



Beneficial Effects of Ascorbic Acid and Alpha-tocopherol on the Locomotor Functional Recovery of Spinal Cord Injured Rats

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Authors' contributions

This work was carried out in collaboration between both authors. Author AAR designed the study, wrote the protocol and wrote the first draft of the manuscript. Author SSA managed the literature searches, editing of manuscript and supervised the study. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To examine the effects of ascorbic acid and alpha-tocopherol on the locomotor functional recovery of rats with incomplete spinal cord injury.

Methods: A total of 80 male Sprague-Dawley rats (180–200 g) were used. Seventy of them were subjected to spinal cord injury (SCI) and given various doses of ascorbic acid and alpha-tocopherol as treatment for 84 days (12 weeks). The remaining 10 rats were used as a control (without SCI, untreated). Basso, Beattie, and Bresnahan (BBB) and Tarlov scores were used to assess the locomotor recovery.

Results: Compared with the baseline value, all the rat groups tested (i.e. 3, 4, 5, 6, 7 and 8) showed gradual improvements in the BBB and Tarlov scores at the end of the first week. These improvements were sustained at all-time points until the completion of the trial period. Besides,

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when compared with disease control, positive improvements were observed in all the groups tested with respect to their BBB and Tarlov scores throughout the trial period. When the low-dose ascorbic acid and alpha-tocopherol groups were compared with the high-dose ascorbic acid and alpha-tocopherol groups, positive differences were observed in the BBB and Tarlov scores. When compared with the ascorbic acid treatment group significant additional improvements were observed in the alpha-tocopherol groups, especially in the high-dose alpha-tocopherol group.

Conclusion: Administration of high-dose alpha-tocopherol enhances the SCI-induced locomotor functional recovery as it is more effective than the ascorbic acid.

Keywords: Antioxidants; ascorbic acid; alpha-tocopherol; spinal cord injury; functional recovery.

1. INTRODUCTION

Spinal cord injury (SCI) is a devastating disease resulting in permanent disability causing extreme anguish to the patients [1]. The ensuing neurological dysfunction and paralysis are proportional to the severity of the trauma itself and is a primary problem in medicine, as it causes a high degree of mortality, severe disability, involving extensive rehabilitation and high costs [1]. Thus far, the management of SCI continues to pose a challenge and no definite treatment has been identified to tackle it. However, huge studies including experimental modeling are being conducted to enable a better understanding of the anatomical and biological consequences of injury and repair, including testing the effectiveness and the risk-to-benefit ratio of the proposed therapy to help resolve this fundamental problem [2,3].

Among the leading mechanisms associated with cell death post SCI, excitotoxicity, oxidative stress, inflammatory response and apoptosis are recognized as the potential targets to prevent tissue damage [4]. The crucial role played by the spinal cord in a broad range of physiological functions is clearly evidenced by the deficits observed post SCI and by the medical conditions that arise during the acute and chronic phases post injury [5-7].

To date, the locomotor deficits have been extensively studied, and several techniques have been designed and developed to encourage and accelerate the recovery of locomotor function [8]. This focuses on locomotor function has been based on several concerns: its clinical relevance and recurring nature, as well as the fact that it is easily and clearly observed and measured. A study also reported that functional outcomes, which, although they vary greatly, depend upon the size and site of the injury, type and timing of intervention, as well as kind of recovery and plasticity evaluated [9].

Over the recent past, several animal models of SCI have been developed, which have considerably enhanced our understanding of the pathophysiology of this condition [10]. A number of approaches to protect the injured spinal cord from secondary pathological processes have been examined experimentally, including the use of antioxidants, membrane stabilizers, glutamate antagonists, anti-inflammatories, caspase inhibitors, calpain inhibitors and other compounds of unclear actions [11-14]. Also, commendable research on the role of free radicals in the occurrence of ischemic damage has been done in short term basis [15,16]. In this study, we tested the hypothesis that ascorbic acid and alpha-tocopherol, as free radical scavengers, were used to investigate their efficiency in the locomotor recovery of rats subjected to incomplete spinal cord injury in long time basis (12 weeks).

