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Protective effect of *Nardostachys jatamansi* in rat cerebral ischemia

Sofiyan Salim, Muzamil Ahmad, Khan Shoeb Zafar, Abdullah Shafique Ahmad, Fakhrul Islam*

Neurotoxicology Laboratory, Department of Medical Elementology and Toxicology, Jamia Hamdard University, Hamdard Nagar, New Delhi 110062, India

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Abstract

The protective effect of *Nardostachys jatamansi* (NJ) on neurobehavioral activities, thiobarbituric acid reactive substance (TBARS), reduced glutathione (GSH), thiol group, catalase and sodium–potassium ATPase activities was studied in middle cerebral artery (MCA) occlusion model of acute cerebral ischemia in rats. The right MCA of male Wistar rats was occluded for 2 h using intraluminal 4–0 monofilament and reperfusion was allowed for 22 h. MCA occlusion caused significant depletion in the contents of glutathione and thiol group and a significant elevation in the level of TBARS. The activities of Na^+K^+ ATPase and catalase were decreased significantly by MCA occlusion. The neurobehavioral activities (spontaneous motor activity and motor coordination) were also decreased significantly in MCA occlusion group. All the alternations induced by ischemia were significantly attenuated by 15 days pretreatment of NJ (250 mg/kg po) and correlated well with histopathology by decreasing the neuronal cell death following MCA occlusion and reperfusion. The study provides first evidence of effectiveness of NJ in focal ischemia most probably by virtue of its antioxidant property.

Keywords: Cerebral ischemia; *Nardostachys jatamansi*; Neuro behaviour; Oxidative stress; Neuroprotection

1. Introduction

Human cerebral ischemia most often results from a transient or permanent occlusion of the middle cerebral artery (MCA). It is one of the leading causes for several neurological deficit and death (Chagnac-Amitai and Connors, 1989). Several processes, such as intracellular calcium accumulation, massive release of excitatory amino acids, free radical formation, inhibition of protein synthesis, etc. have been implicated as initial trigger signals for the induction of ischemia (Bowe and Nadler, 1995; Gutnick et al., 1982; Kilpatrick et al., 1990). Increased free radical formation together with a reduced antioxidant defense causes oxidative stress that may play a pivotal role in the pathogenesis of stroke-associated neuronal injury (McCord, 1985; Braughler and Hall, 1989). Currently, there is evidence from both animal and human studies demonstrating that the oxidative damage to membrane lipids and proteins is increased during cerebral ischemia and reperfusion (Yoshida et al., 1980; Yamamoto et al., 1983). Free radicals are considered to cause lipid peroxidation as well as oxidation of proteins and nucleic acids, leading to membrane damage, enzymatic dysfunction, and finally to cell death (Seis, 1986). The Na^+K^+ ATPase is known to be an early target of oxygen radical induced damage to intact cell (Kim and Akera, 1987; Kako et al., 1988).

Nardostachys jatamansi (commonly known as Sambul lateeb or balchar) is a reputed Indian medicinal plant attributed with many CNS properties (Arora, 1965; Gupta et al., 1994). Recently, it has been shown to possess antioxidant (Tripathi et al., 1996) and GABA enhancing (Prabhu et al., 1994; Houghton, 1999) effects. The latter is known to be value in the treatment of ischemic stroke (Green et al., 2000). This prompted us to probe the plant for neuroprotective action against focal ischemia in rats.

2. Materials and methods

Oubain, adenosine 5′-triphosphate (ATP), thiobarbituric acid (TBA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and sulfosalicylic acid were purchased from Sigma-Aldrich

* Corresponding author. Tel.: +91-11-608-9688x5567; fax: +91-11-608-8874.
E-mail address: fislam2001@yahoo.co.in (F. Islam).

