Research Article

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Naringin ameliorates 5-fluorouracil elicited neurotoxicity by curtailing oxidative stress and iNOS/NF-κB/caspase-3 pathway

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Abstract: This study evaluated the protective effects of naringin (NRG) against 5-fluorouracil (5-FU)-elicited neurotoxicity. The animals were orally administered with NRG and subsequently injected with 5-FU. Injection of 5-FU caused depression in cerebral antioxidant enzymes, including glutathione peroxidase, superoxide dismutase, catalase, reduced glutathione and total protein levels, whereas malondialdehyde and acetylcholinesterase levels/activities were considerably upregulated. In addition, 5-FU-triggered cerebral pro-inflammation was shown via significantly increased levels of pro-inflammatory cytokines, inducible nitric oxide synthase, nuclear factor-κB, and caspase-3. Furthermore, necrotic and inflammatory histopathological lesions were observed in the cerebral tissues. Interestingly, the NRG administration considerably inhibited 5-FU-instigated cerebral oxido-inflammatory and apoptotic parameters in the treated animals. Thus, NRG could mitigate the neurotoxicity of 5-FU via the inhibition of oxido-inflammation and apoptosis in rats. These results suggested that NRG may have a relevant therapeutic importance in the management of 5-FU-elicited neurotoxicity.

Keywords: oxidative stress, naringin, 5-fluorouracil, cancer, apoptosis, inflammation

1 Introduction

The development of various anticancer drugs has provided succor for cancer patients and has significantly enhanced their quality of life and life expectancy [1]. However, the clinical application of most of these anticancer drugs is plagued with various unpleasant side effects that have significantly curtailed their clinical uses [1,2]. 5-Fluorouracil (5-FU) is a fluoropyrimidine (antimetabolite) neoplastic drug widely used as a standard treatment for breast and colorectal cancers [2,3]. Mechanistically, 5-FU inhibits thymidylate synthase and DNA synthesis and alters nucleotide sequences, thus interfering with the protein synthesis leading to cell apoptosis [4,5]. Despite the efficacy of 5-FU, it is undoubtedly associated with cardiotoxicity, diarrhea, myelosuppression, dermatitis, and neurotoxicity [6–8]. 5-FU-induced neurotoxicity is linked with brain toxicity, peripheral neuropathy, and hyperalgesia [6,9,10]. In addition, 5-FU has the potentials to cross the blood–brain barrier (BBB) to induce neurotoxicity via reactive oxygen specie (ROS) and oxidative stress, which subsequently leads to neuroinflammation and neuronal apoptosis [6]. Accordingly, since excess ROS and/or oxidative stress is the major culprit in 5-FU-induced toxicity, antioxidant agents may offer a neuroprotective effect against this toxicity. At the moment, there is no approved agent for mitigating or ameliorating 5-FU-induced toxicity.

Polyphenolic compounds, especially flavonoids, represent a major class of compounds widely distributed in nature, including plants, marine invertebrates, and microbes [11,12]. In plants, flavonoids make up huge phytoconstituents of fruits and vegetables and account for the various potent pharmacological effects of plants, especially their antioxidant and anti-inflammatory properties [12]. Naringin (NRG) is a flavanone glycoside found in citrus fruits, especially in grape fruit. It is more tolerable as a flavonoid than alkaloids in terms of toxicity profile and ability to cross the brain cells [13]. NRG has been associated with several excellent pharmacological properties, including antioxidant, anti-inflammatory, neuroprotective, and cardioprotective properties [14,15]. Meanwhile,
the therapeutic potentials of NRG on oxidative damage have been previously highlighted. NRG inhibits methotrexate-induced renal oxidative injury by modulating inflammation, oxidative stress, and apoptosis [14]. NRG was also shown to exhibit a hepatoprotective effect by suppressing oxidative mediators and fibrosis in cyclophosphamide-elicited toxicity [16]. NRG notably ameliorates neurotoxicity exerted by rotenone via the antioxidant pathway [17]. The protective effects of NRG in models of neurotoxicity has been established; however, the protective role of NRG against 5-FU is unexplored. Hence, this study investigated the protective potentials of NRG against 5-FU-induced neurotoxicity by exploring its effects on inflammatory cascade, oxidative damage, and apoptosis markers.

