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Modulatory Role of *Terminalia chebula* on Erythrocyte Defenses in Young and Aged Rats

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Abstract: The present study was aimed to evaluate the oxidants, enzymatic and nonenzymatic antioxidants, glycoproteins and membrane adenosine triphosphatases (ATPases). The increased levels of oxidants and decreased levels of enzymatic, nonenzymatic antioxidants, glycoproteins and ATPases were observed in erythrocytes of aged rats. After administration of *Terminalia chebula*, the levels were reverted to normal in aged rats. These results indicate that the administration of *T. chebula* acts as a free radical scavenger with potential antioxidant effects in erythrocytes of aged rats.

Key words: Aging, antioxidants enzymes, ATPases, erythrocytes, glycoproteins, *Terminalia chebula*

INTRODUCTION

Aging is the accumulation of changes responsible for the sequential alterations that accompany with advancing age and the age associated progressive increase in the chance of disease and death (Harman, 1992). Also, aging has been proposed to be a result of continuous reactions of the cell components with oxygen free radicals throughout the life span (Reznick *et al*., 1992). These oxygen free radicals can modify DNA, proteins and membrane lipids, leading to cellular degeneration (Olanow, 1992). Erythrocytes have been used as a biological probe in exploring the aging process (Bernabucci *et al*., 2002), which is associated with a loss of ability at the molecular, cellular and whole organism levels (Hall *et al*., 2000). The high polyunsaturated fatty acid content of the erythrocyte membrane and the continuous exposure to high concentrations of oxygen and iron in heamoglobin are factors that make erythrocytes very sensitive to oxidative injury (Bernabucci *et al*., 2002). However, during aging an oxidant challenge exceeds the capacity of the cell's defense system, membrane damage may occur. During such conditions, dietary intake of antioxidants gains immense importance.

Consumption of fruits and vegetables containing high amounts of antioxidative nutraceuticals have been associated with the balance of the free radicals/antioxidants status, which helps to minimize the oxidative stress in the body and to reduce the risk of diseases (Kaur and Kapoor, 2001). Chebulic myrobalan (*Terminalia chebula* Retz.) belonged to the family Combretaceae, a native plant in India and found in the deciduous forests. Its dried ripe fruit tissues have traditionally been used to treat various ailments in Asia. It is a carminative, deobstruent, astringent and expectorant agent (Anonymous, 1978). Its principles constituents contain chebulagic, chebulinic acid and corilagin (Harborne *et al*., 1999). Chebulic myrobalan is highly nutritious and could be an important source of dietary supplement in vitamin C, energy, protein, amino acids and mineral nutrients (Bharthakur and Arnold, 1991). *T. chebula* has been reported to exhibit a variety of biological activity, including anticancer (Saleem *et al*., 2002), antidiabetic (Sabu and Kuttan, 2002), antimutagenic (Kaur *et al*., 2002), antibacterial (Malekzadeh *et al*., 2001) activities. Hence, the present study was designed to examine the effect of *T. chebula* on erythrocyte oxidation products level, antioxidant status, membrane ATPases and glycoproteins in aged rats compared with young rats.

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MATERIALS AND METHODS

Chemicals

1-chloro-2, 4-dinitro benzene (CDNB), 5,5'-dithio-bis (2-nitrobenzoic acid), glutathione (reduced), glutathione (oxidized), 2-thiobarbituric acid were obtained from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade marketed by Sisco Research Laboratories Pvt. Ltd., Mumbai and SD Fine Chemicals Ltd., Mumbai, India.

Preparation of *T. chebula* aqueous Extract

The fully ripe fruits of *Terminalia chebula* were collected from Kolli hills, Tamilnadu, India during the month of January 2005. Hundred grams of dried fruit skins were hammered in to small pieces followed by extraction with 800 mL distilled water for 24 h in water bath at 40°C and repeated for two times. The extracts were then combined, concentrated and finally lyophilized to dry. The final yield of the water extracts was 43.7 g. The extract was re-dissolved in distilled water for further experiments.

