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Original Article

5-fluorouracil-induced neurotoxicity in rat cerebellum granule cells involves oxidative stress and activation of caspase-3 pathway

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Abstract: 5-Fluorouracil (5-FU) is a widely used anticancer drug that acts by blocking DNA replication. Although the side effects of 5-FU are well documented, the mechanism of 5-FU-induced neurotoxicity remains unclear. The current study was performed to investigate the toxicity of 5-FU to rat cerebellum granule cells (CGCs) and to elucidate the corresponding molecular mechanisms. We demonstrated that 5-FU exhibited significant cellular toxicity to CGCs, in a dose-dependent manner, and that it altered intracellular Ca^{2+} levels. The accumulation of intracellular reactive oxygen species in CGCs revealed that 5-FU also induced oxidative stress. Moreover, 5-FU-treated cells showed elevated caspase-3 activity. Furthermore, intraperitoneally administered 5-FU caused slight degenerative changes in the rat cerebellum granular layer. Taken together, these findings revealed that 5-FU substantially impaired the survival of CGCs by inducing oxidative stress and activating the caspase-3 apoptotic pathway.

Keywords: 5-fluorouracil, neurotoxicity, cerebellum granule cells, oxidative stress, caspase

Introduction

5-Fluorouracil (5-FU) is a commonly used chemotherapeutic agent that blocks DNA synthesis and replication via inhibition of thymidylate synthase and incorporation of 5-FU metabolites into RNA and DNA [1]. Presently, 5-FU is still a component of most of the currently applied regimens to treat solid cancers of the gastrointestinal tract, breast, head and neck, and pancreas [2]. Derivatives of 5-FU, including carmofur, tegafur, and capecitabine (prodrugs which are metabolized to 5-FU, their only active product *in vivo*), are also often used clinically as anticancer drugs [3-5].

Most anticancer agents that kill cancer cells also have a low therapeutic index, thus affecting a diverse range of normal cell types. This leads to a myriad of adverse side effects on multiple organ systems and may severely limit their activity. Such effects on normal tissues have been observed for almost all classes of anticancer drugs, including alkylating agents [6], antimetabolites [7], and even antihormonal agents [8]. Typical side effects of 5-FU include myelosuppression, nausea, vomiting, diarrhea, and stomatitis [9]. Previous studies have shown that 5-FU has the potential to induce toxicity in various tissues. Cardiotoxicity is a well-defined side effect of 5-FU, which often occurs as myocardial ischemia, and to a lesser extent, as cardiac arrhythmias, hyper- and hypotension, left ventricular dysfunction, and cardiac arrest mediated by multifactorial pathophysiological mechanisms [10]. Additionally, 5-FU was demonstrated to be genotoxic, as indicated by chromosomal damage in animals treated with 5-FU [11]. McQuade *et al*. reported that 5-FU induced gastrointestinal dysfunction and enteric neurotoxicity *in vivo* [12]. Furthermore, 5-FU was also found to cause hepatic damage from overproduction of free radicals and inflammatory mediators [13].

5-Fluorouracil not only causes adverse effects in primary organs where it accumulates, but also in secondary locations such as the central nervous system (CNS) upon systemic circulation [14]. Mustafa *et al*. have shown earlier that 5-FU readily crosses the blood-brain barrier (BBB) and affects spatial working memory and newborn neurons in the adult rat hippocampus. Moreover, there is some evidence that 5-FU can cross the BBB by simple diffusion and cause neurotoxicity including nystagmus, ataxia, dysarthria, and epilepsy [15, 16]. 5-Fluorouracil has also been associated with both acute and delayed CNS toxicities [17]. Acute CNS toxicities, such as pancerebellar syndrome and subacute encephalopathy with severe cognitive dysfunction, and delayed cerebral demyelinating syndrome reminiscent of multifocal leukoencephalopathy, have been increasingly reported following 5-FU therapies [18]. Similar neurologic syndromes, such as cerebellum dysfunction, encephalopathy, and multifocal inflammatory leukoencephalopathy, have also been observed in cancer patients treated with 5-FU derivatives [5, 19].

