Research Article

Role of *Urtica dioica* in prevention of ischemia-reperfusion induced cerebral damage and neurobehavioral alterations in rats

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Abstract

Objective: The present study was carried out to evaluate cerebro-protective potential of *Urtica dioica* against ischemia-reperfusion induced cerebral damage and neurobehavioral alterations in rats. **Material and methods:** In order to evaluate the cerebroprotective activity, we have chosen *in vivo* bilateral common carotid artery occlusion induced cerebral ischemia-reperfusion injury model (30 min ischemia and 24 h reperfusion) and *Urtica dioica* (0.2, 0.4, 0.8 ml/kg) treatment was given for seven days prior to induction of ischemia and reperfusion. Cerebral damage was determined using TTC staining technique. We employed Elevated plus maze test to assess memory and Locomotor activity, Inclined beam walking test (neurological score) and Hanging wire test for evaluation of motor co-ordination. **Results:** Severe cerebral damage was observed in rats subjected to ischemia-reperfusion. *Urtica dioica* treatment dose dependently offered cerebro-protection by significantly reducing the infarct size. Marked impairment of memory and motor co-ordination in parallel to the significant increase in the infarction size was observed in rats subjected to ischemia-reperfusion injury. Pretreatment with *Urtica dioica* significantly attenuated ischemia-reperfusion induced neurobehavioral alterations in the rats. **Conclusion:** This work suggests that *Urtica dioica* may improve the therapeutic outcome of stroke, if administered in conjunction with thrombolytic therapy.

Keywords: *Urtica dioica,* cerebro-protective, ischemia-reperfusion, neurobehavioral

Introduction

Stroke and cardiac arrest are major causes of mortality and disability worldwide, affecting millions of people and accounting for the highest health-care expenses of all diseases. Stroke is a cerebrovascular disorder characterized by the sudden disruption of blood supply to the brain, which, if not resolved quickly, results in severely ischemic brain tissue, eventually leading to cell death (Beal, 2010). According to the World Health Organization's most recent statistics, around 15 million individuals worldwide suffer from a stroke. One-third of these people died (3 million women and 2.5 million men) and one-third are permanently disabled (Thrift et al., 2017).

Part of the ischemic tissue in which the cells are irreversibly damaged is called "infarction". The severity of stroke outcome depends on the extent of infarct size (Lipton, 1999). Reperfusion therapy, such as thrombolytics or bypass surgery, can help to reduce the pathological consequences of a stroke. However, several animal and clinical investigations have demonstrated that overproduction of reactive oxygen species and excessive inflammatory response occurs in the early reperfusion period, which potentiates the ischemic damage (Leira et al., 2006; Pan et al., 2007). As a result, oxidative stress and inflammation are thought to be important contributors to cerebral ischemia-reperfusion injury. The inhibition of reactive oxygen species formation, inflammatory cell activation, pro-inflammatory cytokine release and apoptotic gene expression has shown to protect against cerebral ischemia-reperfusion injury (Lin et al., 2016).

Ischemia-reperfusion induced brain damage is linked to a wide range of neuro-psychiatric problems, including motor, behavioural and cognitive disorders (Jenkins et al., 1981). Memory deficits and sensorimotor abnormalities have been observed in global cerebral ischemia subjects in preclinical and clinical studies (Yan et al., 2007; Gaur et al., 2009). At a four-year follow-up, around one-third of stroke survivors screened during hospitalization were found to fulfil dementia criteria and about 80% of patients with acute stroke present with focal weakness or paralysis (Chemerinski and Robinson, 2000).

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In diverse experimental settings, a number of traditional medicines with potent antioxidant and anti-inflammatory activities have been shown to reduce brain damage caused by ischemia-reperfusion. Plants, such as *Nigella sativa, Ginkgo biloba, garlic, Tanshinone, Panax ginseng, Bacopa monnieri, Withania somnifera, Centella asiatica, Curcumin,* and *Ocimum sanctum* been shown to protect against ischemia-reperfusion induced brain damage in various experimental stroke models (Wu et al., 2010).