Animals and Treatment

Young (3-4 months, 120-150 g) and aged (22-24 months, 380-410 g) Wistar male albino rats were used for the experiments. The rats were housed in polypropylene cages on a 12L:12D cycle and fed *ad libitum* on commercial laboratory food pellets and water. The animals were divided into four groups namely, Group I: Control young rats were received water only. Group II: Young rats were treated with *T. chebula* aqueous extract, 200 mg dose kg^{-1} body weight in 1.5 mL of water through gastric incubations for 4 weeks. Group III: Control aged rats were received water only. Group IV: Aged rats were treated with *T. chebula* aqueous extract, 200 mg dose kg^{-1} body weight in 1.5 mL of water through gastric incubations for 4 weeks.

Preparation of Erythrocyte and Erythrocyte Membranes

On completion of the experimental period, animals were anaesthetized with Thiopentone sodium (50 mg kg^{-1}) and the blood was collected with EDTA as anticoagulant. The erythrocyte membrane was isolated according to the procedure of Dodge *et al*. (1963) with a change in buffer according to Quist (1980). Preparation of the haemolysate was carried out as described by Beutler (1984).

Determination of Oxidation Products in Erythrocyte Membranes

Lipid peroxidation was assessed biochemically by determining the level of malondialdehyde (MDA) (Beuge and Aust, 1978). MDA levels were expressed as nmoles of MDA formed/mg protein using 1,1,3,3-tetraethoxypropane as standard. The protein carbonyl (PCO) content was analyzed using 2,4-dinitrophenylhydrazine (DNPH) as described by Levine *et al*. (1990).

Assay of Enzymatic Antioxidants in Erythrocyte Lysate

Copper, zinc superoxide dismutase (Cu, Zn-SOD) activity was measured by the method of Kakkar *et al*. (1984) using NADH-PMS-NBT. Catalase (CAT) activity was measured according to the method of Beers and Sizer (1952). Glutathione peroxidase (GPx) was estimated by Rotruck *et al*. (1973), glutathione reductase (GR) activity by the procedure of Staal *et al*. (1969), glucose-6-phosphate dehydrogenase (G6PDH) by Korenberg *et al*. (1955) and glutathione-s-transferase (GST) according to Habig *et al*. (1974).

Estimation of Non-enzymatic Antioxidants in Erythrocyte Lysate

Reduced glutathione (GSH) was measured as described by Ellman and Archs (1959) using 5, 5-dithiobis- (2-nitrobenzoic acid) (DTNB) reagent. Oxidized glutathione (GSSG) was measured by

masking GSH with 2-vinylpyridine by the same procedure of GSH estimation. Redox state was determined by the redox index: (GSH+2×GSSG)/(2×GSSG×100). Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) contents were assayed according to Omaye *et al*. (1979) and Desai (1984), respectively.

Determination of Glycoproteins in Erythrocyte Membranes

In the erythrocyte membrane pellets the lipids were extracted according to the method of Folch *et al*. (1957) using chloroform-methanol mixture (2:1 v/v). The resulting defatted residue was suspended in sodium acetate buffer (containing 2 mg cysteine HCl mL^{-1}, final pH 7.0) and deproteinized by 4-5 volumes of ethanol, evaporated to dryness in the cold under a vaccum and subjected to hydrolysis by heating with 2 mL of 4 N HCl for 4-6 h. The hydrolyzed material was neutralized with 4 N sodium hydroxide and used for estimating erythrocyte sialic acid (Warren, 1959), hexose (Niebes, 1972) and hexosamine (Wagner, 1979).

Determination of Membrane ATPases in Erythrocyte Membranes

Sodium potassium ATPase (Na^+ K^+-ATPase), calcium ATPase (Ca^{2+}-ATPase) and magnesium ATPase (Mg^{2+}-ATPase) were determined by the method of Bonting (1970), Hjerten and Pan (1983) and Ohnishi *et al*. (1982), respectively. In all the three cases the enzyme activity was expressed as a function of inorganic phosphorous liberated, which is due to the breakdown of ATP.

Estimation of Haemoglobin

Haemoglobin content was determined using the cyanmethemoglobin method (Fairbanks and Klee, 1999).

Statistical Analysis

The values are expressed as mean±Standard Deviation (SD). The results were computed statistically (SPSS software package) using one-way analysis of variance. Post hoc testing was performed for intergroup comparisons using the least significance (LSD) test. A p-value<0.05 was considered significant.

