Radioprotective Role of *Grewia asiatica* in Mice Blood

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Summary

The aim of the present study was to evaluate the radioprotective effect of *Grewia asiatica* fruit pulp extract (GAE) on Swiss albino mice against radiation induced hematological and biochemical alterations. Swiss albino mice (6–8 weeks) were divided into four groups. Group I (normal) without any treatment. Group II (Drug) was orally supplemented (GAE) once daily at the dose of 700 mg / kg. b.wt / day for 15 days. Group III (control) only irradiated group. GroupIV (Drug+IR) was administered same as group II, then exposed to 5Gy of gamma radiation. Mice were sacrificed at 24 and 72 hours post irradiation. Radiation induced deficit in different blood constituents GSH, GSH-Px, sugar, and protein levels in serum could be significantly increased, whereas radiation induced elevation of lipid peroxidation and cholesterol level was markedly decreased in GAE pre-treated animals than control group. It showed that GAE provides protection against radiation-induced alterations in blood of Swiss albino mice.

Key words: *Grewia asiatica*, Antioxidant, Radioprotection, reduced glutathione, lipid peroxidation.

Introduction

Over the past 50 years, radiation research has focused on screening a plethora of chemical as well as biological radioprotectors [1–3]. Synthetic protectors against oxidative damage to tissue have toxicity. This limits their value in the clinical field. In the recent years there has been increasing interest in antioxidant activity[4] of plant origin compounds. Also public interest in phytoceuticals to inhibit chronic diseases and aging is gathering momentum. Reactive oxygen species such as hydroxyl (OH*) and peroxyl radicals (ROO*) and the superoxide anion (O_2*) are constantly produced as a result of metabolic reactions in living systems. [5] Living systems are protected from oxidative damage by these reactive species by enzymes such as superoxide dismutase and glutathione peroxidase and by antioxidant compounds such as ascorbic acid, tocopherols, and carotenoids. [6] However, when free-radical production exceeds the antioxidant capacity of the organism, these radical species attack lipids, proteins, and DNA, thus damaging structural integrity and function of cell membranes, enzymes, and genetic material.[7] A growing body of evidence indicates that various pathological conditions, including cardiovascular disease, arthritis, various cancers, and Alzheimer's disease, are associated, at least in part, with the damaging effects of uncontrolled free-radical production.[7] Many foods contain nonnutritive components such as flavonoids and other phenolic compounds that may provide protection against chronic diseases through multiple effects, which are as yet poorly understood. [8] Nutritional intervention to increase intake of phyto-antioxidants may reduce the threat of free radicals. These compounds may act as antioxidants by reacting with free radicals and thus interrupting the propagation of new free radical species, or by chelating metal ions such as Fe^{2+}, which catalyze lipid oxidation to alter their redox potentials. In addition, it has been shown that antioxidant supplements can significantly improve certain immune responses. [9] Recent research has indicated that the people who eat higher amounts of fruits and vegetables have about one half the risk of cancer and less mortality from cancer.[10-11]

India has a rich heritage of medicinal plants many of which have been explored for the various bioactivities since ages, but the radioprotective potential of the plants have been hardly explored. In this context *Grewia asiatica* (Phalsa) cultivated on a commercial scale mainly in the northern and western states of India, [12-13] is known for its medicinal properties. The fruit is astringent and stomachic. Morton[14] reported that unripe phalsa fruit alleviates inflammation and is administered in respiratory, cardiac and blood disorders, as well as in fever reduction. Furthermore and infusion of the bark is given as a demulcent, febrifuge, and treatment for diarrhea. *Grewia asiatica* contains anthocyanin type cyanidin 3- glucoside, [15] vitamin C, minerals and dietary fibers etc.[16]

Exposure of animals to ionizing radiation causes a series of physiological changes known as acute radiation syndrome that is dependent on the exposure dose and may lead to death. The damage to the hematopoietic system is a major factor in the mortality following an acute radiation exposure.[17] The present study is an attempt to investigate the possible protective role of *Grewia asiatica* fruit extract (GAE) against radiation induced oxidative stress on the hematopoietic constitutes and biochemical indices.