2. METHODS

2.1 Animals

A total of 80 adult male Sprague-Dawley (SD) rats weighing 180-200 g were used in the study. The rats were housed in polycarbonate cages with sawdust bedding. They were kept in a temperature controlled room (23±1°C) and maintained on 12-hours light/dark cycles, with free access to standard laboratory food and tap water.

2.2 Drugs

Alpha-tocopherol and ascorbic acid used as an antioxidants were procured from Aldrich chemical company, Germany and chloral hydrate was purchased from Merck chemical company, Germany for anaesthetizing the rats.

2.3 Spinal Cord Injury

Out of 80 rats, seventy of them were anaesthetized with chloral hydrate (450 mg kg⁻¹ body weight) by intraperitoneal (IP) injection and

Table 1. Group descriptions

Groups	Treatment
1	Control (without SCI, untreated)
2	Disease control (saline)
3	Ascorbic acid 500 mg/kg body weight
4	Ascorbic acid 1000 mg/kg body weight
5	Alpha-tocopherol 500 mg/kg body weight
6	Alpha-tocopherol 1000 mg/kg body weight
7	Ascorbic acid 250 mg/kg body weight+ Alpha tocopherol 250 mg/kg body weight
8	Ascorbic acid 500 mg/kg body weight+ Alpha tocopherol 500 mg/kg body weight

laminectomy performed at T 7-8 level leaving the dura intact. A compression plate (2.2 x 5.0 mm) was loaded with a weight of 35 g placed on the exposed cord for 5 minutes in order to create the incomplete SCI. The remaining 10 rats were used as a control (without SCI, untreated).

2.4 Groups

The group descriptions of the animals are shown in Table 1 above. Eighty SD rats were divided into eight groups of ten rats each with similar functional activity. The rats in group 1 served as control (without SCI, untreated), group 2 served as disease control (SCI + saline). Rats in group 3 and 4 received intraperitoneal administration of ascorbic acid daily, while group 5 and 6 rats received oral administration of alpha-tocopherol daily. Group 7 and 8 rats received combination of ascorbic acid and alpha-tocopherol for 84 days (12 weeks).

2.5 Body Weight

During the study period, before locomotor measurements, the body weights of the rats (in grams) were measured baseline to day 84.

2.6 Locomotor Rating Scales

2.6.1 Basso, Beattie, and Bresnahan (BBB)

The Basso, Beattie and Bresnahan (BBB) locomotor rating scale is the most widely used open field test and has been accepted as a valid way to assess locomotor function after spinal cord contusion injury in the rats [17,18]. Prior to injury, the rats were acclimated to the open field where behavioral observations were conducted. The protocol was similar to the BBB Open Field Training Procedures. It is an ordinal 21-point scale, 0 being no observable hind limb movement and 21 being normal rat locomotion.

The scale takes into consideration of the limb movement, trunk and abdomen position, paw placement and position, walking, and trunk instability. Behavioral data were collected during 4-min testing periods beginning with day 1 to day 14. Two investigators scored each hind limb individually according to the BBB scales separately [17,18].

2.6.2 Tarlov scale

The Tarlov scale was used to measure and record the locomotor recovery following injury. The Tarlov scale ranged from a score of 0 to 4, where 0 reflects spastic paraplegia and no movement of the lower limbs; 1, spastic paraplegia and slight movement of the lower limbs; 2, good movement of the lower limbs but unable to stand; 3, able to stand but unable to walk normally; and 4, complete recovery [19].

2.7 Ethical Approval

The protocol of this study was approved by the research ethics committee of the institution. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

2.8 Statistical Analysis

Data analysis was carried out using Microsoft Excel 2010) Microsoft Corporation, Seattle, WA, USA) and the Statistical Package for Social Sciences version 16) SPSS Inc., Chicago, IL, USA).

The Kolmogorov-Smirnov test was performed for equal variances across the groups. The activity and recovery scores were analyzed by analysis of variance with repeated measurements over time and tukey–kramer multiple comparisons test. *p*-values <0.05 were taken as statistically significant.