Urtica dioica (UD) is a well-known ethnomedicinal plant, is also known as common stinging nettle. It has a long history of use in Indian traditional folk medicine (Mamta and Preeti, 2014). In several experimental animal models and clinical studies, it has been shown to have potent antioxidant and anti-inflammatory properties (Upton, 2013). However, no research on neuroprotective effect of Urtica dioica on ischemia-reperfusion brain injury has ever been done. Thus, it was considered worthwhile to investigate the potential role of *Urtica dioica* on ischemia-reperfusion induced cerebral injury in rats. Therefore, in the present investigation we have planned to explore cerebro-protective role of *Urtica dioica* against ischemia-reperfusion induced cerebral damage and, neurobehavioral alterations.

Materials and methods

Plant extract

Ethanolic extract of UD in the form of homeopathic mother tincture was purchased from a local medical store (manufactured by 'Bhandaris Pvt. Ltd', Batch no. M2/07, manufacturing date 11/2012, with 48% ethanol as vehicle .drug strength 1/10).

UD is prescribed as a mother tincture in homeopathy. The whole plant is used to make a homoeopathic. A mother tincture is essentially an alcoholic extract prepared according to the procedure described in Homoeopathic Pharmacopoeia (Sinha and Saha, 1980). Both the US Homoeopathic Pharmacopoeia and the German Homoeopathic Pharmacopoeia cover it. A mother tincture, despite being a homoeopathic formulation, is equally effective as an alcoholic extract often used by researchers in phytochemical or biological studies (Kumar and Sharma, 2005; Nimgulkar et al., 2011; Khuda-Bukhsh et al., 2011; Ghosh et al., 2013).

Dose Selection

In this study, 3 doses (0.2, 0.4, 0.8 ml/kg) of *Urtica dioica* were used. The doses were chosen based on previous experimental studies in animals with *Urtica dioica* and extrapolation from the doses used for clinical use. The use of UD has been reported safe in acute and chronic toxicity studies in mice, at the dose levels of 250, 500, 1000, 2000 mg/kg body weight. Typical daily dosages of UD for clinical use include, 360 mg aqueous extract, 460 mg dried extract, and 600 mg freeze-dried preparation. Tincture of the plant is taken at a dose of 1/2-1 tsp (2-5 mL) three times daily (Mamta and Preeti, 2014).

Animals

Wistar male albino rats (230–300g) were obtained from Srinivasa Enterprises, Hyderabad, Telangana, India. The animals were kept under standard laboratory conditions, maintained on 12 h light/dark cycle, had free access to food and water. Animals were acclimatized to laboratory conditions before the test. Each animal was used once in the experiment. Experimental protocol was approved by the Institutional Animal Ethics Committee and was conducted according to the CPCSEA guidelines for the use and care of experimental animals (Regd. No. 516/PO/C/01/CPCSEA).

Experimental Protocol

The rats were divided into seven groups of 6 rats each and following experimental protocol was used. Group 1:Normal control; Group 2: Sham control, received surgery, without BCCA occlusion; Group 3: I/R control, received 30 min BCCA occlusion and 24 hours reperfusion; Group 4: Vehicle control, received 0.8 ml/kg of 24% ethanol (double dilution of tincture) for 7 days prior to induction of ischemia and reperfusion; Group 5: Urtica dioica (0.2 ml/kg) Group 6: Urtica dioica (0.4 ml/kg) Group 7 : Urtica dioica (0.8 ml/kg). Prior to induction of ischemia and reperfusion, the rats in the treatment groups (Group-5, 6 and 7) received respectively 3 doses of Urtica dioica (0.2, 0.4, 0.8 ml/kg) intraperitonealy daily for seven days.

At the end of experimental procedure, all the rats were assessed for neurobehavioral outcome. Then the rats were sacrificed, % cerebral infarction was measured.

Induction of Cerebral Ischemia-Reperfusion (I/ R) injury in rats

Induction of cerebral ischemia-reperfusion injury was carried as modified method of Jingtao (Jingtao et al., 1999). Animals were anesthetized by giving thiopentone sodium (45 mg/kg, i.p). Both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternocleidomastoid and the sternohyoid muscles parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. A cotton thread was passed below each carotid artery. The induction of ischemia was performed by occluding bilateral common carotid arteries (BCCAO) for 30 min. After 30 min of ischemia arteries were freed from occlusion by removing threads. Then it was confirmed for reflow of blood. The skin was closed with stitches using waxed silk suture. Reperfusion was allowed for 24 hours. During the BCCAO, animals were observed for the following criteria: maintenance of dilated pupils, absence of a corneal reflex

when exposed to strong light stimulation, and maintenance of rectal temperature at (37°C). Animals which did not match these criteria and showed seizures were excluded from the study. Sham control animals received surgery, without BCCAO.