2 Materials and methods

2.1 Chemicals

NRG and 5-FU were supplied by Sigma-Aldrich (St Louis, MO, USA) and Celon Laboratories PVT Ltd (Telangana, India), respectively. Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Randox, UK, and Cusabio Technology, China.

2.2 Experimental animals

These experimental procedures used in this study were ratified by the ethics review committee of the institution (EBSU/REC/BMS/1808/02/001). The research was conducted in accordance with the stipulated guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty-four littermate male Wistar rats were used in this study and weighed between 150 and 190 g. The rats were kept in the cage (length = 45 cm, breath = 35 cm, and height = 40 cm) in a well-aerated room at standard temperature (25 ± 3°C), natural light/dark cycle, and humidity. All the animals fed on normal rat chow and clean tap water ad libitum. The animals were allowed to adapt to the new environment for 14 days before the commencement of this study.

2.3 Experimental design

Upon the completion of the acclimatization, simple randomization method was adopted to divide the rats into four groups (n = 6 rats/group); the vehicle-treated control were given normal saline for 10 days, the NRG-treated group were given 100 mg/kg of NRG per os (p.o.) daily for 10 days [18], the 5-FU group were given a single dose of 150 mg/kg of 5-FU intraperitoneal (i.p.) injection on the eighth day [19,20], and the NRG + 5-FU group were given 100 mg/kg of NRG p.o. daily for 10 days plus a single dose of 150 mg/kg of 5-FU i.p. on the eighth day.

The animals were anesthetized using diethyl ether and sacrificed by decapitation on the 11th day. The cerebrum was carefully harvested, washed with phosphate buffered saline (PBS), weighed instantly, and preserved frozen in sample bottles at −80°C.

Some parts of the cerebral tissues were homogenized in PBS (1:5) and centrifuged at 4,000 rpm for 10 min to acquire the supernatant that was used for biochemical analysis. The cerebrum index was determined by dividing the weight of each brain with body weight. The remaining cerebral tissues were kept in 10% formal saline for histopathological evaluation.

2.4 Acetylcholinesterase (AChE) activity measurement

The activity of the cerebral AChE was estimated using a previously reported method [21]. The yellow color absorbance produced in the reacting system of cerebral AChE (40 µL), acetylthiocholine iodide (0.07 mol/L, 20 µL), and 100 µL of dithiobisnitrobenzoate ion (DTNB reagent, 0.01 mol/L) was measured spectrophotometrically at 405 nm.

2.5 Total protein (TP) measurement

The TP content in the cerebral supernatant was estimated following the Bradford method [22].

2.6 Superoxide dismutase (SOD) activity

The SOD activity was determined based on a previously reported protocol [23]. SOD estimation was based on the transmutation of superoxide anion into hydrogen peroxide and oxygen. The absorbance was read at 420 nm.

2.7 Catalase (CAT) activity measurement

CAT was estimated colorimetrically using the protocol of Aebi [24]. The reaction mixture consists of 100 µL of the
supernatant, 0.5 mL of 10 mM PBS, and 0.4 mL of 0.2 M H$_2$O$_2$. The constituted mixture was incubated at 37°C for 60 s. Each unit of CAT decomposes 1 μM of H$_2$O$_2$ per minute.

2.8 Glutathione peroxidase (GPx) activity

The activity of GPx was evaluated using a previously reported protocol of Flohe and Gunzler [25]. The GPx analysis followed the glutathione oxidation by cumene hydroperoxide (1 mL of 20 mmol/L). The redox reactions of glutathione (GSH) in the presence of NADPH and glutathione reductase convert NADPH to NADP$^+$. The reduction in the absorption was determined at 340 nm by spectrophotometer.

2.9 Glutathione estimation

GSH was determined following the previous protocol [26]. The sample supernatant (0.4 mL) and 20% tricarboxylic acid (0.4 mL) were mixed and centrifuged for 20 min at 10,000 rpm. After centrifugation, 0.25 mL of the supernatant was mixed with 0.2 M phosphate buffer (pH 8.0) and 2 mL of 0.6 mM DTNB. The absorbance of the resulting solution was read at 412 nm.