Materials and Methods

Animal care and handling:

The animal care and handling was done according to the guidelines set by World Health Organization, Geneva, Switzerland and INSA (Indian National Science Academy,

New Delhi, India). The departmental animal ethical committee approved this study. Swiss albino mice, 6–8 weeks old weighing 23±2 gm, from an inbred colony were used for the present study. These animals were maintained under controlled conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. They were provided standard mice feed (procured from Hindustan Levers Ltd., India) and water ad libitum. Tetracycline water once a fortnight was given as preventive measures against infections.

Extract preparation (Drug)

Fresh fruits of *Grewia asiatica* collected locally in summer season were washed, shade dried and powdered after removal of seeds. Methanolic extract was then prepared by refluxing for 48 hours (3x12) at 40^{o}C. The extract thus obtained was vacuum evaporated so as to get in powdered form. The extract was redisolved in doubled-distilled water (DDW) just before the oral administration. For the various concentrations, a known amount of GAE was suspended in DDW and 50 µl of GAE suspension was given to each mouse by oral gavage as given by Ahaskar *et al*.[18)]

Source of irradiation

The cobalt teletherapy unit (ATC-C9) at Cancer Treatment Center, Radiotherapy Department, SMS Medical College and Hospital, Jaipur, Rajasthan, India was used for irradiation. Unanaesthestized animals were restrained in well-ventilated perspex boxes and whole body exposed to gamma radiation at a distance (SSD) of 77.5cm from the source to deliver the dose rate of 1.07 Gy/ min.

Dose selection

The dose selection of *Grewia asiatica* was done on the basis of drug tolerance study in our laboratory. Various dose of *Grewia asiatica* (100, 400, 700, 1000, 1300 mg/kg b.wt. of animal) were tested for their effects on the tolerance to 10 Gy gamma irradiation in Swiss albino mice. Thus 700 mg/kg b.wt. /day was obtained as optimum dose and used for further experimentation.

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Experimental design

Mice selected from an inbred colony were divided into 4 groups (30 animals in each Group).
Group I (normal): Mice of this group did not receive any treatment.
Group II (drug): Mice of this group were administered with GAE (700mg/kg of b.wt. /day) for 15 consecutive days once daily.
Group III (control): Mice received DDW (volume equal to *Grewia asiatica* solution) for 15 days and were then exposed to 5Gy of gamma-radiation.

Group IV (Experimental): In this group oral administration of GAE (700mg/kg of b.wt. /day) was made once daily for 15 consecutive days. One hour after administration of last dose of GAE, mice were whole body exposed to single dose of 5 Gy gamma-radiation as in group third.

Six mice from each group were necropsied at 24 and 72 hour of post irradiation.These animals were killed by cervical dislocation, and their blood was collected from orbital sinus by heparinised needle, to estimate various hematological and biochemical parameters

Hematological study

Blood was collected from caudal vein in a vial containing 0.5 M EDTA. The total numbers of erythrocytes, leucocytes, differential leucocytes count, hematocrit, hemoglobin and erythrocyte sedimentation rate were estimated by adopting standard procedures.

Biochemical Assay

Reduced glutathione (GSH) assay: The glutathione content in the blood was measured spectrophotometrically using DTNB as a coloring reagent, according to the method of Beutler *et al* [19].

GSH-Px assay: GSH-Px estimation was carried out by the method of Hochstei and Utely[20].

Lipid peroxidation (LPO) assay: LPO was measured by the method of Buege and Aust[21].

Protein assay: Estimation of protein was based on the method proposed by Bradford.[22]

Cholesterol assay: Total cholesterol was estimated by Burchard method [23]

Statistical Analysis

The results obtained in the present study were expressed as mean ± SEM. The statistical difference between various groups were analysed by the Student's *t*-test and the significance was observed at the $p < 0.02$, $p < 0.01$and $p < 0.001$ level.

Results

Hematological studies

Drug supplementation for 15 consecutive days (Group II) did not show any appreciable significant change in various hematological constituents (erythrocytes, leucocytes, monocytes, eosinophills, hemoglobin, and erythrocyte sedimentation rate) in comparison to the normal (Group I). On the other hand, lymphocyte, neutrophils and haematocrit showed significant change in comparison to the normal. (Table 1).