Although the phenomenon of 5-FU-induced neurotoxicity is well recognized, its mechanisms remain unclear and need to be elucidated. Given the large number of individuals treated for cancer, it is imperative to fully uncover the molecular mechanisms of 5-FU neurotoxicity to develop safer pharmacological strategies to treat cancer. Cerebellum granule cells (CGCs) possess similar characteristics as neural tissues and have been widely used as an *in vitro* model to elucidate the mechanism of action of a wide range of therapeutic agents [20]. In this study, we showed that 5-FU induced apoptosis in CGCs by stimulating calcium influx, oxidative stress, and caspase activation. Furthermore, the neurotoxicity of 5-FU was demonstrated by histopathological analysis using Sprague-Dawley rats in an *in vivo* model.

Materials and methods

Preparation of primary cell cultures

Primary cultured CGCs were isolated as described previously [21]. Dissociated cerebella from 7-day-old Sprague-Dawley rats were cultured in high-glucose Hank's balanced salt solution (GIBCO, Grand Island, NY, USA) and seeded at 5×10^5 cells/mL in 96-well plates, or at 1×10^6 cells/mL in 6-well plates (Corning Inc., Corning, NY, USA), which had been coated with poly-L-lysine (0.01%; Sigma-Aldrich, St. Louis, MO, USA). Subsequently, CGCs were cultured in Dulbecco's modified Eagle's medium/F12 (HyClone, Logan, UT, USA) supplemented with 5% horse serum (GIBCO), 5% fetal bovine serum (GIBCO), 25 mM KCl (Sigma-Aldrich), 1% L-glutamine (HyClone), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO), under standard culture conditions (37°C, 5% CO_2). After 18-22 h, 10 μM arabinofuranosylcytosine (Fluca, St. Louis, MO, USA) was added to inhibit proliferation of non-neuronal cells. Cells were cultured for seven additional days until synapses formed.

Evaluation of cell viability

To assess the cytotoxicity of 5-FU on CGCs, cells were treated with increasing concentrations of 5-FU (0.01-100 μM). After 24 h, cell viability was determined by Alamar Blue (AB) assay. Fluorescence intensity was measured at 590 nm upon excitation at 530 nm by using a multi-well fluorometric reader (Thermo Scientific, Waltham, MA, USA). Dose-response curves were generated to calculate the half maximal inhibitory concentration (IC_{50}).

Measurement of intracellular free Ca^{2+} level

Cerebellum granule cells grown on plates were loaded with 5 μM Fura-2/AM dye (Sigma-Aldrich) for 30 min in PBS without Ca^{2+} and Mg^{2+} at 37°C and 5% CO_2. Cells were then treated with three different concentrations of 5-FU (0.1, 1, and 5 μM). The increase in intracellular Ca^{2+} level was expressed in terms of ratio of fluorescence intensity upon excitation at 340 and 380 nm (F340/F380), which is proportional to Ca^{2+} concentration. The F340/F380 ratio was measured at an emission wavelength of 510 nm every 50 s. The experiments were carried out in the dark.

Assessment of intracellular reactive oxygen species (ROS) accumulation

The production of intracellular ROS was measured spectrophotometrically by using the fluorescent probe 2,7-dichlorofluorescindiacetate (DCFH-DA), which passively enters cells and reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly,

CGCs were incubated with 100 μM DCFH-DA (Sigma-Aldrich) for 30 min at 37°C and 5% CO_2, before treatment with different concentrations of 5-FU (0.01-5 μM) for 1 h, 4 h and 12 h, respectively. Hydrogen peroxide (H_2O_2, 1 mM) was used as a positive control. DCF fluorescence was measured using a microplate spectrofluorometer (Thermo Scientific) at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Western blot

After treatment with different concentrations of 5-FU for 12 h or 24 h, CGCs were collected and lysed on ice for 30 min in RIPA lysis buffer containing a mixture of protease inhibitors (EDTA-free Protease Inhibitor Cocktail Tablets; Roche, Basel, Switzerland). CGC samples containing equal amounts of total proteins were analyzed by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Pall, Port Washington, NY, USA). For immunoblotting, the nitrocellulose membrane was incubated with TBS-T containing 5% non-fat milk for 1 h, and then blotted with an anti-caspase-3 primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After washing with TBS-T, the blots were incubated with horseradish peroxidase-labeled secondary antibody (goat anti rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 25°C. Finally, the bound antibodies were incubated with a signal substrate (Pierce, Waltham, MA, USA) and exposed to X-rays films (Kodak, Rochester, NY, USA).