RESULTS

The results obtained in erythrocytes showed that the levels of MDA and PCO were found significantly increased 43.75 and 31.73%, respectively in aged control rats as compared with young control rats (Table 1). In *T. chebula* treatment to aged rats, levels of MDA and PCO were significantly decreased ($p<0.001$, $p<0.01$) with comparison of age-matched controls. In drug treated young rats showed 22.22% ($p<0.05$) decrease in MDA levels.

The activities of the enzymatic antioxidants SOD, CAT, GPx, GR, G6PDH and GST were significantly ($p<0.001$) decreased in erythrocytes of aged control rats when compared with young control rats. *T. chebula* aqueous extract treatment to aged rats showed an increase ($p<0.001$) in these erythrocytes antioxidant enzymes at 24.79, 40.56, 26.45, 42.29, 43.59 and 31.90%, respectively (Table 2). In the young drug treated rats, *T. chebula* showed an increase in SOD, CAT and GPx activities at 17.28, 11.32 and 11.25% as compared with young control rats.

A significant decrease (43.13%) in GSH level with increase (25%) of GSSG levels in erythrocytes of aged rats, these alterations decreased ($p<0.001$) the GSH/GSSG ratio and redox ratio in erythrocytes of aged rats. *T. chebula* treatment increased the GSH content in erythrocytes of young and aged rats at 14.55 and 42.34%, respectively and decreased ($p<0.001$) the GSSG level with increased GSH/GSSG ratio (51.33% in aged and 14.56% in young) and redox ratio at $p<0.001$ (Table 3).

Table 1: Effect of *T. chebula* treatment on oxidation products in erythrocytes of young and aged rats

Treatments	Group I	Group II	Group III	Group IV
MDA	8.19±0.92	6.37±1.06^{a**}	14.56±1.14^{a***}	9.94±1.07^{b***}
PCO	8.49±1.01	8.24±0.94	12.78±1.24^{a***}	10.28±1.27^{b***}

Values are expressed as mean±SD of six rats; Units: MDA nmoles of MDA formed mg^{-1} protein; PCO µmoles of DNPH mg^{-1} protein; Statistical comparison: :^{a}Compared with Group I; :^{b}Compared with Group II; *: p<0.05, **: p<0.01, ***: p<0.001

Table 2: Effect of *T. chebula* treatment on enzymatic antioxidants in erythrocytes of young and aged rats

Treatments	Group I	Group II	Group III	Group IV
SOD	4.02±0.34	4.86±0.37^{a***}	2.73±0.32^{a***}	3.63±0.29^{b***}
CAT	51.17±5.11	58.35±4.36^{a*}	25.92±2.74^{a***}	43.61±3.19^{b***}
GPx	7.34±0.53	8.27±0.44^{a***}	5.06±0.37^{a***}	6.88±0.39^{b***}
GR	2.73±0.22	2.82±0.17	1.31±0.18^{a***}	2.27±0.20^{b***}
G6PDH	1.45±0.14	1.51±0.11	0.66±0.16^{a***}	1.17±0.21^{b***}
GST	7.02±0.52	7.24±0.54	4.14±0.33^{a***}	6.08±0.38^{b***}

Values are expressed as mean±SD of six rats; Units: SOD: 50% nitroblue tetrazolium reduction min^{-1} mg^{-1} Hb; CAT: µmoles of H_2O_2 consumed min^{-1} mg^{-1} Hb; GPx: µmoles of GSH consumed min^{-1} mg^{-1} Hb; GR: µmoles of NADPH oxidized min^{-1} mg^{-1} Hb; G6PDH: U mg^{-1} Hb; GST: mmoles of GSH-CDNB conjugated min^{-1} mg^{-1} Hb; Statistical comparison: ^{a}: Compared with Group I; ^{b}: Compared with Group II; *: p<0.05, **: p<0.01, ***: p<0.001

Table 3: Effect of *T. chebula* treatment on non-enzymatic antioxidants in erythrocytes of young and aged rats