Total erythrocytes count decreased significantly ($p<0.001$) at 24 and 72 hrs following radiation (Group III) from the normal(Group I). Maximum decrease in total erythrocytes count by 45.5% was recorded on 72 hrs *p.i.* in control group, In group IV (GAE + IR), a significant ($p< 0.01$) increase in red cell count with respect to Group III (control) was noticed during the entire period of study. (Table 1).

Table 1. Variations in hematological parameters of mice after 5 Gy of gamma irradiation with (experimental) or without (control) *Grewia asiatica* Fruit extract.

Parameter Studied	Group I	Group II	Post- irradiation Autopsy Intervals	Group III	Group IV
Hemoglobin	13.8±0.96	13.2±0.89^{n}	24 Hrs	10.3±0.66	12.6±0.73^{d}
			72 Hrs	9.89±0.56	12.01±.0.47^{c}
RBC (10^6/mm^3)	5.27±0.28	5.29±0.23^{n}	24 Hrs	3.61±0.19	4.28±0.15^{c}
			72 Hrs	2.87±0.14	3.79±0.17^{b}
WBC (10^3/mm^3)	5.61±0.15	5.58±0.08^{n}	24 Hrs	2.87±0.13	3.51±0.11^{b}
			72 Hrs	2.59±0.11	2.99±0.06^{b}
Lymphocyte (%)	39.34±0.47	41.25±0.72^{d}	24 Hrs	31.46±0.87	36.63±0.73^{b}
			72 Hrs	27.69±1.42	35.14±.0.53^{a}
Monocytes (%)	3.18±0.25	2.82±0.26^{n}	24 Hrs	0.786±0.13	1.58±0.18^{b}
			72 Hrs	0.917±0.11	1.73±.0.12^{a}
Neutrophills (%)	57.16±0.18	56.42±0.19^{c}	24 Hrs	42.12±0.32	52.14±0.36^{a}
			72 Hrs	41.22±0.26	50.78±.0.31^{a}
Eosinophills (%)	2.11±0.13	2.08±0.17^{n}	24 Hrs	1.17±0.14	1.22±0.11^{n}
			72 Hrs	1.06±0.10	1.39±0.13^{e}
Haematocrit (%)	44.12±0.49	45.34±0.22^{d}	24 Hrs	33.93±0.36	37.49±0.57^{a}
			72 Hrs	28.44±0.62	32.17±0.28^{a}
ESR (mm/hr)	4.64±0.17	4.71±0.08^{n}	24 Hrs	5.94±0.18	4.74±0.24^{b}
			72 Hrs	6.24±0.27	4.58±0.16^{a}

Table 2. Variations in LPO, GSH, GSSG, GPx I blood of mice after 5 Gy of gamma irradiation with (experimental) or without (control) *Grewia asiatica* fruit extract.

Parameter Studied	Group I	Group II	Post- irradiation Autopsy Intervals	Group III	Group IV
LPO (n mol/ml)	2.69±0.29	2.67±0.23^{n}	24 Hrs	6.99±0.43	4.93±0.33^{b}
			72 Hrs	7.17±0.31	5.28±0.27^{a}
GSH (n mol/ml)	65.12±3.8	65.48±3.2^{n}	24 Hrs	23.96±2.6	31.58±2.1^{d}
			72 Hrs	20.12±2.8	29.26±2.9^{d}
GSH-Px (U/gm Hb)	5.947±0.11	5.986±.07^{n}	24 Hrs	3.796±0.06	5.148±0.09^{a}
			72 Hrs	3.725±0.03	5.184±0.05^{a}

Hemoglobin concentration in irradiated mice (group III) showed decrease of 25.36% and 28.33 % from the normal mice (Group I) on the 24 and 72 hours, respectively. Animals supplemented with GAE prior to irradiation (group IV) exhibited a higher hemoglobin concentration than group III. Haematocrit percentage was found to be significantly ($p< 0.001$) lower in irradiated group III with a maximum decline at 72 hrs (28.44±0.62). In group IV, hematocrit values were significantly higher ($p< 0.001$) than Group III (control). (Table 1).

In group III, erythrocyte sedimentation rate exhibited a significant rise over normal. In GAE pre-treated animals (group IV), the increase in erythrocyte sedimentation rate was significantly lesser than group III and values were not much higher than normal. (Table 1).