Measurement of caspase-3 protein level

Intracellular protein levels of activated caspase-3 were measured using the Caspase-Glo® 3/7 assay according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, CGCs were exposed to various concentrations of 5-FU (0.01-5 μM) for 12 h or 24 h, prior to addition of the Caspase-Glo reagent. The activated caspase products were quantified using a luminometer (Thermo Scientific).

Animal treatment regimen

Male Sprague-Dawley rats (aged 6 weeks) were obtained from the Peking University Health Science Center. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Peking University. All animals were housed in the animal care facility in a temperature-, humidity-, and light-controlled environment with a 12 h light/dark cycle, and were given free access to standard laboratory diet and water. The rats were randomly divided into two groups of six rats each, and received 5-FU (20 mg/kg) in three consecutive injections every other day intraperitoneally; control rats received equal amounts of 0.9% saline. The body weight of rats was measured daily. The animals were sacrificed at the end of the experiment on day 7 (rats were first injected with 5-FU on day 1).

Histopathological analysis

Hematoxylin & eosin (H&E) staining and immunohistochemistry (IHC) evaluation were performed on paraffin-embedded sections of the cerebellums that were dissected on day 7. Rats were deeply anaesthetized by intraperitoneal injection of ketamine before perfusion fixation. Then, the cerebellum was immediately collected and cut into 5-μm-thick sections, and subsequently processed with H&E staining. After morphological screening, the paraffin embedded slides were submitted to IHC evaluation by deparaffinization, dehydration, antigen retrieval and blocking. A primary antibody to glial fibrillary acidic protein (GFAP) (Abcam, UK, 1:1000) was used for overnight incubation. Subsequently, sections were washed and incubated with a secondary horseradish peroxidase (HRP)-labeled antibody (goat anti rabbit IgG, Santa Cruz, USA, 1:5000) for 30 min. Then, the slides were washed and developed with the substrate (1% DAB and 0.3% H_2O_2, 1:1) for 5 min and counterstained with hematoxylin. The imagines were photographed with a laser scanning confocal microscope (Leica, TSC SP5, USA).

Statistical analysis

Results are expressed as the mean ± SD. The data were analyzed via Student's *t*-test or ANOVA. A P-value < 0.05 was considered statistically significant, while a P-value < 0.01 was considered highly significant.

Results

5-FU induced CGC cytotoxicity

To evaluate the cytotoxic effects of 5-FU on CGCs, we measured cell viability after 24 h exposure to 5-FU (0.01-100 μM) using an AB

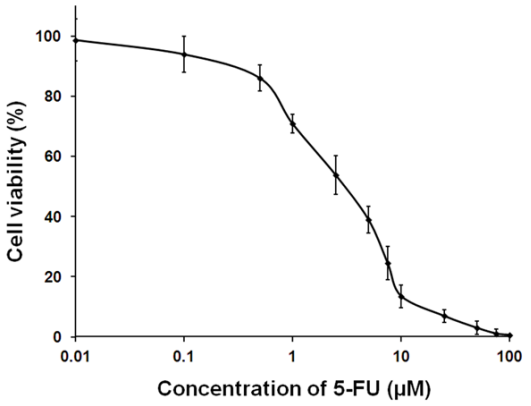

Figure 1. Dose-dependent effects of 5-FU on CGC proliferation. The viability of CGCs treated with 5-FU for 24 h was evaluated by an Alamar Blue (AB) assay and expressed as percentage viability relative to that of untreated cells. The experiments were repeated at least three times; the figure shows a representative experiment.