2.10 Lipid peroxidation level

Malondialdehyde (MDA) was evaluated by the reaction of thiobarbituric acid reactive substances (TBARS) using Ohkawa et al.’s method [27]. The acidic medium reaction between thiobarbituric acid (1 mL of TBAR 0.67%) and MDA yielded a pink color solution. The absorbance was determined by spectrophotometer at 534 nm.

2.11 Determination of pro-inflammatory mediators and nitric oxide (NO)

The neuroinflammatory mediators were determined in the cerebral using rat ELISA technique. The assays were performed in consistent with instructions included in the manufacturer’s protocols. The levels of tumour necrosis factor alpha (TNF-α) (Cat No: CSB-E11987r), interleukin 1 beta (IL-1β) (Cat No: CSB-E08053r), interleukin 6 (IL-6) (Cat No: CSB-E04640r), and nuclear factor kappa B (NF-κB) (Cat No: CSB-E13539h) were evaluated using ELISA kits from Cusabio Technology, China. NO was determined spectrophotometrically based on the previously reported method [28]. The assay protocol involved the conversion of nitrate to nitrite in the presence of sulfanilic acid and N-(1-naphthyl) ethylenediamine. The absorbance of the deep purple azo dye was measured at 548 nm. The activity of inducible nitric oxide synthase (iNOS) (Cat No: CSB-E08326m) was estimated by ELISA kit.

2.12 Caspase 3 estimation

The level of caspase-3 (Cat No: CSB-E08857r) in the cerebral tissues was estimated via ELISA kit from Cusabio Technology, China.

2.13 Histopathological analysis

The 10% formalin-fixed cerebral tissues were dehydrated in graded alcohol, embedded in paraffin, and stained with hematoxylin & eosin (H&E) stain. The stained tissue sections were observed under a light microscope based on the established protocols [4]. The lesions in sections were scored semi-quantitatively following the previously established criteria [4].

2.14 Statistical analyses

The results were analyzed using Graph Pad Prism statistical package version 9.0. To compare the study groups, analysis of variance was used, followed by Tukey’s post hoc test. The results were displayed as mean ± standard deviation (SD). A significance level of $p < 0.05$ was considered.

3 Results

3.1 Effect on body and cerebral weights

It was observed that 5-FU insignificantly reduced the body weight of 5-FU group rats compared with the healthy control. However, the cerebral weight and index were notably decreased in the 5-FU group when compared to the healthy control group. NRG considerably ($p < 0.05$) improved the cerebral weight and index in the treated animals in comparison with the 5-FU group (Table 1).
3.2 Effect on oxidative stress markers

The exposure of the rats to 5-FU notably decreased the cerebral activities of CAT, SOD, and GPx as well as GSH levels when juxtaposed with the healthy control group, while MDA level was significantly increased. Nevertheless, pretreatment with NRG exerted notable increases in the activities/levels of antioxidant parameters followed by a marked decrease in the level of MDA when compared to the untreated group (Table 2).

3.3 Effect on TP and AChE

It was observed that the TP content was markedly decreased, while AChE activity in the cerebrum was markedly increased after 5-FU injection in comparison with the healthy control. Nevertheless, the oral dose of NRG remarkably abrogated the altered level and activity of TP and AChE, respectively, when juxtaposed with the untreated 5-FU group (Figure 1).

3.4 Effect on neuroinflammatory mediators

The cerebral levels of pro-inflammatory cytokines, including IL-6, IL-1β, TNF-α, and NO, were conspicuously increased in the 5-FU group relative to the healthy control animals (Figure 2). Conversely, it was observed that NRG prevented the cerebral pro-inflammatory cytokines surge in the NGR-treated group when juxtaposed to the untreated 5-FU group (Figure 2).

3.5 Effect on iNOS, NF-κB, and caspase-3

5-FU administration dramatically upregulated the cerebral expression of iNOS, NF-κB, and caspase-3 compared to the healthy control group (Figure 3). As a protective or curative agent, NRG inhibited cerebral inflammation and apoptosis, which provoked remarkable decreases in iNOS, caspase-3, and NF-κB expressions in the treated group compared to the 5-FU rat group (Figure 3).