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Table 1
Effect of cerebral ischemia on neurobehavioral activities and its protection by NJ

Parameters	SHAM	MCAO	MCAO + NJ	NJ alone
Locomotor time (s)	157.33 ± 10.31	80.83 ± 11.88 ** (48.6%)[a]	126.17 ± 7.24[†] (19.8%)[a] (38.5%)[b]	139.33 ± 9.13 (11.4%)[a]
Rest time (s)	142.67 ± 10.31	219.17 ± 11.88 ** (53.6%)[a]	173.83 ± 7.24[†] (21.8%)[a] (20.7%)[b]	160.67 ± 9.13 (12.6%)[a]
Distance traveled (cm)	3127.50 ± 251.49	1473.66 ± 212.76 ** (52.9%)[a]	2367.66 ± 140.50 **,[†] (24.3%)[a] (60.7%)[b]	2690.33 ± 187.09 (14.0%)[a]
Average speed (cm/s)	625.17 ± 50.39	294.17 ± 42.58 ** (52.9%)[a]	473.16 ± 28.18 *,[†] (24.3%)[a] (60.8%)[b]	540.33 ± 37.34 (13.6%)[a]
Rota-rod	172.83 ± 3.98	54.73 ± 6.70 ** (68.3%)[a]	99.8 ± 6.65 **,[†] (42.2%)[a] (73.2%)[b]	123.3 ± 7.24 ** (28.6%)[a]

Values are expressed as mean ± S.E.M. Significance was determined by ANOVA followed by Dunnett's test.
[a] Values in parentheses indicate the percentage change vs. sham.
[b] Values in parentheses indicate the percentage change vs. MCAO.
* $P < .05$.
** $P < .01$ vs. sham.
[†] $P < .05$ vs. MCAO.

Foreign Holding Chemical, India. Other chemicals were of analytical grade.

The root of NJ De Jones (Valerianaceae) was procured from herbal market of New Delhi. It was identified and authenticated by the taxonomist of Department of Environmental Botany (Jamia Hamdard, New Delhi). The specimen has been kept in the Herbarium Department of Ilmul Advia (Faculty of Medicine) under voucher specimen No. JH/T-05/1. The roots were dried in shade, cleaned and powdered to prepare the alcoholic extract as earlier described by Prabhu et al. (1994). One kilogram of moderately powdered roots of NJ was extracted by refluxing with 95% ethyl alcohol in Soxhlet extractor for 6–8 h. The extract was evaporated to dryness under reduced pressure and temperature using rotatory vacuum evaporator (Radhakrishnan et al., 2001) and dried residue was weighed and stored at 4 °C. The yield of dry extract from crude powder of NJ was 10%. The alcoholic extract of NJ was suspended in a mixture of ethanol:Tween 80:distilled water in a ratio of 1:2:5.

The animals were used in accordance with the procedure approved by the Animal Ethics Committee of Jamia Hamdard. Male Wistar rats weighing 250–300 g (Central Animal House, Jamia Hamdard) were housed under constant environmental conditions and allowed free access to pelleted food and water ad libidum. Animals were divided into four groups each having six animals. The first group served as sham, second was MCAO, i.e., ischemia, was induced for 2 h followed by 22 h of reperfusion, third was pretreated with NJ for 15 days followed by MCAO, i.e., MCAO + NJ group, and fourth was treated with drug alone, i.e., NJ alone with a dose of 250 mg/10 ml (vehicle)/kg b.w. through oral route for a period of 15 days. The animals of each group were sacrificed 22 h after reperfusion and brains were dissected out for biochemical estimation. The neurobehavioral activity was evaluated before the sacrifice.

The right MCA occlusion was produced using an intraluminal filament model (Longa et al., 1989). The rats were anesthetized with chloral hydrate (400 mg kg^{-1} ip), a 4–0 nylon monofilament, the tip of which was rounded with the use of an open flame, was inserted into the external carotid artery and advanced into the internal carotid artery until a slight resistance was felt. Such resistance indicated that the filament had passed beyond the proximal segment of the anterior cerebral artery. At this point, the intraluminal filament blocked the origin of the MCA and occluded all sources of blood flow from the internal carotid artery, anterior cerebral artery and the posterior cerebral artery. Throughout the procedure, a thermocouple was inserted into the rectum and body temperature was maintained at 37 ± 0.5 °C with a thermostatically controlled infrared lamp. Two hours after the induction of ischemia, the filament was slowly withdrawn until tip reached external carotid artery. Animals were then returned to their cages and closely monitored until they recovered from anesthesia. In sham-operated rats, the external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted.