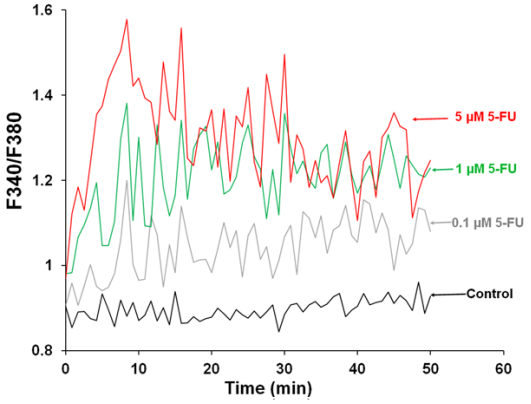

Figure 2. Changes in intracellular Ca^{2+} levels in CGCs in response to 5-FU treatment. The levels of Ca^{2+} were determined from the ratio of fluorescence intensity upon excitation at 340 and 380 nm. Each line represents the F340/F380 fluorescence ratio of a representative cell population.

assay. As shown in **Figure 1**, cell viability declined with increasing 5-FU concentrations. From the dose-response curves, we also calculated the IC_{50}, which on average was equal to 2.7 ± 0.5 μM.

5-FU exposure increased intracellular free Ca^{2+} levels in CGCs

To investigate the molecular mechanisms of 5-FU toxicity in CGCs, we assessed the influence of 5-FU on intracellular calcium influx by measuring the changes in intracellular free Ca^{2+} levels. **Figure 2** shows that 5-FU treatment increased Ca^{2+} levels in a dose-dependent manner; the rise in Ca^{2+} level in CGCs was immediate and the maximal fluorescence intensity was reached after 8-9 min. The maximum response in the group treated with 5 μM 5-FU was approximately two-fold of that in the negative control group. The results clearly demonstrated that 5-FU treatment elevated intracellular Ca^{2+} levels in CGCs.

5-FU induced ROS production in CGCs

It is known that Ca^{2+} signals activate enzymes associated with ROS generation, thus contributing to oxidative stress and neuronal damage [22]. Hence, the potential of 5-FU to induce oxidative stress was assessed by measuring ROS levels in CGCs. As shown in **Figure 3A**, 5-FU had little effect on intracellular ROS production upon 1 h treatment. However, when the exposure time was extended to 4 h (**Figure 3B**), 5-FU treatment at concentrations higher than 0.1 μM induced a significant increase in intracellular production of ROS. After 12 h treatment (**Figure 3C**), the intracellular ROS levels increased continuously in CGCs treated with lower concentrations of 5-FU (0.01-0.5 μM), and the highest ROS generation in CGCs was observed at 0.5 μM. Nevertheless, the generation of intracellular ROS was reduced when the drug concentration was higher than 1 μM. Taken together, the marked enhancement in fluorescence intensity in the 5-FU-treated groups, indicating an accumulation of intracellular ROS, suggested that 5-FU induced oxidative stress in CGCs by interfering with the cellular antioxidant mechanism.

5-FU stimulated caspase-3 activation in CGCs

To determine whether oxidative stress caused by 5-FU treatment could stimulate apoptosis, we investigated the activation of caspase-3, from a caspase-3 precursor (32 kD) into the 17 kD activated caspase-3 fragment by western blot, as previously described [21]. As shown in **Figure 4A**, 5-FU had little effect on caspase-3 activation in CGCs after 12 h treatment, as only the precursor band was detected in treated as well as control groups. However, when the exposure time was extended to 24 h (**Figure 4B1**), both the intact caspase-3 precursor and the cleaved product (activated caspase-3) were detected in 5-FU-treated cells, as compared to