Measurement of percentage cerebral infarction

Animals were sacrificed by cervical dislocation and the brain was removed immediately. Then it was washed with ice cold saline. The brain was wrapped in aluminium foil and kept at −4°C. Frozen brain was sliced into uniform sections of 2 mm thickness. The slices were incubated in 1% 2,3,5-Triphenyl tetrazolium chloride (TTC), purchased from Sigma Aldrich, India, dissolved in phosphate-buffered saline (pH 7.4) at 370C for 30 min. TTC is converted to red formazone pigment by nicotinamide adenine dinucleotide (NAD) and dehydrogenase present in living cells. Hence viable cells were stained deep red. The infarcted cells loose these enzymes and, thus remained unstained 110 (Bederson et al., 1986). Pale, necrotic infarcted tissue was separated and weighed. Percentage cerebral infarction was calculated.

Assessment of neurobehavioral parameters

Locomotor activity

The Locomotor activity (ambulatory activity) was recorded by using actophotometer. Before Locomotor task, the animal was placed individually in the activity meter for 3 min for habituation. Thereafter, Locomotor activity was recorded using actophotometer for a period of 5 min. It was recorded and expressed in terms of total photo beam count per 5 min (Reddy and Kulkarni, 1998).

Hanging wire test

This task was used to measure gripping and forelimb strength of the rats after ischemiareperfusion induced brain injury. In this test, animals were suspended by the forelimbs on a wire stretched between 2 posts 60 cm above a foam pillow. The time (in seconds) until the animal fell was recorded. The cut off time was taken as 90 Sec (Hunter et al., 2000).

Inclined beam walking test (Neurological Score)

The inclined beam walking test was used to evaluate fore and hind limb motor coordination. Each animal was individually placed on a wooden bar, inclined at an angle of 60° from the platform. The motor performance of rats was scored on a scale ranging from 0 to 4. A score of 0 was assigned to an animal that could readily traverse the beam. Score 1, 2 and 3 were given to animals demonstrating mild, moderate and severe impairment, respectively. Score 4 was assigned to the animals completely unable to walk on the beam (Feeney et al., 1981).

Elevated plus maze test for memory

Elevated plus maze was used to assess memory dysfunction. It consists of two opposite open arms (50×10 cm), crossed with two closed arms of the same dimensions with 40 cm high walls. The arms are connected with the central square (10×10 cm). Acquisition of memory was assessed on day 6th, day just before the surgery. The rat was placed individually at one end of an open arm facing away from the central square. The time taken by animal to move from open arm and enter into one of the closed arms was recorded as initial transfer latency (ITL). The rat was allowed to explore the maze for 30 s after recording ITL and returned to its home cage (Hock, 2015).

Statistical analysis

All the values are expressed as mean ± S.E.M. Wilcoxon rank sum test was used for the neurological scores. The data of all the other experiments were analyzed using One way analysis of variance (ANOVA) followed by Tukey's post hoc test. In all the tests, criterion for statistical significance was $P < 0.05$.

Results

Effect of *Urtica dioica* on percentage cerebral infarction

Percentage cerebral infarction in vehicle control rats was 62.95 ± 2.460. It was significantly reduced by 24%, 56%,

Table 1. Effect of *Urtica dioica* on Percentage Cerebral Infarction

Groups (n=6)	% Cerebral Infarction	% Reduction of Infarction
Normal	1.690 ± 0.335	
Sham control	3.192 ± 0.375	
I/R control	62.47 ± 2.340$^{###}$	
Vehicle control	62.95 ± 2.460	
Urtica dioica (0.2 ml/kg)	47.88 ± 2.561***	24
Urtica dioica (0.4 ml/kg)	35.20 ± 1.337***	56
Urtica dioica (0.8 ml/kg)	23.87 ± 1.335***	62