Treatments	Group I	Group II	Group III	Group IV
GSH	3.64±0.28	4.26±0.22^{a***}	2.07±0.24^{a***}	3.59±0.27^{b***}
GSSG	0.09±0.01	0.09±0.01	0.12±0.01^{a***}	0.09±0.01^{b***}
GSH/GSSG ratio	40.44±3.78	47.33±4.16^{a*}	17.25±4.38^{a***}	35.44±3.94^{b***}
Redox index	0.21±0.03	0.24±0.02	0.10±0.02^{a***}	0.19±0.02^{b***}
Vitamin C	6.74±0.52	7.51±0.44^{a**}	4.83±0.47^{a***}	6.36±0.45^{b***}
Vitamin E	2.26±0.17	2.30±0.16	1.43±0.19^{a***}	2.06±0.22^{b***}

Values are expressed as mean±SD of six rats; Units: GSH and GSSG- µmol g^{-1} Hb; Vitamin C and E- µmol d L^{-1}; Statistical comparison: ^{a}: Compared with Group I; ^{b}: Compared with Group II; *: p<0.05, **: p<0.01, ***: p<0.001

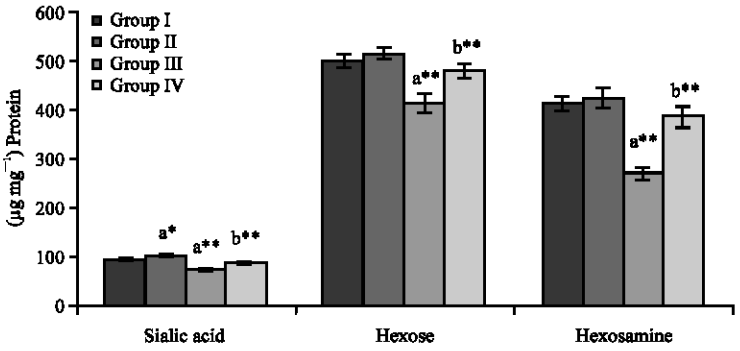

Fig. 1: Effect of *T. chebula* treatment on glycoproteins in erythrocytes of young and aged rats. Each bar represents the mean±SD of six rats. Statistical comparison: ^{a}: Compared with Group I; ^{b}: Compared with Group II; *: p<0.05, **: p<0.001

The levels of sialic acid, hexose and hexosamine in aged control rats showed significant decrease in p<0.001 (Fig. 1). *T. chebula* administration to aged rats showed a significant increase in 18.16, 13.69 and 30.05%, respectively. The increased level of sialic acid in young drug treated rats also observed at 7.14% (p<0.05).

Figure 2 shows that the levels of membrane ATPases such as Na^+K^+ATPase (25.73%), Ca^{2+}ATPase (35.55%) and Mg^{2+}ATPase (53.04%) were decreased in erythrocytes of aged control rats. Administration of *T. chebula* to aged rats increased the levels of Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase, respectively at 20.93, 31.93 and 47.78%.

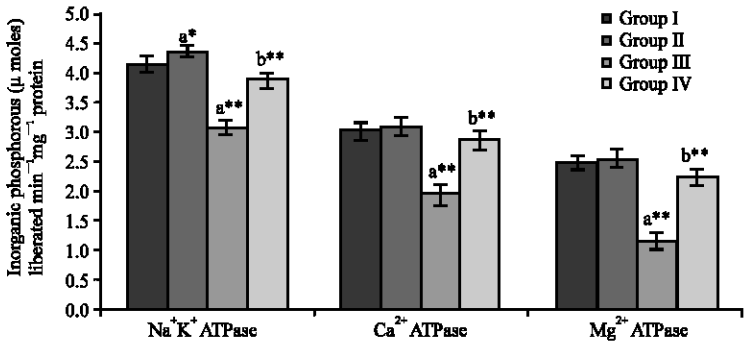

Fig. 2: Effect of *T. chebula* treatment on membrane ATPases in erythrocytes of young and aged rats. Each bar represents the mean±SD of six rats. Statistical comparison: [a]: Compared with Group I; [b]: Compared with Group II; *: $p<0.05$, **: $p<0.001$

DISCUSSION

According to the free radical theory of ageing, ageing is caused by the accumulation in the cell of macromolecules, such as DNA, proteins and lipids, that have been damaged by free radicals leading to the common final pathway for cell death (Sohal *et al*., 1995). Because of continuous generation of free radicals by the oxidation of hemoglobin, erythrocytes are exposed to continuous oxidative stress (Irene *et al*., 1992). There were positive correlations between the oxidative markers-MDA and PCO levels and aging. Nohl (1991) reported that accumulation of lipid peroxidation products increased during aging. These results are parallel with present findings. Upon *T. chebula* treatment, a decrease in MDA and PCO levels were observed. This may be due to reducing the amount of hydroxyl radicals and also as a scavenger of peroxide and superoxide radicals by several thiols (Haenen *et al*., 1989).