3.6 Effect on cerebral histology

We observed the normal cerebral histological findings after gross examination of the cerebrum from the control group. Similar normal tissue structural findings were also confirmed in the NRG group. On the contrary, 5-FU induced fatty changes, inflammatory alterations, eosinophilic material, and neuronal necrosis in the 5-FU group. The aforementioned cerebral alterations were reduced by the administration of NRG in the NRG + 5-FU rat group (Figure 4).

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<th>Table 1: Effect of naringin on body weight, cerebrum weight, and index of 5-FU-intoxicated rats</th>
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5-FU: 5-Fluorouracil; values are mean ± SD (6 rats/group). *Significant when compared to control group in the same column (p < 0.05); #significant when compared to the 5-FU group in the same column (p < 0.05).

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<th>Table 2: Effect of NRG on cerebral SOD, CAT, and GPx activities (U/mg protein), and GSH and MDA levels (ng/mg protein) in 5-FU-intoxicated rats</th>
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5-FU: 5-fluorouracil; values are mean ± SD (6 rats/group). *Significant when compared to control group in the same column (p < 0.05); #significant when compared to the 5-FU group in the same column (p < 0.05).
4 Discussion

This investigation explored the neuroprotective effects of NRG against 5-FU-induced neurotoxicity. The dose adopted in this study has been found to be 10-fold lesser than the no-observed-adverse-effect-level (1,250 mg/kg/day) for NRG in rats according to Li et al. [29]. NRG is a potent bioflavonoid from citrus fruits with immense neuroprotective,

**Figure 1**: Effect of NRG on cerebral levels of TP and AChE in 5-FU-intoxicated rats. NRG: naringin; 5-FU: 5-fluorouracil. Values are expressed as mean ± SD (n = 6). *Significant when compared to control (p < 0.05); #significant compared to the 5-FU group.

**Figure 2**: Effect of NRG on cerebral levels of inflammatory cytokines and NO in 5-FU intoxicated rats. NRG: naringin; 5-FU: 5-fluorouracil. Values are expressed as mean ± SD (n = 6). *Significant when compared to control (p < 0.05); #significant compared to the 5-FU group.
antioxidant, anti-inflammatory, and anti-apoptotic properties [30,31]. The results portrayed that NRG offered a potent protection against neurotoxicity induced by 5-FU by inhibiting lipid peroxidation and pro-inflammatory cytokines and offering antioxidant effect.

Several studies have indicated that memory decline is associated with cholinergic dysfunction, and neurotoxicity impairs cholinergic functions leading to cognitive disabilities [32]. In previous literature, it has been shown that chemotherapy-induced toxicity can incur deficits in cholinergic function and increases the activity of AchE [1,32]. In agreement with these previous studies, 5-FU significantly increased AchE activity, suggesting impaired neuronal transmission. Treatment with NRG attenuated AchE activity in the treated rats. In support, the effect of NRG on AchE activity was previously highlighted by Oladapo et al. [33], who showed that NRG mitigated AchE activity in stress-induced neurobehavioral deficits.

Excess ROS and/or oxidative stress is a consistent feature implicated in chemotherapy-induced neurotoxicity, including 5-FU-induced toxicity. Specifically, 5-FU is metabolized into active substances that ensue ROS production and subsequent oxidative stress [2,4]. Furthermore, the sustained ROS accumulation instigated by 5-FU can lead to the upregulation of several pro-inflammatory and apoptotic mediators that ultimately led to neuronal cell death [4,34]. However, SOD, CAT, GSH, and GPx are the hallmarks of antioxidant defense mechanisms employed by cells/ organs to deter oxidative damage, and these enzymes have been shown to be depleted in several models of 5-FU and anticancer drug-induced toxicity [34–37]. In this study, NRG supplementation notably increased cerebral GSH, SOD, CAT, and GPx and reduced lipid peroxidation marker, MDA, suggesting the antioxidant prowess of NRG. Oral administration of NRG leads to NRG metabolism by enzymes to yield a major metabolite naringenin, the main absorbable form in the intestine [29]. It could cross the BBB to exert its traditional antioxidant effect demonstrated in this study [15]. The beneficial antioxidant properties of NRG were also highlighted by Zhang et al. [15]. It was
Figure 4: Effect of NRG on histopathological examination of the cerebrum. The control and NRG groups show the normal cerebrum histology with normal neuron cells; the 5-FU group illustrates the vacuolation of neutrophil (black arrow), necrotic neuron (yellow arrow), and eosinophilic material (red arrow). The alterations were alleviated in the NRG + 5-FU group showing mild eosinophilic material (green arrow). H & E (×400). Values are expressed as mean ± SD (n = 6). *Significant when compared to control (p < 0.05); #significant compared to the 5-FU group.
reported that NRG suppressed ROS and MDA, while the serum SOD level was increased in chronic obstructive pulmonary disease animals [15].