I/R (ischemia/reperfusion). Results were represented as mean ± S.E.M. (n = 6). ANOVA and Tukey's post hoc test was used for statistical analysis of data. ### Significantly different from Sham control group at $P < 0.001$. *** Significantly different from I/R control group at $P < 0.001$

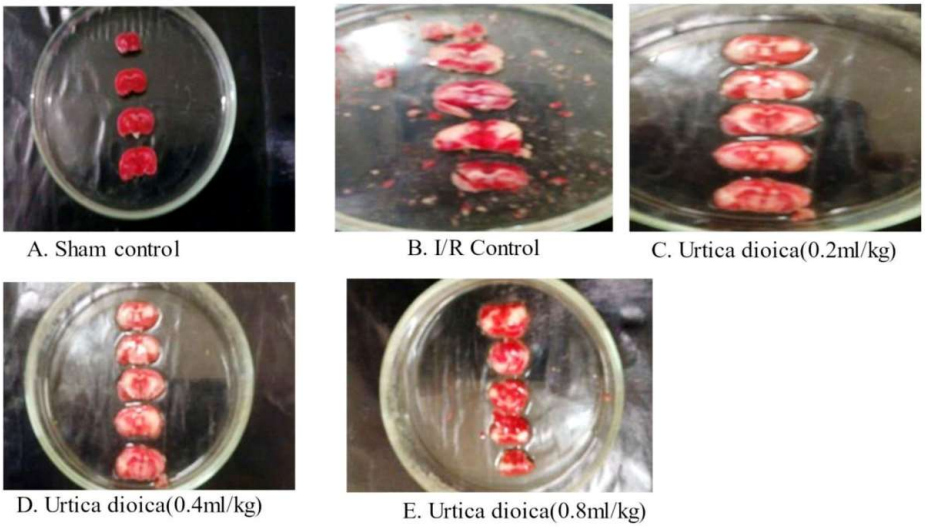

Figure 1. Photograph of brain sections stained with TTC Pale, unstained section indicates infarcted tissue and darkly stained section indicates viable (non-necrotic) tissue

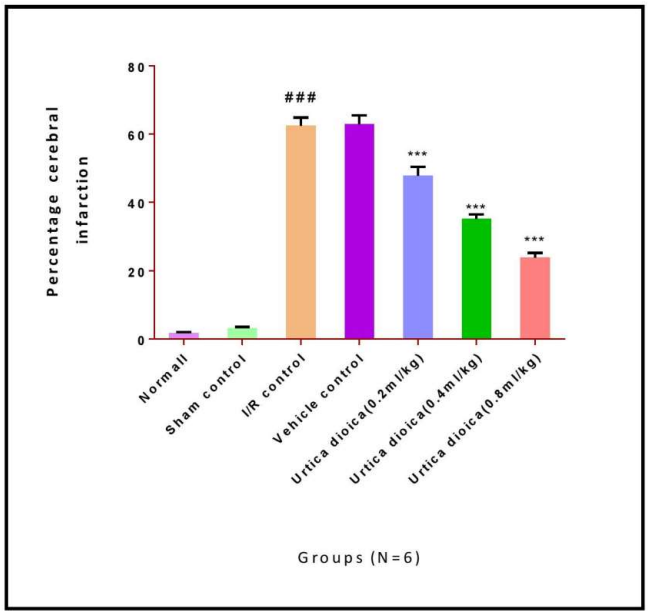

Figure 2. Effect of *Urtica dioica* on brain infarction volume in rats exposed to cerebral ischemia/reperfusion. I/R (ischemia/reperfusion). Results were represented as mean ± S.E.M. (n = 6). ANOVA, and Tukey's post hoc test was used for statistical analysis of data. ### Significantly different from Sham control group at $P < 0.001$. ***Significantly different from I/R control group at $P < 0.001$.

and 62% respectively with the treatment of 0.2, 0.4, 0.8 ml/kg doses of Urtica dioica for 7 days prior to induction of ischemia and reperfusion, indicating the cerebro-protective action of Urtica dioica (Figure 1, Figure 2 and Table 1).