3. RESULTS

3.1 Body Weight

The effectiveness of spinal cord injury on animal body weight are shown in Fig. 1. The group 2 rats (disease control group, SCI + saline) showed a gradual decline in body weight from day 2 until day 7 when compared with the baseline value. However, a gradual increase in the body weight as observed from day 8 (second week onwards) which was maintained until the study was completed. Similar results were observed in all the groups tested (i.e. 3, 4, 5, 6, 7 and 8), in which the improvements were noted to begin on day 6 for groups 6 and 8. Compared with the low-dose ascorbic acid (group 3) and alpha-tocopherol rat groups (group 5), positive enhancements in body weight were observed in the rats treated with high-dose ascorbic acid (group 4) and alpha-tocopherol (group 6). Further, when compared with the rats given ascorbic acid treatment notable improvements were observed in the body weight in rat groups on alpha-tocopherol, especially in those on high-dose alpha-tocopherol.

3.2 BBB Score

Effectiveness of different doses of ascorbic acid and alpha-tocopherol on BBB score of spinal cord injured rats are shown in Table 2. At the end of the first week, groups 3, 4, 5, 6, 7 and 8 showed gradual improvement in the BBB scores, when compared with the baseline value, which were maintained at all time points until the completion of the trial period (week 12). Compared with the disease control, positive improvements in BBB were observed in all the groups tested. Compared with the rats on low-

dose ascorbic acid (group 3) and alpha-tocopherol group (group 5), positive BBB improvements were observed in the rats on high-dose ascorbic acid (group 4) and alpha-tocopherol (group 6). Besides, when compared with rats given ascorbic acid treatment, significant additional improvements were observed in the rat groups given alpha-tocopherol, mainly group 6 (i.e. alpha-tocopherol 1000 mg/kg body weight). Compared with the initial weeks, the recovery rates of all the groups tested were lower during last four weeks (i.e. weeks 9-12).

3.3 Tarlov Score

The effectiveness of different doses of ascorbic acid and alpha-tocopherol on Tarlov score of spinal cord injured rats are shown in Table 3. Compared with the baseline value, groups 3, 4, 5, 6, 7 and 8 demonstrated gradual improvement in the Tarlov scores at the end of the first week, which were maintained at all time points until the trial period was completed (week 12). Compared with the disease control positive improvements in the Tarlov scores were observed in all the groups tested. Compared with the rats on low-dose ascorbic acid and the alpha-tocopherol groups (groups 3 and 5), positive differences were recorded in the Tarlov scores in the rats on high-dose ascorbic acid (group 4) and alpha-tocopherol (group 6). However, when compared with those given ascorbic acid treatment significant additional improvements were observed in the alpha-tocopherol groups, especially group 6 (i.e. alpha-tocopherol 1000 mg/kg body weight). Compared with the initial weeks, the recovery rates of all the rat groups tested were less over the last four weeks (i.e. weeks 9-12).

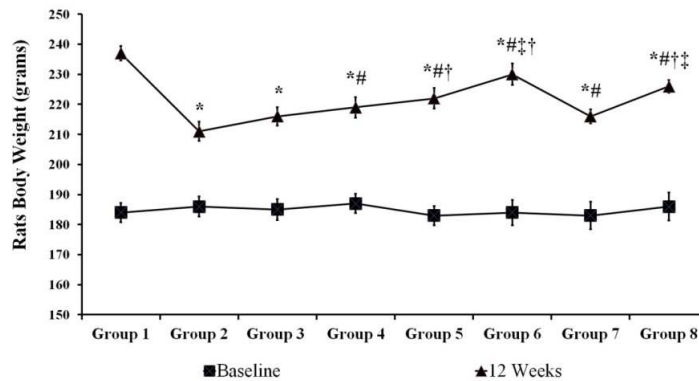


Fig. 1. Effectiveness of spinal cord injury on animal body weight (grams)

Values are presented as mean \pm standard deviation, Groups compared: * Compared to baseline, # vs 3, 4, 5, 6, 7, 8. † 3 vs 4, 5, 6, 7, 8. ‡ 4 vs 5, 6, 7, 8. † 5 vs 6, 7, 8. ¶ 6 vs 7, 8

Table 2. Effectiveness of different doses of ascorbic acid and alpha-tocopherol on BBB score of spinal cord injured rats