Imbalance between radical production and catabolism of oxidant during aging would shift the cells towards oxidative stress resulting in alterations of membrane properties and cell dysfunction. Cu, Zn-SOD is one of the major antioxidant enzymes in erythrocytes where superoxide radials ($O_2^{\bullet -}$) are continuously generated by the autooxidation of hemoglobin (Bernabucci *et al*., 2002). The decrement in Cu, Zn-SOD activity in the present study may be due to the inactivation of this enzyme by its product H_2O_2 (Ceballos-Picot *et al*., 1992). The increase in SOD activity in erythrocytes of young and aged rats on *T. chebula* treatment may be due to the potential quenching of free radicals by its phenoxyl radial, which significantly decrease the superoxide radicals level (Rice-Evans *et al*., 1996). CAT catalyses the reduction of H_2O_2 to H_2O and O_2 (Ceballos-Picot *et al*., 1992). According to present results, a significant decrease in CAT activity in erythrocytes with aging could be the decrease in NADH, since NADH is necessary for the conversion of CAT from its inactive state. Treatment with *T. chebula* increased the CAT activity in erythrocytes via the increase of G6PDH activity, which produces NADH by pentose pathway in erythrocytes. A major function of GP_X in erythrocytes may be the disposal of organic peroxides and the maintenance of protein thiols in their reduced states (Mueller *et al*., 1997). In the present study the GP_X activity was decreased in erythrocytes of aged rats, this may be the accumulation of the superoxide anion which inactivates GP_X by reacting with the selenium at the active site of the enzyme (Prabhu, 2002). *T. chebula* administration increased the GP_X activity by the protection of sulphydryl groups in glutathione from oxidative damages.

GR is an important enzyme for maintaining the intracellular concentration of reduced glutathione. The lowered activity of GR in erythrocytes of aged rats might be attributed to the excessive production of oxidized glutathione, which fails to match the capacity of GR, to reduce oxidized glutathione

(Sen *et al*., 1993). Oxidized glutathione is produced when peroxides are detoxified by GP_X and is recycled back to the reduced form by GR at the expense of NADPH (Mc Intyre and Curthoys, 1980). After administration of *T. chebula* aqueous extract, the GR activity was restored, which is indicative reduced oxidative stress. Also, the most important enzyme in the defense of the erythrocyte against oxidative attack is G6PDH, which catalyses the initial step of the pentose phosphate pathway and whose most important function is the reduction of nicotinaide adenine dinucleotide phosphate ($NADH^+$) to NADPH (Lord-Fontaine and Averill-Bates, 2002). In this study, the G6PDH activity was decreased in aged rats may be due to the oxidation of the active site of G6PDH, which contains an essential lysine residue (Naylor *et al*., 1996). *T. chebula* supplementation increased the activity of G6PDH in aged rats by producing more reducing equivalents and by the reduction of oxidized glutathione to reduced glutathione. The multifunctional enzyme, GSTs are performs several roles in the detoxification of broad spectrum of electrophilic reactive substances and drug metabolize (Jodynis-Liebert *et al*., 2000). GST catalyzes the transformation of peroxides to less toxic products conjugating them to reduced glutathione, it can be considered an antioxidant enzyme as well (Yi, 1990). In the present study the GST activity was lowered in erythrocytes of aged rats might be attributed to increased lipid peroxidation and depletion of glutathione status (Huang *et al*., 2003). GST activity was increased in *T. chebula* treated aged rats by inhibiting lipid peroxidation and increased availability of GSH from GSSG by the enzyme GP_X.