Effect of *Urtica dioica* on neurological score (Inclined beam walking test)

This test was used to evaluate fore and hind limb motor co-ordination. In the rats subjected to 30 min of BCCA occlusion followed by 24 hours of reperfusion there was significant elevation of neurological score, indicating impairment in motor co-ordination as compared to the sham control rats. Neurological Score was not significantly influenced with Urtica dioica (0.2 142 ml/kg) pretreatment as compared to the vehicle control rats. On the contrary, Urtica dioica (0.4, 0.8 ml/kg) significantly and dose dependently improved neurological score, the motor impairment as compared to the vehicle control rats (**Table 2**).

Effect of *Urtica dioica* on locomotor activity

Challenging the animals with 30 min of BCCA occlusion followed by 24 h of reperfusion significantly impaired locomotor activity as compared to the sham control rats. Pretreatment with Urtica dioica (0.2, 0.4, 0.8 ml/kg) significantly improved the locomotor activity in a dose dependent manner as compared to vehicle control rats (**Table 2**).

Effect of *Urtica dioica* on hanging wire latency time

This task was used to measure gripping and forelimb strength of the rats. In the rats subjected to 30 min of BCCA occlusion followed by 24 hours of reperfusion there was significant decrease in hanging wire latency time, indicating impairment in grip strength as compared to the sham control rats. Urtica dioica (0.4, 0.8 ml/kg) pretreatment showed significant and dose dependent increase in the hanging wire latency time as compared to the vehicle control rats. However, Urtica dioica (0.2 ml/kg) did not influence hanging wire latency time significantly as compared to the vehicle control rats (Table 2).

Table 2. Effect of *Urtica dioica* on Neurobehavioral Parameters

Groups (n=6)	Neurological score	Locomotor Activity (count/5 min)	Hanging wire Latency time (sec)	Transfer latency time (sec)
Normal	0.33 ± 0.211	253.8 ± 2.496	71.50 ± 2.405	36.67 ± 1.308
Sham control	0.33 ± 0.211	251.5± 3.253	69.50 ± 2.579	38.33 ± 1.542
I/R control	2.83 ± 0.307$^{###}$	146.3 ± 2.418$^{###}$	24.00 ± 1.949$^{###}$	77.00 ± 1.414$^{###}$
Vehicle control	2.67 ± 0.211	150.5± 2.432	23.50± 2.487	78.67 ± 1.706
Urtica dioica (0.2 ml/kg)	2.33 ± 0.211	167.0± 2.708**	32.33 ± 1.892	66.83 ± 1.600**
Urtica dioica (0.4 ml/kg)	1.67 ± 0.211*	208.7± 3.029***	43.17 ± 1.701***	56.83 ± 1.621***
Urtica dioica (0.8 ml/kg)	0.83 ± 0.307***	235.7 ± 2.963***	56.17 ± 2.151***	49.83 ± 1.302***

I/R (ischemia/reperfusion). Results were represented as mean ± S.E.M. (n = 6). Results were analyzed by one way ANOVA, followed by Tukey's multiple comparison test. ### Significantly different from Sham control group at P < 0.001. ** Significantly different from I/R control group at p < 0.01. *** Significantly different from I/R control group at P < 0.001. * Significantly different from I/R control group at P < 0.05.

Effect of *Urtica dioica* on transfer latency time (Elevated plus maze test)

This test was used to assess memory function in rats. Challenging the animals with 30 min of BCCA occlusion followed by 24 h of reperfusion resulted in significant increase in the transfer latency time, indicating memory impairment as compared to the sham control rats. Pretreatment with Urtica dioica (0.2, 0.4, 0.8 ml/kg) significantly attenuated the memory impairment in a dose dependent manner as compared to the vehicle control rats (Table 2).

Discussion

Many animal models have been developed to induce cerebral ischemia-reperfusion injury. A model of incomplete global cerebral ischemia, achieved by BCCA occlusion of rats for 30 min, followed by reperfusion for 24 hours was used in this study. Such induced partial ischemia, without affecting the collateral circulation, reflects the events occurring during transient ischemic attacks and clinical cerebral infarction (Jingtao et al., 1999).

We have measured extent of ischemia-reperfusion induced cerebral damage in terms of percentage cerebral infarction. TTC staining was used to differentiate infarcted tissue from non-necrotic tissue (Benedek et al., 2006).