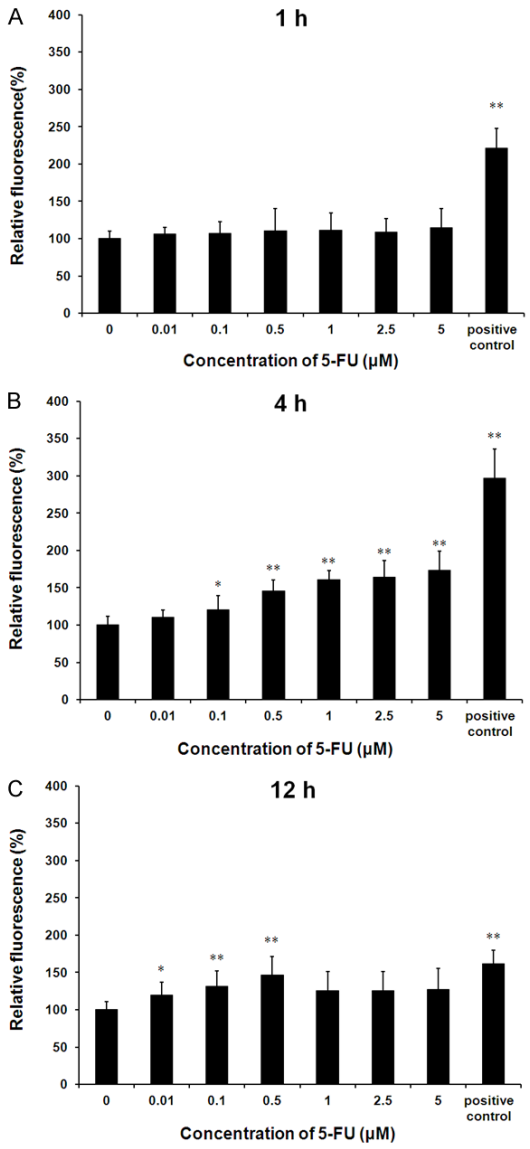

Figure 3. ROS formation in CGCs upon 5-FU treatment. CGCs were loaded with the oxidative stress-sensitive dye DCFH-DA and then exposed to different concentrations of 5-FU (1 mM H_2O_2 served as the positive control) for 1 h (A), 4 h (B) and 12 h (C), respectively. Relative fluorescence intensity was recorded using a microplate spectrofluorometer. All values are relative to that of the negative control (set to 100%). *P < 0.05 and **P < 0.01, versus negative control.

control untreated cells which showed only the 32 kD caspase-3 precursor band. Moreover, the levels of the caspase-3 precursor decreased, while those of the activated form increased in a 5-FU dose-dependent manner. The up-regulation of activated caspase-3 was indicative of 5-FU-induced apoptosis. Quantification of activated caspase-3 in CGCs revealed that activated caspase-3 levels in the groups treated with 5-FU (0.01-5 μM) were 1.2~4.0-fold higher than those in the negative control group (**Figure 4B2**). These results indicate that 5-FU-induced neurotoxicity in CGCs is mediated through a caspase-dependent signaling pathway involving caspase-3 activation.

5-FU induced neurotoxicity in rat cerebella

To evaluate the potential overall toxicity of 5-FU *in vivo*, rats were exposed to 5-FU for seven days by intraperitoneal injection every other day. During the experimental period, we did not observe any significant differences in either survival or food/water intake, between control and 5-FU-treated rats. The health of the animals was monitored on a daily basis by measuring their body weight. The average body weight at the end of the experiment (day 7) was 294 ± 10 g and 289 ± 8 g for control and treated rats, respectively (**Figure 5A**), indicating that 5-FU did not cause any general toxicity at the concentrations tested.

To look more specifically whether 5-FU could generate some kind of neurotoxicity, cerebellar sections were stained with H&E to assess neuronal injury (**Figure 5B**). The morphology of the granular layer in the cerebellar tissue of control rats was normal, with numerous small granule cells (**Figure 5B1**). However, the cerebellar sections from 5-FU treated rats, revealed pathological changes in granule cells (**Figure 5B2**). Those cells appeared deeply stained due to their shrunken size, and exhibited slight degenerative changes with loose and separated structure. Immunohistochemistry analyses of cerebellar cortex from 5-FU-treated animals (**Figure 5C**) showed an increase in the intensity of GFAP staining in the glial-rich molecular layer, as compared to control animals, suggesting 5-FU were capable of stimulating either astrocyte activation or proliferation. Due to their dense nucleus, granule cells can be characterized by compact blue staining in morphology when hematoxylin is used. As shown in **Figure 5C2**, an obvious decrease in the intensity of blue-stained granule cells was observed in 5-FU-treated rats. These histological findings indicate that 5-FU induced a mild neurotoxic effect on granule cells *in vivo*.