	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6	Group-7	Group-8
Baseline	21	1.7±0.5	1.6±0.5	1.7±0.6	1.6±0.5	1.7±0.6	1.7±0.4	1.7±0.5
Week-1	21	2.1±0.5	2.2±0.6	2.2±0.5	2.2±0.7	2.6±0.7	2.2±0.7	2.5±0.5
Week-2	21	3.2±0.6*	3.4±0.7*	3.4±0.7*	3.5±0.8*	4.2±0.9*	3.5±0.8*	4±0.6*
Week-3	21	4.8±0.7*	5.2±0.7*	4.9±0.7*	5.2±0.9*	5.9±0.7*	5.1±0.5*	5.6±0.9*
Week-4	21	5.6±0.9*	6.3±0.7*	6.2±0.8*	6.9±1.1*	7.4±0.9*	6.4±0.9*	6.9±0.8*
Week-5	21	6.4±0.8*	7.1±0.9*	7.3±0.9*	8.1±1.3*	9.2±1.2*	7.3±1.1*	8.5±1.1*
Week-6	21	7.2±0.9*	8.1±1.2*	8.4±1.1*	9.3±1.4*	10.3±1.3*	8.3±1.3*	10.1±1.3*
Week-7	21	8.7±1.2*	8.9±1.3*	9.4±1.4*	10.5±1.5*	12.4±1.4*	9.2±1.7*	11.2±1.5*
Week-8	21	9.2±1.1*	9.8±1.6*	10.8±1.3*	11.9±1.8*	13.9±1.6*#† f	10.1±2.1*	12.3±1.7*
Week-9	21	10±1.2*	11.2±1.7*	11.9±1.5*	12.8±1.9*	15.5±1.8*#†‡ f	11.8±1.4*¶	13.6±2.1*#†
Week-10	21	10.5±1.3*	12.1±1.9*	12.7±1.6*	13.1±2.1*	15.9±1.9*#†‡ f	12.6±2.3*¶	14.5±2.1*#†
Week-11	21	10.8±1.4*	12.9±2.1*	13.1±2.1*	13.7±2.4*#†	16.6±2.2*#†‡ f	13.1±2.1*¶	15.1±2.3*#†
Week-12	21	11.4±1.9*	13.1±2.2*	13.5±2.4*	14.3±2.3*#†	17.3±2.4*#†‡ f	13.7±2.4*¶	15.6±2.1*#†

Values are presented as mean ± standard deviation.

Groups compared: * Compared to baseline, # 2 vs 3, 4, 5, 6, 7, 8. † 3 vs 4, 5, 6, 7, 8. ‡ 4 vs 5, 6, 7, 8. f 5 vs 6, 7, 8. ¶ 6 vs 7, 8

Table 3. Effectiveness of different doses of ascorbic acid and alpha-tocopherol on Tarlov score of spinal cord injured rats

	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6	Group-7	Group-8
Baseline	4	0.3±0.1	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.1	0.4±0.1	0.4±0.1
Week-1	4	0.8±0.2	0.9±0.3	1±0.3	0.9±0.3	0.9±0.1	0.8±0.3	1.1±0.4
Week-2	4	1.3±0.4*	1.2±0.5*	1.4±0.4*	1.2±0.3*	1.3±0.3*	1.2±0.4*	1.7±0.5*
Week-3	4	1.7±0.4*	1.7±0.5*	1.6±0.4*	1.5±0.4*	1.5±0.4*	1.4±0.4*	1.9±0.4*
Week-4	4	1.8±0.5*	2±0.5*	1.8±0.5*	1.7±0.5*	1.7±0.5*	1.6±0.5	2.1±0.4*
Week-5	4	1.9±0.5*	2.1±0.4*	1.9±0.6*	1.8±0.5*	1.9±0.4*	1.8±0.6*	2.2±0.5*
Week-6	4	1.9±0.6*	2.1±0.4*	2.1±0.5*	2±0.4*	2.1±0.5*	1.8±0.5*	2.3±0.5*
Week-7	4	1.9±0.5*	2.2±0.5*	2.2±0.5*	2.1±0.5*	2.4±0.5*	1.9±0.6*	2.3±0.6*
Week-8	4	2±0.4*	2.2±0.6*	2.3±0.5*	2.2±0.5*	2.7±0.6*#† f	2±0.5*	2.4±0.5*
Week-9	4	2.1 ± 0.5*	2.2±0.5*	2.4±0.5*	2.3±0.5*	2.7±0.5*#†‡ f	2.1±0.6*	2.5±0.6*#†
Week-10	4	2.1 ± 0.5*	2.2±0.6*	2.4±0.6*	2.4±0.6*	2.8±0.6*#†‡ f	2.2±0.5*	2.6±0.6*#†
Week-11	4	2.2 ± 0.6*	2.3±0.6*	2.5±0.4*	2.5±0.5*	2.9±0.5*#†‡ f	2.3±0.6*	2.7±0.5*#†
Week-12	4	2.2 ± 0.6*	2.3±0.6*	2.5±0.5*	2.7±0.6*	2.9±0.6*#†‡ f	2.4±0.6*	2.7±0.6*#†