The brain was weighed and homogenized in ice-cold Tris–HCl buffer (pH 7.2) and centrifuged at 1000 rpm for 3 min at 4 °C. The supernatant contained crude membranes

Table 2
Effect of cerebral ischemia on TBARS, GSH and thiol group and protection with alcoholic extract of NJ

Parameters	SHAM	MCAO	MCAO + NJ	NJ alone
TBARS (nmol/g tissue)	39.14 ± 2.13	58.23 ± 1.78 ** (48.0%)[a]	46.20 ± 1.41 *,[††] (18.0%)[a] (20.6%)[b]	37.78 ± 1.14 (3.5%)[a]
GSH (μmol/g tissue)	1.50 ± 0.08	0.61 ± 0.04 ** (59.3%)[a]	1.17 ± 0.05 **,[††] (22.0%)[a] (91.8%)[b]	1.53 ± 0.04 (2.0%)[a]
Thiol group (μmol/g tissue)	10.2 ± 0.80	5.6 ± 0.60 ** (45.1%)[a]	8.6 ± 0.40[††] (15.7%)[a] (53.6%)[b]	10.9 ± .0.40 (6.8%)[a]

Values are expressed as mean ± S.E.M. Significance was determined by ANOVA followed by Dunnett's test.
[a] Values in parentheses indicate the percentage change vs. sham.
[b] Values in parentheses indicate the percentage change vs. MCAO.
* $P < .05$.
** $P < .01$ vs. sham.
[††] $P < .01$ vs. MCAO.

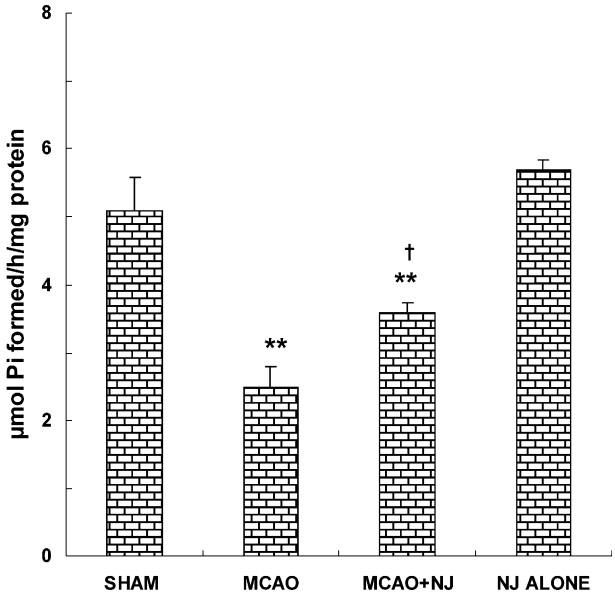

Fig. 1. Brain Na^+K^+ ATPase activity and its protection with NJ in cerebral ischemia–reperfusion. The unit of enzyme is expressed as µmol pi formed/h/mg protein. Values are expressed as mean ± S.E. Significance was determined by ANOVA followed by Dunnett's test. $^{**}P<.01$ vs. sham, $^{\dagger}P<.05$ vs. MCAO.

were used for the estimation of Na^+K^+ ATPase, TBARS and thiol groups. The remaining supernatant was again centrifuged at 10,000 rpm for 15 min at 4 °C. The post-mitochondrial supernatant was used for the study of catalase activity. The Na^+K^+ ATPase activity was determined as inorganic phosphorous (pi) release, using the method of Sovoboda and Mossinger (1981). The method of Utley et al. (1967) was used with slight modification as described by Shah and Vohara (2002) for the estimation of TBARS formation. The catalase activity was determined by the method of Claiborne (1985). The method of Jollow et al. (1974) was used for the estimation of GSH. The thiol group was determined by the method of Sedlak and Lindsay (1968). The neurobehavioral activity in MCA occlusion model of focal ischemia with slight modification (Yamamoto et al., 1988) was monitored in video path analyzer (Coulbourn Instrument, USA) by measuring the locomotion, rest, distance traveled and speed of the animal. Motor in coordination was evaluated using Rota-rod apparatus (Omneteed Columbus, OH, USA). Animals were kept on the rotor and the speed was maintained at 10 rpm. The motor in coordination was evaluated as the ability of rat to hold the rotating rotor. The time for which the rat held the rotor was calculated and the results were expressed in seconds. The cut-off time was 3 min. For histopathological study, the brains of control and experimental groups in focal ischemia were perfusion-fixed as previously described by Nakayama et al. (1988) with a mixture of formaldehyde (40%), glacial acetic acid and methanol (1:1:8 v/v). The tissues were cut into 3-mm thickness and its blocks were embedded in paraffin. Sections 4–5-µm-thick were cut in the coronal plane and stained with haematoxylin and eosin.