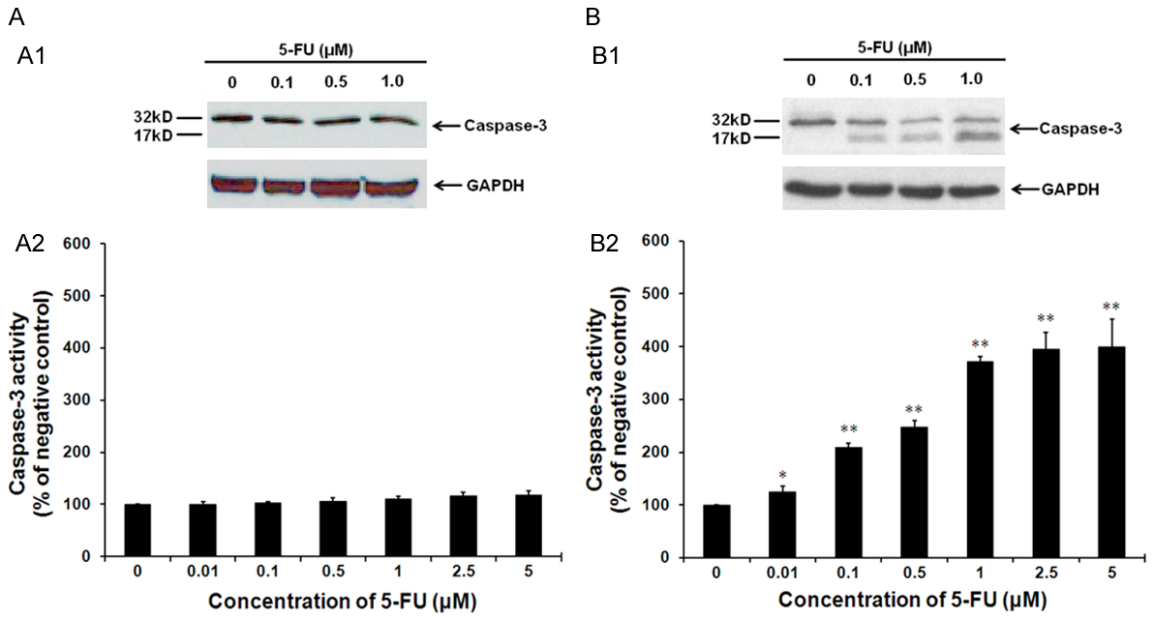

Figure 4. Activation of caspase-3 in CGCs upon 5-FU treatment. Expression and activity of capsase-3 was analyzed by western blot in CGCs treated with 5-FU for 12 h (A) and 24 h (B). (A1, B1) Representative immunoblots of caspase-3 cleavage. Precursor and activated caspase-3 proteins were detected using specific antibodies. (A2, B2) Intracellular protein levels of activated caspase-3 in 5-FU-treated CGCs based on the Caspase-Glo assay. All values are relative to that of the negative control (set to 100%). $*P < 0.05$ and $*P < 0.01$, versus negative control.

Discussion

Although the tissues that constitute the CNS are very important in the human body, they are unable to regenerate after serious damage. Consequently, the side effects of anticancer drugs on the CNS are a major concern. It has become increasingly clear that systemic chemotherapy for non-CNS cancers, such as breast cancer [23], can also induce a wide range of undesirable neurological damage. Despite the fact that multiple clinical studies have reported an association of 5-FU with several neurologic syndromes [24, 25], little is known about the cellular and molecular mechanisms underlying these effects. In this study, we demonstrated that 5-FU induced neurotoxicity in rat CGCs, involving oxidative stress and activation of the caspase-3 pathway.

It has been proven that 5-FU can cross the BBB and exert toxic effects on neurons [14, 15]. Our cytotoxicity assay revealed that 5-FU induced toxicity in CGCs with an IC_{50} value of 2.6 ± 0.5 μM, which is in agreement with previous studies on neural progenitor cells and oligodendrocytes [17]. However, in the 0-10 μM dose range, 5-FU had insignificant effects on several cancer cell lines such as MCF-7 and MDA-MB-231 breast cancer cells, ES-2 ovarian cancer cells, and T98 glioblastoma multiforme cells, whereas oligodendrocyte-type-2 astrocyte progenitor cells, oligodendrocytes, and glial-restricted precursor cells were sensitive to 5-FU [17]. In addition, the IC_{50} values of 5-FU for other cell types were higher than that for CGCs. For instance, the IC_{50} value was 400 μM in rat cardiomyocytes (H9c2) after treatment with 5-FU for 72 h [26], suggesting that CGCs are vulnerable to even low levels of 5-FU *in vitro*.