A marked decline in total leucocyte count was also observed by 2.87±0.13 and 2.59±0.11 at 24 and 72 hrs of post irradiation in group III(irradiated) as compared to group I (5.61±0.15). In animals of GAE pretreated irradiated (group IV), these cells were scored as significantly higher ($p<0.01$) than the corresponding control group III. (Table 1).

In differential leucocyte counts, maximum decrease in monocytes (0.786±0.13) was observed at 24 hrs ***p.i.*** as compared to group I (3.18±0.25), whereas maximum decrease in lymphocyte (27.69±1.42) was observed at 72 hr in group III as compared to group I (39.34±0.47). In animals pretreated with GAE, this decrease was less pronounced in comparison to group III. (Table 1).

Neutrophil percentage showed a significant decrease ($p< 0.001$) in group III animals and was the lowest on 72 hr (41.22±0.26) when compared to group I (57.16±0.18). In group IV, such increase in neutrophilic counts was significantly higher in comparison to control (Group III). (Table 1).

Eosinophils showed a significant decline after radiation exposure, which was found maximum at 72 hrs (1.06±0.10) as compared to group I (2.11±0.13).In GAE pretreated irradiated animals (group IV), such decrease was lesser in comparison to control during entire period of study (Table 1).

Biochemical Studies:

No significant difference in blood lipid peroxidation levels was observed in GAE alone treated animals (group II) as compared to normal (group I). A significant increase ($p< 0.001$) in blood lipid peroxidation levels was noted in gamma irradiated animals (group III) as compared to normal (Group I). However, such level was found to be declined significantly in GAE pretreated irradiated (group IV) animals in comparison to control group (Table 2).

No significant alterations in glutathione contents of blood were observed between normal and GAE- treated animals. However, a statistically significant ($p< 0.001$) decrease in glutathione was noted in control (group III) animals as compared to group I. In GAE pretreated irradiated (group IV) animals exhibited a significant elevation ($p< 0.05$) in glutathione as compared to group III, but the values remained below normal (Table 2).

The changes in the level of blood GSH-Px in Table 2 shows that the activities of GSH-Px significantly inhibited in blood following radiation exposure. GAE pretreatment significantly ($p<0.001$) increased the level of GSH-Px then compared to control.

In only GAE treated animals (group II), levels of cholesterol markedly decreased by 10.26% then compared with normal mice. Highly augmented levels of cholesterol in the control group declined by administration of GAE by approximately 6.97% and 22.56% at 24 and 72 hr post-irradiation, respectively (Table 3).

There was a significant continuous reduction in protein content in control (group III) The values of protein content in the experimental group (groupIV) were significantly higher ($p<0.01$) than corresponding control mice at 24 and 72 hrs post irradiation but still lower then normal level . (Table 3)

A sharp decrease of 56.44 % and 60.58% in amount of blood sugar compared to the normal is observed in control groups at 24 and 72 hrs respectively, whereas in experimental group(Group IV), blood sugar is raised significantly then there corresponding control group but still lower then normal levels.(Table 3). Total blood sugar levels were markedly lowered in the experimental group after diet supplementation with GAE.

Table 3. Variations in biochemical parameters of mice after 5 Gy of gamma irradiation with (experimental) or without (control) *Grewia asiatica* fruit extract.

Parameter Studied	Group I	Group II	Post-irradiation Autopsy Intervals	Group III	Group IV
Protein (gm %)	8.3±0.97	7.9±0.84[n]	24 Hrs	5.9±0.32	7.4±0.31[b]
			72 Hrs	5.7±0.38	7.2±0.21[b]
Cholesterol	120.83±0.89	108.43±0.78[a]	24 Hrs	158.21±1.19	147.17±1.38[a]
			72 Hrs	169.53±1.04	131.28±1.27[a]
Blood Sugar	122.21±1.15	126.33±1.29[c]	24 Hrs	53.23±0.79	87.43±1.27[a]
			72 Hrs	48.17±1.09	93.18±0.87[a]