Aside from the upregulated level of lipid peroxidation, 5-FU toxicity also resulted in increased cerebral levels of NO and iNOS. NO acts as an important messenger molecule in the CNS; however, at a very high concentration, it can act as a neurotoxic agent. ROS and reactive nitrogen species (RNS) can collectively alter the protein, lipid, and carbohydrate components of cells resulting in cell death [38,39]. Accumulating pieces of evidence have reported that chemotherapeutic agents, including 5-FU, can induce nitrosative/oxidative stress, which mediate the underlying mechanisms of toxicity [4,40]. Treatment with NRG reduced the NO and iNOS levels in the cerebral tissues of 5-FU-administered rats. This result agrees with a previous report that shows the NRG administration decreases the NO production in cyclophosphamide-induced erythrocytotoxicity [16].

Inflammation plays a pivotal role in the physiopathology of 5-FU-induced neurotoxicity. 5-FU toxicity increases systemic inflammation through cytokine-induced neuroinflammation instigated by the overgeneration of ROS. In addition, 5-FU can cross the BBB and activates microglia and astrocytes, which potentially results in the upregulation of NF-kB pathway and inflammatory cytokines release [41,42]. Growing number of reports have shown that 5-FU-mediated multiorgan toxicity occurs partly due to increased inflammatory responses [35,43,44]. In this regard, 5-FU induced significant neuroinflammation via NF-kB cerebral activation and increased cerebral levels of cytokines TNF-α, IL-1β, and IL-6. However, NRG showed a marked reduction in the expression of these inflammatory mediators, suggesting anti-inflammatory effects of NRG in this study. Moreover, the anti-inflammatory efficacy of NRG has also been highlighted in renal, hepatic, neuronal, and intestinal tissues [16,17,45].

Moreover, this study shows that 5-FU administration resulted in increased cerebral levels of apoptosis as indicated by higher level of caspase 3. Chemotherapy-induced multiorgan toxicity is associated with the induction of several pro-apoptotic-related genes [35,46]. Caspase 3 is a vital effector of apoptosis, and activation of caspases has been implicated in 5-FU-induced toxicity [47,48]. Furthermore, it has been shown that increased levels of ROS and oxidative stress enhance apoptosis, including increasing Bax and caspases [1,49]. As such, the inhibition of ROS, oxidative stress and inflammation may subsequently abort apoptosis. In this study, 5-FU-exposed animals showed significantly increased cerebral caspase 3 activity. By implication, 5-FU instigated cerebral apoptosis via ROS and oxidative stress pathways, which corroborate with the previous studies [47]. However, NRG treatment ameliorated caspase 3 activity, confirming the anti-apoptotic effects of NRG. These findings were supported by previous studies [50,51].

5 Conclusion

In conclusion, this study has deciphered that NRG substantially attenuated 5-FU elicited cerebral neurotoxicity by
impeding oxido-inflammatory and apoptosis markers. Furthermore, NRG significantly increased cerebral antioxidant enzyme activities, suppressed pro-inflammatory cytokines, and reduced AChE and caspase 3 activities. Collectively, as shown in the schematic diagram of Figure 5, these results suggested that NRG could have potentials as a nutraceutical for toxicities induced by chemotherapy.

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Conflict of interest: None declared by the authors.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on a reasonable request.

References