Calcium ions are ubiquitous secondary messengers involved in many cellular processes and are tightly regulated within a narrow physiological range [27]. Calcium homeostasis disruption may have deleterious consequences for cells, particularly neurons, leading to necrosis and/or apoptosis and subsequently to neurodegenerative diseases such as Alzheimer's [27]. Our data showed that 5-FU treatment elevated intracellular Ca^{2+} level in CGCs; similar results have also been reported with colon carcinoma cells [28]. This observation is consistent with the view that disturbance in calcium homeostasis is associated with 5-FU toxicity in CGCs.

Reactive oxygen species are produced continuously as natural by-products of the normal metabolism of oxygen and can cause oxidative

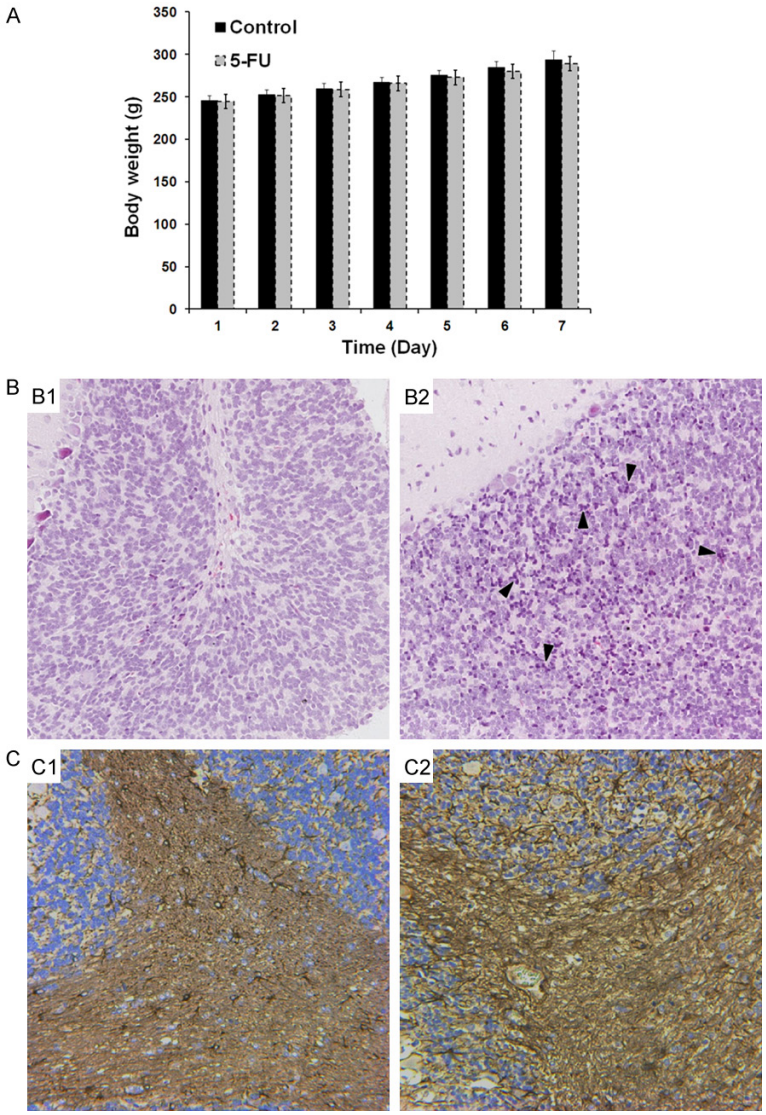

Figure 5. 5-FU induced neurotoxicity in rat cerebella. (A) Daily body weight measurements during the 7-day experimental period. (B) Histological analyses of cerebellar sections by H&E staining. (B1) Representative cerebellar section of control rats showing normal granule cell characteristics. (B2) Representative cerebellar section of rats treated with 5-FU exhibiting granule cells slightly shrunken in size with deeply stained nuclei (black arrows). (C) Cerebellum tissues of control (C1) or 5-FU treated rats (C2) immunostained with an anti-GFAP antibody (400×). 5-FU treated group showed an increase in the intensity of GFAP staining in the glial-rich molecular layer, with an obvious decrease in the intensity of blue-stained granule cells. The rats received 5-FU (20 mg/kg) in three consecutive injections every other day intraperitoneally; control animals received equal amounts of 0.9% saline. Day 1 was the day of the first 5-FU injection; day 7 was the end-point of the experiment.