The data were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's test.

3. Results

Table 1 shows the effect of MCAO and its protection by NJ on locomotion, rest, distance traveled, average speed and motor in coordination. The above neurobehavioral activities were decreased significantly ($P<.01$) in MCAO group and increased significantly ($P<.05$) in MCAO + NJ group as compared to their corresponding controls. The NJ alone group has shown a non significant depletion in the above neurobehavioral activities as compared to sham.

Table 2 shows the levels of TBARS, GSH, thiol group and their protection with NJ. The content of TBARS was significantly increased in MCAO ($P<.01$) as compared to sham but its level was attenuated significantly ($P<.01$) in MCAO + NJ group as compared to MCAO group. The content of GSH was depleted significantly ($P<.01$) in MCAO group as compared to sham. On the other hand, GSH level was found to be elevated significantly ($P<.01$) in MCAO + NJ group as compared to the MCAO group. The level of thiol group was depleted significantly ($P<.01$) in MCAO group as compared to sham and its level was protected significantly ($P<.01$) in MCAO + NJ group as compared to MCAO group.

Fig. 1 shows the effect of MCAO on Na^+K^+ ATPase activity and its protection by NJ. The Na^+K^+ ATPase activity was depleted significantly in MCAO group

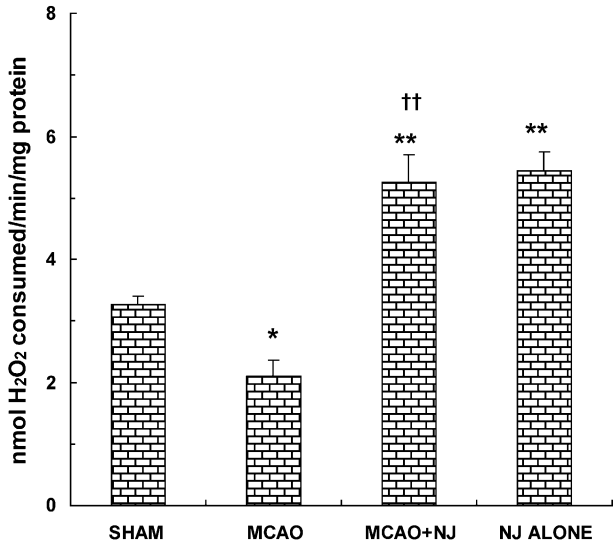

Fig. 2. Brain catalase activity and its protection with NJ in cerebral ischemia–reperfusion. The unit of enzyme is expressed as nmol H_2O_2 consumed/min/mg protein. Values are expressed as mean ± S.E. Significance was determined by ANOVA followed by Dunnett's test. $^{*}P<.05$ and $^{**}P<.01$ vs. sham, $^{\dagger\dagger}P<.01$ vs. MCAO.

(51.1%) as compared to sham. Pretreatment of MCAO group with NJ for 15 days has protected the activity significantly (44.2%) in MCAO + NJ group as compared to MCAO group. In the NJ alone group, the activity of Na^+K^+ ATPase was increased nonsignificantly (11.8%) as compared to sham. The catalase activity was depleted significantly (35.3%) in MCAO group as compared to sham and its activity was enhanced significantly in MCAO + NJ group (148.8%) when compared with corresponding control (Fig. 2). Also, the activity of the catalase was increased significantly (67.2%) in NJ alone group as compared to sham.

Fig. 3 shows the histopathological changes after 2 h of occlusion and 22 h of reperfusion and protection with NJ (250 mg/kg b.w. for 15 days) in focal cerebral ischemia. The neurodegeneration was observed after ischemia/reperfusion but the pretreatment with NJ markedly reverse these changes more comparable to sham group.