Discussion

In the present study, there was a considerable decrease in the hematological values (erythrocytes, leucocytes, differential leucocyte count, hematocrit and hemoglobin) after irradiation as compared to normal. However, significant rise in these parameters was evident in GAE pre-treated animals. Sharp decline in erythrocytes, leucocytes, hematocrit, hemoglobin and non-neutrophilic granules was observed at 72 hrs following irradiation. Micke et al. [24] [25] reported a significant decrease of human neutrophilic granulocyte function at 3.5 and 4.0 Gy sub-lethal radiation dose. The decrease in the values of hematological parameters following radiation exposure may be assigned to a direct damage caused by lethal dose of radiation[25] [26]. Whole-body irradiation of moderate dose-range (5–10 Gy) leads to a decreased concentration of all the cellular elements in blood. This can be due to direct destruction of mature circulating cells, loss of cells from circulation by hemorrhage or leakage through capillary walls and less production of cells.[17] Hematocrit is the percentage of whole blood that is made up of cells and decrease in its value below normal indicates anaemia. Another measure of anaemia is decrease in hemoglobin percentage[26]. In the present investigation, it has been observed that hemoglobin level declined significantly following radiation exposure. These observations are in accordance with the findings of others[27-28]. The decrease in hemoglobin content can be attributed to decline in the number of red blood cells. In GAE pretreated animals, hemoglobin values were higher then control mice, which show a significant protection of erythrocytes by GAE. The synthesis of hemoglobin begins at 12 h after binding of erythropoietin to its receptor[29] due to increased iron pool by erythropoietin[30]. Decrease in hematocrit percentage is also observed in the control mice, which can be attributed to the failure of erythropoiesis, destruction of mature cells or increased plasma volume[31]. The decrease in the number of WBC as well as erythrocytes in the present study also supports the view of decreased erythropoiesis as the cause of decline in hematocrit values. GAE protects bone

marrow and blood erythropoietic cells and try to maintain the normal percentage of hematocrit.

One of the basic mechanisms of radiation damage is production of free radicals leading to the formation of peroxides and oxidative reactive species. These peroxides, via lipid peroxidation, damage the cell membrane and other components of cell. Free radicals such as superoxide anion (O_2^-), the hydroxyl radical (OH_2), and hydrogen peroxide (H_2O_2) are typically triggered by the exposure of living tissue to ionizing radiation.

Lipid peroxidation is a highly destructive process and cellular organelles and whole organism, lose biochemical function and/or structural and architecture, [32] which may lead to damage or death of cell. The preservation of cellular membrane integrity depends on protection or repair mechanisms capable of neutralizing oxidative reactions. The presence of antioxidants in the plants suppresses the formation of free lipid radical and thus prevents the formation of endoperoxidation. In the present study, however, GAE treatment did not significantly alter the lipid peroxidation level in unirradiated animals, but it significantly lowered the radiation-induced lipid peroxidation in terms of malondialdehyde.The inhibition of lipid peroxidation in biomembranes can be caused by antioxidants [33-34].

Under normal conditions, the inherent defense system including glutathione and antioxidant enzymes protects against the oxidative damage. GSH offers protection against oxygen derived free radicals and cellular lethality following exposure to ionizing radiation. [35] GSH is a versatile protector and executes its radioprotective function through free radical scavenging, restoration of the damaged molecule by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state. [36] The present study demonstrates a significant reduction in blood GSH following exposure. This could be due to the enhanced utilization of the antioxidant system as an attempt to detoxify the free radicals generated by radiation. Oral administration of GAE did not significantly influence the endogenous GSH level either in blood, but its presence during radiation exposure protects the endogenous GSH depletion due to irradiation. The lower depletion of blood GSH in the GAE pre-treated irradiated animals could be due to the higher availability of GSH, which increases the ability to cope up with the free radicals produced by irradiation. The increased GSH level suggests that protection by GAE may be mediated through the modulation of cellular antioxidant levels. The basic effect of radiation on cellular membranes is believed to be the peroxidation of membrane lipids. Radiolytic products, including hydroxyl and hydroperoxyl radicals, can initiate lipid peroxidation[37]. Glutathione peroxidase prevents the accumulation of oxidized lipids in mitochondrial cell membranes and also detoxifies H_2O_2 by utilizing reduced glutathione as a co- substrate[38]. In the present investigation, higher GSH-Px and GSH activities in blood were observed in the mice of experimental group then control group .The increased GSH-Px might be due to enhanced utilization of GSH which acts as a substrate for GSH-Px, since the intracellular level of GSH may be the major mechanism for regulation of GSH-Px [39]. The reduction in the amount of MDA content and the elevation in GSH and GSH-Px in the GAE-treated animals suggests that GAE may scavenge the free radicals formed during oxidative stress.