GSH plays a pivotal role in defending against oxidative haemolyses. Conditions that perturb intracellular levels of GSH have been shown to result in significant alterations in cellular metabolism. In the present study, the decreased levels of GSH may be the result of an increased oxidation of GSSG, increased degradation or decreased synthesis. *T. chebula* administration increase the levels by providing NADPH for the reduction of oxidized glutathione into reduced glutathione, catalyzed by GR and G6PDH. GSSG acts as a physiological indicator of the intracellular defense system against free radicals (Ding *et al*., 2002). An increase in GSSG levels in erythrocytes of aged rats was supported that enhanced oxidative challenge, such as by lipid peroxides, would be expected to result in depletion of the cellular GSH pool and a corresponding increase in GSSG (Aw, 1999). Administration with *T. chebula* decreased the GSSG levels by recycling of GSH from GSSG by the enzyme GR using NADPH as a cofactor. Measurement of GSH/GSSG levels has been used to estimate the redox environment of a cell (Schafer and Buettner, 2001). We observed a significant decrease in the redox index in aging, which may be related to the decreased GSH levels and increased GSSG concentrations. *T. chebula* treatment reverted to near normalcy in aged rats.

The decrease in the levels of vitamin C and E in aged control rats may be increased oxidative stress with aging. The decrease of ascorbate damages the erythrocyte membrane, since they are involved in regeneration of tocopherols, the lipophilic membrane antioxidant from its oxidized form. *T. chebula* reversed the decline of vitamins due to the increased ascorbic acid absorption, reduction of dehydroascorbate to ascorbic acid and preventing the involvement of hemoglobin iron in lipid peroxidation processes (Maffei Facino *et al*., 1996).

Oxidative stress increases MDA (Halliwell and Chirico, 1993) and the cytotoxic effects of MDA are well known than can induce a reduction of membrane fluidity (Chen and Yu, 1994) and increase erythrocyte membrane fragility (Spickett *et al*., 1998). A decreased erythrocyte membrane glycoproteins content were found in the present study in aged subjects compared with the young and a significant negative correlation was found between erythrocyte glycoproteins content and subject age. Sialylated glycoproteins are responsible for the negative charge of the erythrocyte membrane surface (Izumida *et al*., 1991). Therefore, the reduction in glycoproteins content might lead to decreased intercellular electrostatic repulsion and enhanced erythrocyte aggregation in the aging. *T. chebula* administration restored the glycoproteins in erythrocytes of aged rats might be due to either enhanced sialic acid synthesis or decreased sialidase activity.

Maintenance of membrane fluidity within narrow limits is a perquisite for proper functioning of RBCs and lipids and membrane-bound ATPases play a key role in this connection. Erythrocyte membrane bound enzymes are sensitive indices of altered cellular environment and responsible for

unidirectional activated transport of Ca^{2+} and Mg^{2+} ions. Na^{+} ions are extruded against the concentration gradient. Due to peroxidation of membrane lipids, the osmotic stability of the erythrocytes is altered in the presence of the divalent metal. The causative factor for the shortened survival and decreased deformability can be closely related to inhibition of the membrane-bound ATPases. Aging has been shown to reduce ATPases activity and K^{+} transport and to produce membrane changes in red cells. These changes may be more damaging to the cell and a more important cause of haemolysis than haemoglobin denaturation. Erythrocyte Ca^{2+}-ATPase is also reported to be potentially vulnerable to auto-oxidative modification because it has free thiol groups and resides in close proximity to unsaturated membrane lipids (Hebbel *et al*., 1986). Consequently this conclusion may be pertinent to $Na^{+}K^{+}$ATPase that has an environment in common with Ca ^{2}ATPase. In the present study, the inhibition of $Na^{+}K^{+}$ATPase, Mg^{2+}ATPase and Ca^{2+}ATPase was observed in the erythrocytes of aged control animals. Administration of *T. chebula* brought about a significant increase in the activities of these enzymes and this might be due to the ability of *T. chebula* to act as a reducing agent, thus helping to maintain membrane thiols essential for the activity of these enzymes in their reduced state.

All these results suggest that *T. chebula* is highly protective against oxidative damage and ageing. It balances the antioxidant system and stimulates metabolism of oxidative wastes. Thereby, *Terminalia chebula* can be acts as a potent drug for preventing age and age related degenerative diseases and improving the normal life.

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