Values are presented as mean ± standard deviation.

Groups compared: * Compared to baseline, # 2 vs 3, 4, 5, 6, 7, 8. † 3 vs 4, 5, 6, 7, 8. ‡ 4 vs 5, 6, 7, 8. f 5 vs 6, 7, 8. ¶ 6 vs 7, 8

4. DISCUSSION

Animal models play a crucial role in the development of experimental therapies for SCI. The rat model has been the most consistently studied and standardized model. It has received great attention by scientists for the assessment of locomotive performance and determination of the functional consequences of the initial injury, the spontaneous recover of function and the value of the therapeutic strategies [20-23]. Currently, several assessment methods, including the BBB locomotor rating scale, modified Tarlov scoring system, grid-walk test, narrow beam test and inclined plane test, have been developed to evaluate the recovery of movement post SCI [17,23-26]. Further, the locomotor functional outcome in the experimental SCI models is the most significant factor for evaluating the degree of injury and treatment efficacy. It is directly related to the extent of neuronal damage in the gray matter at the injury site, the loss of the ascending and descending axons in the white matter, and the reorganization of the rest of the nervous system [27,28]. The aim of this study was to examine the effects of ascorbic acid and alpha-tocopherol on the locomotor functional recovery of rats subjected to incomplete spinal cord injury. From the observations of the present study, SCI-induced body weight reduction in all the control, disease control and experimental rat groups during the first week (first 7 days) followed by a slow recovery. This decrease is partly due to fluid loss during the surgical procedure and the great stress imposed upon the body at the time of the initial trauma, triggering the body metabolism to work faster to supply the required energy and nutrients to hasten healing and combat infections. This results in the weight decrease in the animals [29-32]. The results of this study revealed gradual recovery over a 12-week period post SCI, compared with the baseline readings. This observations concur with the records of previous studies which reported that SCI reduced animal body weight [31-34].

Research has shown that alpha-tocopherol is an antioxidant with protective properties enabling the reduction or prevention of oxidative damage. Alpha-tocopherol is a lipid-soluble antioxidant, which prevents the lipid peroxidation chain reactions in the cellular membranes by interfering with the propagation of the lipid radicals [12,14]. In this study, all the rat groups tested for alpha-tocopherol (groups 5 and 6) were observed to show a gradual improvement in the BBB and