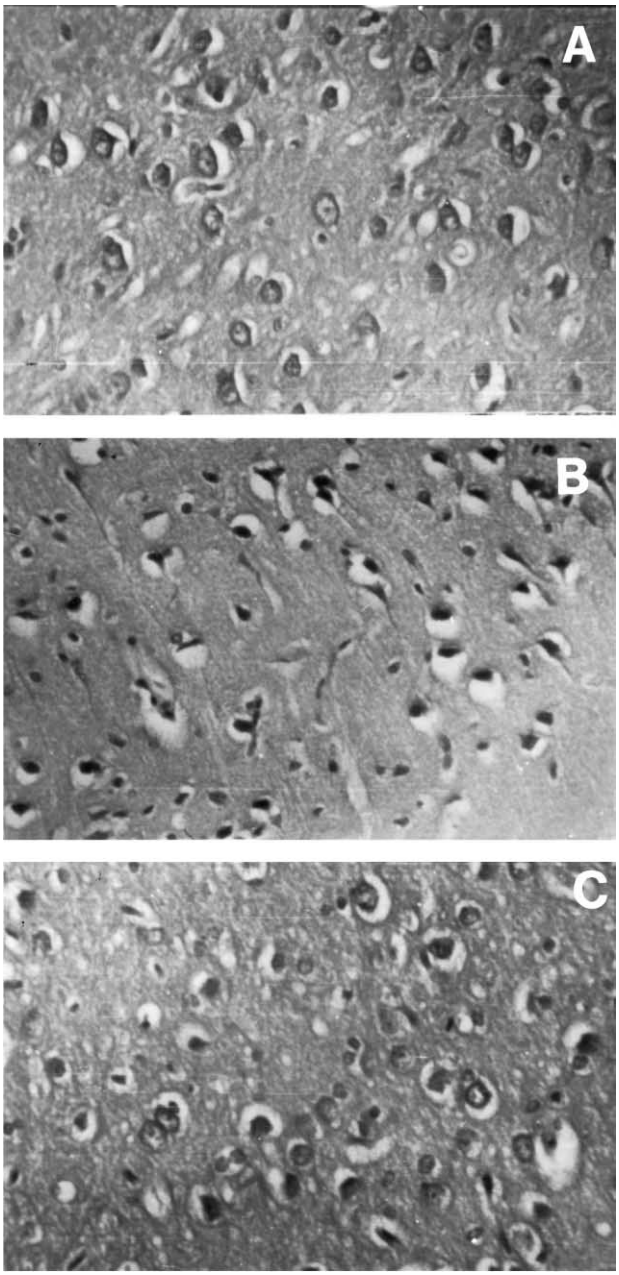

Fig. 3. Representative photographs of coronal sections of brain after 2 h of occlusion and 22 h of reperfusion in MCA-occluded rats. (A) Sham (B) ischemia/reperfusion only, (C) ischemia/reperfusion with 15 days pretreatment of NJ 250 mg/kg b.w., respectively. Each section was stained with haematoxylin and eosin. Magnification (A–C) × 20.

4. Discussion

The pretreatment with alcoholic extract of NJ in cerebral ischemia for 15 days has shown a significant improvement in motor coordination and spontaneous motor activity after 2 h of occlusion and 22 h of reperfusion. The mechanism by which NJ exerts its effect is not clearly known but there is substantial evidence that drug enhances the function of γ-amino butyric acid (GABA) (Hamied et al., 1962, Prabhu et al., 1994; Houghton, 1999) and it has been suggested that GABA potentiate the treatment of acute ischemic stroke (Green et al., 2000). In cerebral ischemic injury, excessive accumulation of glutamate takes place in the extracellular fluid (Choi, 1988; Benveniste, 1991), which can induce excessive activation of *N*-methyl-D-aspartate (NMDA) or 2-amino-3-hydroxy-5-isoxazolepropionate (AMPA)/kainate receptors resulting in accumulation of intracellular fluid, sodium and calcium ions (Coyle and Puttfarcken, 1993). Enhancement of GABA function may hyperpolarize the neurons thereby protecting them from excitotoxicity. It is proposed that NJ stimulates the GABA ergic neurons, which antagonizes the effect of glutamate and protects the neurons from cerebral ischemia.