We found that sections in the sham control rats were uniformly and darkly stained, indicating non-necrotic and viable tissue. Whereas, in vehicle control rats, a part of the tissue was found pale and unstained, indicating necrosis of brain tissue due to ischemia and reperfusion. Therefore, we confirmed that BCCA occlusion model was established successfully.

Infarction volume in the brain is an important determinant in assessing the consequences of cerebral ischemia-reperfusion injury. Therefore, we have measured cerebral damage in terms of percentage cerebral infarction. We found that BCCA occlusion of rats for 30 min, followed by reperfusion for 24 hours (vehicle control group) produced a percentage infarction of 62.95 ± 2.460. Pretreatment with Urtica dioica (0.2, 0.4, 0.8 ml/kg) for 7 days prior to induction of ischemia and reperfusion reduced the percentage cerebral infarction significantly and dose dependently.

Our results are in accordance with earlier studies carried out in the same experimental models (Gaur et al., 2009; Raghavendra et al., 2009). Moreover, our findings are in harmony 137 with previous studies demonstrating that natural herbal medicines could protect the brain from damage caused by ischemia and reperfusion (Hatware et al., 1999; Jiang et al., 2007).

Cerebroprotective action of *Urtica dioica* observed in our study is strongly supported by previous studies conducted on *Urtica dioica*. Vafaee et al. (2012) investigated the neuroprotective effect of the immunomodulary drug Setarud, which is composed of herbal extracts including Rosa canina, Urtica dioica and Tanacetum vulgare, supplemented with selenium against focal ischemia-reperfusion injury. Therefore, the results in the present study propose that Urtica dioica has significant cerebroprotective action as shown by a significant decrease in percentage cerebral infarction.

Global cerebral ischemia and reperfusion induced behavioral alterations in experimental animals are similar to clinical symptoms in stroke patients (Dobkin, 1991). Similarly, global cerebral ischemia has been demonstrated to cause a marked decrease in grip and muscle strength of the limbs and memory impairment in several experimental models (Mukherjee et al., 2007; Rehni and Singh, 2007).

In the present study, BCCA occlusion for 30 min followed by 24 hours of reperfusion showed significant impairment of memory, demonstrated by increased transfer latency time 146 (Elevated plus maze test) and a marked decrease in muscle and grip strength (Locomotor activity, Hanging wire test and Inclined beam walking test), which were significantly attenuated by Urtica dioica pretreatment, suggesting the

therapeutic potential of Urtica dioica against ischemia-reperfusion injury.

These findings are similar to earlier studies, which also reported motor and memory deficits in the animals following BCCA occlusion and reperfusion injury (Rehni and Singh, 2007; Zarruk et al., 2011). In support, several studies have reported the protective effect of natural compounds against ischemic-reperfusion induced neurobehavioral alterations. Our findings are also supported by the previous studies, where natural compounds reversed the ischemia-reperfusion injury induced neurobehavioral alterations, due to their antioxidant and anti-inflammatory potential (Choi et al., 2004; Saleem et al., 2006; Jiang et al., 2007).

Cerebroprotective action of *Urtica dioica* observed in our study is strongly supported by previous studies conducted on *Urtica dioica*. Vafaee et al. (2012) reported the protective effect of the immunomodulary drug Setarud, containing extracts of *Rosa canina*, *Urtica dioica* and *Tanacetum vulgare,* supplemented with selenium against ischemic cerebral damage. It has been shown to reduce cerebral infarct volume, and improve the motor function of rats with cerebral ischemia (Vafaee et al., 2012).

Conclusion

Urtica dioica showed cerebroprotective potential by limiting the infarct size in ischemia reperfusion injured rats. In this experiment we found that *Urtica dioica* showed dose dependent cerebroprotective effect with the selected doses of 0.2, 0.8 ml/kg. Marked impairment of memory and motor co-ordination in parallel to the significant increase in the infarction size was observed after BCCA occlusion for 30 min followed by 24 hours reperfusion in rats. Pretreatment with Urtica dioica significantly and dose dependently attenuated ischemia-reperfusion induced neurobehavioral alterations in the rats. Present study suggests the protective effect of *Urtica dioica* and its therapeutic potential against ischemia-reperfusion induced neurobehavioral alterations in rats.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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