Tarlov scores at the end of the first week, which were maintained at all time points until the completion of the trial period (week 12) when compared with the baseline value. Compared with disease control positive improvements were recorded in all the alpha-tocopherol groups tested, the high dose of alpha-tocopherol groups in particular. Prior studies performed to evaluate the efficacy of alpha-tocopherol on compression injury in the spinal cords of rats reported greatly reduced motor disturbance induced by SCI with alpha-tocopherol supplementation [33,34]. Post injury, the spinal cord evoked potentials that revealed greater recovery, in both amplitude and latency, in the rat group supplemented with alpha-tocopherol than in the control group [33,34]. Acute SCI produces tissue damage that continues to progress for days and even weeks after the initial insult, with related functional impairments. A reduction in the degree of progressive tissue loss (neuroprotection) post SCI should enable better recovery [35]. It has been suggested that free-radical generation and subsequent lipid peroxidation contributes to delayed tissue damage post traumatic SCI. Ubiquinols (reduced coenzyme Q), ascorbate (vitamin C) and alpha-tocopherol are known endogenous antioxidants; therefore, any decrease in the levels of these compounds in the tissues may reflect ongoing oxidative reactions [36]. In this study, it is clear that the SCI-induced motor disturbance was greatly reduced by alpha-tocopherol. This observation concurs with the results of other investigators, who also reported that alpha-tocopherol may exert reparative effects on SCI [33,34]. Further, studies reported that high-dose alpha-tocopherol oral supplementation for five days prior to SCI attenuates the progressive post-traumatic decrease in white matter spinal cord blood flow as well as significantly enhances hind-limb motor function compared with the rats not given the supplementation [33,37-39]. Also, alpha-tocopherol deficiency has been known to increase post-SCI lipid peroxidation and attenuate motor functional recovery [34]. However, despite these effects, the importance of alpha-tocopherol as an acute treatment for SCI is limited by the fact that it requires weeks to achieve a significant increase in the tissue parenchymal central nervous system levels [40]. However, long-term, high-dose alpha-tocopherol oral supplementation may offer effective prophylactic neuroprotection against SCI [40].

In the present study, ascorbic acid treated group 3, 4 showed a gradual improvement in BBB and

Tarlov scores at week one, and such improvement was maintained at all-time points until the end of the trial period (week 12) as compared with the baseline value. Compared with the disease control group positive improvements were observed in all the rat groups tested for ascorbic acid. Water-soluble ascorbic acid present in the cytosol and extracellular fluid directly interacts with the free radicals to prevent oxidative damage [12,14]. Earlier studies reported the antioxidative function of ascorbic acid and its effect on reducing ischemia post experimental compression injury [36]. The pretreatment of spinal cord injured animals with a single dose of ascorbic acid has been reported to support spinal cord blood flow, which is confirmed by the present study [41,42]. In our study we also found, when compared with rats given ascorbic acid treatment, revealed significant additional improvements were observed in the alpha-tocopherol groups, mainly those on the alpha-tocopherol 1000 mg/kg body weight, which is concur with the previous findings [11,14].

Research support that administration of high-dose ascorbic acid and alpha-tocopherol are effective for SCI treatment and spinal cord injury-induced motor disturbance was found to be greater in the ascorbic acid and alpha-tocopherol deficient rats [41]. Studies also reported that high-dose ascorbic acid and alpha-tocopherol administration during the acute post SCI phase caused a significant reduction in secondary injury-induced tissue necrosis and improved the functional performance in these rats [12-14,20,43]. In our study, results clearly indicated that compared with the low-dose ascorbic acid (group 3) and alpha-tocopherol group (group 5), positive improvements in the BBB and Tarlov scores were observed in the rat groups on high-dose ascorbic acid (group 4) and alpha-tocopherol (group 6).

Studies reported that a stronger association between SCI, and combined administration of ascorbic acid and alpha tocopherol are thus evident. Latest evidence shown that supplementation alpha-tocopherol and ascorbic acid may protect against dementia and improve cognitive function in later life [44]. Alpha-tocopherol and ascorbic acid combined supplementation reduces oxidative stress and improves antioxidant enzymes and positive muscle work in chronically loaded muscles of rats [45]. However, in a literature review, combined use of alpha-tocopherol and ascorbic acid in different SCI studies result in different

functional outcomes. A study reported that the use of ascorbic acid and alpha-tocopherol did not improve the neurological performance in SCI rats. However, their histopathological examination showed that the inflammatory response was less intense following the administration of a combination of ascorbic acid and alpha-tocopherol, despite the discrepancy regarding this fact in the scientific literature [41,43,46]. In the present observation showed that notable differences were observed in combined groups (group 7 and 8). However, these improvements were lesser than the same dose of alpha-tocopherol alone treated groups.

5. CONCLUSION

In conclusion, the outcome of both the BBB and Tarlov scales clearly indicates that the alpha-tocopherol and ascorbic acid enhance the locomotor functional recovery of rats with spinal injury. However, the administration of high-dose alpha-tocopherol improves the locomotor recovery against SCI which is more effective than ascorbic acid.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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