Reduction in rate of the protein synthesis may be due to unfavorable conditions like unavailability of one or more essential enzymes and/or reduction in sites of protein synthesis. [40] The decrease of protein noted may be due to its lyses, by X-irradiation or may be at the synthesis level, also may be the depression of enzyme involved in the activation of amino acid and transferring to t-RNA, [41] or by the inhibition of release of synthesized polypeptides from polysomes.[42] Increased protein concentration recorded in our study, shows that GAE supplemented irradiated mice is a beneficial effect. This proves an improvement in ribosomal activities, which enhance the protein synthesis, can be treated as

antiradiation effect. The present study shows a decrease in blood glucose level in control group. Hypoglycemia might be due to disturbance in enzymatic mechanism in the liver which does not produce the requisite amout of glucose needed by animal. While GAE pre-treated irradiated animal blood sugar is higher then control group, shows that GAE may protect the enzymatic mechanism in the liver and may be source of blood glucose.

Fruits like ber, phalsa, apple and strawberry have been shown to possess moderate antioxidant activity ranging from 12-64 mM FRAP. [43)] Matsumoto *et al* [44)] have shown that the antioxidative activity of plasma lasted longer than the presence of anthocyanin glycosides in the plasma. They assumed that anthocyanins were converted into some metabolites having antioxidant activity. Like other flavonoids, anthocyanins and anthocyanidins (the aglycone form) have antioxidant properties.[45)] The antioxidant potency of anthocyanin extracts is concentration dependent. [46)] The positive effects of anthocyanin pigments could be related to their potent antioxidant activity demonstrated in various in vitro and in vivo studies. [44-45, 47-49)] Anthocyanins possesses bactericidal, antiviral, and fungistatic activities. They exhibit a strong antioxidant activity that prevents the oxidation of ascorbic acid, provides protection against free radicals, shows inhibitory activity against oxidative enzymes, and has been considered as important agents in reducing the risk of cancer and heart disease. [50)] All evaluated anthocyanins were better agents against lipid peroxidation than α-tocopherol (up to seven times). Also, it was demonstrated that anthocyanins have scavenging properties against ·OH and O_2. [51)]

Haldar *et al*[52)] examined effects of vegetarian diet and variations in the habitual intakes of foods and nutrients on blood antioxidants. Total intake of fruits, vegetables and fruit juices was positively associated with plasma levels of several carotenoids and vitamin C.Mechanisms of antioxidative action of vitamin C are direct scavenging and blocking of ROS, as well as regeneration of other antioxidative systems[53)]

The biological benefits of certain carotenoids may be due to their potent antioxidant properties attributed to specific physico-chemical interactions with membranes. McNulty *et al* [54)] test this by measuring the effects of various carotenoids on rates of lipid peroxidation and correlated these findings with their membrane interactions, as determined by small angle X-ray diffraction approaches. The findings indicate distinct effects of carotenoids on lipid peroxidation due to membrane structure changes. These contrasting effects of carotenoids on lipid peroxidation may explain differences in their biological activity.

Earlier studies in our laboratory demonstrated that oral administration of 700 mg/k.g. b.wt/day dose of GAE, prior to radiation exposure (10 Gy), was found to be effective in terms of survivability then other higher and lower doses of GAE. The radioprotective effect of GAE was also determined by calculating the dose reduction factor (DRF), which was 1.53. Protective role of GAE in mice brain against 5Gy gamma radiation was also studied by Ahaskar *et al*[18)].

The results of the present investigation demonstrate that GAE pretreatment protects the hematopoietic system of mice against radiation-induced damage by inhibiting the glutathione depletion, decreasing lipid peroxidation level, and increasing hematological constituents in peripheral blood. The protection afforded with GAE in hematological and biochemical parameter of blood in the present study may be due to synergistic effects of its antioxidative constituents, which may prove to be beneficial for the clinical use of such dietary compounds as radio protector.

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