo contributed equally to this work.

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