Glutamate is also associated with oxidative stress in neurodegenerative disorders (Coyle and Puttfarcken, 1993), while production of reactive oxygen species is one of the main causes of ischemic brain injury. Accumulation of neurotoxic free radicals and consequent neurodegeneration results from decreased function of free radical scavenging antioxidants including ascorbate, superoxide dismutase and GSH in ischemia (Fridovich, 1978; Adlard et al., 1973; Folbergrova et al., 1979). The GSH which may serve functions in intra- and intercellular signaling in brain such as: (a) uptake, synthesis and release of glutamate and GABA (Weber, 1999; Yang et al., 1995); (b) a substrate (Chang et al., 1987) and an allosteric modulator (Li et al., 1997) of eicosanoid biosynthesis; (c) regulation of NMDA and non-NMDA glutamate receptors activity (Ogita et al., 1995; Oja et al., 2000); (d) neuronal depolarization by acting at ionotropic receptors of its own (Bians et al., 1997; Oja et al., 2000); and (e) activation of transcription factors (Hauzi et al., 1999). It is note worthy that genetic deficiencies in GSH-related enzymes lead to marked impairment of nervous system function (Cooper and Meister, 1992). Besides, GSH metabolism suffers changes in cognitively impaired

animals that can be attenuated by neurotrophic impairment (Cruz-Auguado et al., 1998).

Besides its role in behavioural activity (Cruz-Auguado et al., 2001), it also acts as one of the guarding factors against oxidative stress (Lovine, 1982). Thus, GSH inhibition in cerebral ischemia would increase the susceptibility of plasma membrane towards peroxide attacks. However, the main cause of GSH loss during oxidative stress in brain ischemia is the formation of protein glutathione mixed disulphides (PrSSG) and loss of thiol proteins (Ravindranath and Reed, 1990). The loss of GSH and formation of PrSSG in the brain results the various membrane dysfunction, such as inhibition on Na^+K^+ ATPase activity (Reed, 1990). The enzyme is essential for cellular excitability and is very susceptible to free radical reaction and lipid peroxidation because it is embedded in cell membrane and requires phospholipids for the maintenance of its activity (Cooper et al., 1980; Furui et al., 1990; Ildan et al., 1996). While the catalase, which removes H_2O_2 and its depletion, is known to be a factor that contributes to brain injury and cerebral oedema following cerebral ischemia (Patt et al., 1988), found to be decreased significantly in vehicle treated group. Furthermore, in the presence of Fe^{3+} and superoxide anion, hydrogen peroxide is partly converted to hydroxyl radicals, which are highly reactive with the membrane constituents (Mak et al., 1992) may elevate lipid peroxidation.

The glutathione, which affects the neurobehavioral activity and guards the membrane from peroxidative attacks, were increased significantly with NJ treatment, which may reduce the TBARS formation in ischemic/reperfusion. On the other hand, it has increased the activities of catalase and Na^+K^+ ATPase significantly in MCAO + NJ group. The significantly increased catalase activity in MCAO + NJ group has utilized the production of H_2O_2, which was produced during ischemia/reperfusion may be another factor of TBARS depletion in the brain.

The present results demonstrate that pretreatment with NJ for 15 days protected postischemic histological, behavioral and biochemical changes in MCAO + NJ group. The effect appears to be mediated through (a) improving the glutathione content, (b) inhibiting lipid peroxidation and (c) effects on Na^+K^+ ATPase and catalase activity. These observations correlated well with histopathological changes by attenuating the postischemic neuronal cell death in MCAO + NJ group vs. the sham-operated group. This is in agreement with earlier reports (Hunter et al., 1998; Sinha et al., 2002). The observed depletion of GSH, total-SH, catalase, Na^+K^+ ATPase and elevation of TBARS in MCAO group leading to neurodegeneration is confirmed by histopathological differences between the treated and control groups. This suggests that antioxidant property of NJ (Tripathi et al., 1996) may be a possible mechanism in protecting the neurons, by increasing the endogenous defensive capacity of the brain to combat ischemia reperfusion-induced oxidative stress.

Thus, NJ may be considered as a potential candidate in the armamentarium of drugs for the prophylactic treatment in patients which are prone to stroke.

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