damage to biomolecules, resulting in cell injury or death [29]. It has been reported that overproduction of ROS can result in oxidative stress, a pathophysiological process that can lead to cardiovascular disease, hypertension, atherosclerosis, diabetes mellitus, neurodegenerative diseases, and cancer [30]. Although the main anticancer mechanism of 5-FU is blocking DNA synthesis and replication, oxidative stress has also been proposed to play a key role [31]. Indeed, previous reports have revealed that 5-FU increases intracellular levels of ROS in cancer cells [32]. Furthermore, oxidative stress is also thought to account for many of the toxic effects of 5-FU. It has been previously reported that 5-FU inhibited activities of cytoplasmic antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, in the myocardium [33]. This reduced the efficiency of the antioxidant defense system to cope with free radical attacks, and was thought to be responsible for 5-FU-induced cardiotoxicity. Moreover, 5-FU chemotherapy was also shown to increase superoxide radical and hydrogen peroxide production in the neutrophils of cancer patients [34]. In the present study, we found that 4 h 5-FU treatment induced oxidative cell damage in CGCs by stimulating ROS production. Other reports have also demonstrated a similar deleterious mechanism of 5-FU in cardiomyocytes and liver cell lines [13, 26]. When the exposure time was extended to 12 h, the generation of intracellular ROS was reduced in CGCs treated with higher concentrations of 5-FU (1-5 μM), which may be explained by the following two reasons. Firstly, excessive ROS generated at the shorter induction time induced necrotic cell death, damaging the cell membrane, which led to leak of fluorescent DCF. Secondly, CGCs used defense mechanisms to

counterbalance ROS production, thus explaining the lower fluorescence after 12 h 5-FU treatment. A similar effect of the exposure time on ROS generation has been previously shown to occur in another cell line [35].

It is common knowledge that ROS generation and oxidative stress ultimately lead to apoptotic cell death [36]. It is also well documented that the signaling pathways leading to apoptosis involve the sequential activation of cysteine proteases known as caspases, with caspase-3 activation often regarded as the point of no return during apoptosis [37]. Our western blot analysis (**Figure 4A**) and quantification of activated caspase-3 (**Figure 4B**) confirmed that caspase-3 was involved in 5-FU-induced neurotoxicity in CGCs. The caspase-dependent pathway has also been previously shown to be activated in response to 5-FU therapies in renal and oral cancer cells [38, 39].

The ability to cross the BBB by passive diffusion enables 5-FU to affect the brain when administered systemically [15]. Several clinical studies have demonstrated that various neurologic syndromes, including multifocal inflammatory leukoencephalopathy, cerebellar dysfunction, encephalopathy, peripheral neuropathy and oculomotor disturbance, have been reported in cancer patients treated with 5-FU [24, 25]. In our study, histological analysis revealed that 5-FU administered by intraperitoneal injection caused slight degenerative changes in the rat cerebellar granular layer. Other brain regions have also been shown to be affected by 5-FU-induced neurotoxicity. For instance, Mustafa *et al.* demonstrated that 5-FU chemotherapy reduced neurotrophin levels and disrupted neurogenesis in the murine hippocampus [14]. Furthermore, Groves *et al.* reported that 5-FU decreased spine density and altered spine morphology throughout the hippocampus [40]. It was also demonstrated that delayed degenerative damage to white matter could be caused by systemic application of 5-FU [17].

In conclusion, the present study demonstrates that 5-FU treatment induced histological alterations in the cerebellum granular layer of Sprague-Dawley rats. In addition, we showed that 5-FU exhibited significant cytotoxicity to rat CGCs involving oxidative stress and activation of the caspase-3 pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

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