

ORIGINAL ARTICLE

Antioxidative therapy in contusion spinal cord injury

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Introduction: Some studies have made use of the antioxidative capabilities of high doses of vitamins C and E with the aim of neutralizing the noxious effects of free radicals following spinal cord lesion.

Objectives: To evaluate the effects of vitamins C and E, separately and together, on the functional performance of rats that were subjected to standardized spinal cord contusion.

Materials and methods: Forty male Wistar rats were used, divided into four groups of 10 animals each. Group 3 received vitamin C $100 \text{ mg kg}^{-1} \text{ day}^{-1}$ intraperitoneally; Group 2 received vitamin E $100 \text{ mg kg}^{-1} \text{ day}^{-1}$ orally; Group 1 received vitamins C and E, at the same dosages; and Group 4 was the control. The vitamin therapy was administered for 1 month and then the animals were killed. A direct contusional injury was caused and functional evaluation was performed using the Basso, Beattie and Bresnahan rating scale. The rats were evaluated on the second postoperative day and weekly thereafter, until the end of the experiment.

Results: The results were evaluated by means of the one-tailed, non-paired and non-parametric Mann-Whitney test, comparing the groups two by two. No significant difference in functional performance was observed between the groups.

Conclusion: The use of vitamins C and E in these rats did not improve their neurological performance. However, histopathological examination showed that the inflammatory response was less intense following administration of the combination of vitamins C and E.

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Keywords: spinal cord injury; Wistar rats; antioxidants; vitamin

Introduction

Spinal cord injury is known to be followed by a cascade of events that contribute toward worsening the spinal injury. These events are called secondary injury, and include spinal cord ischemia and free radical, among other events.^{1,2}

It can be inferred that there is an association between free radicals and spinal cord injury, from observations that these products present increased concentrations following spinal injury, that their production can be reduced using steroid antioxidants³ and that there are lower concentrations of endogenous antioxidants following the injury.⁴

Among the various types of free radicals, it seems that those of interest in relation to spinal cord injury look are oxygen derived (O_2 , H_2O_2 or OH).^{5,6} It has already been proven that vitamin E is important for the structural and functional maintenance of nerve tissue, to avoid free radical

action on cell membranes.^{1,2} However, it is also supposed that vitamin E administration to individuals with acute spinal cord injury would have no effect, as its absorption rate is low and chronic use before the injury would be required, for protective effects to be achieved.⁶

Vitamin C returns vitamin E to its antioxidant status through reductive action, and may boost its effects. It also participates in the elimination of oxygen free radicals.⁷ We believe that administration of high doses of antioxidants that are naturally present in the organism (vitamins C and E) may have protective effects with regard to spinal cord injury. This may be achieved without presenting the side effects that are found in some classes of drugs, particularly glucocorticoids.^{8–11} In this light, the aim of this study was to evaluate the possibility of using vitamins C and E for treating contusional spinal cord injury.

Objectives

This study had the aim of evaluating the influence of treatment with vitamins C and E, separately and together,

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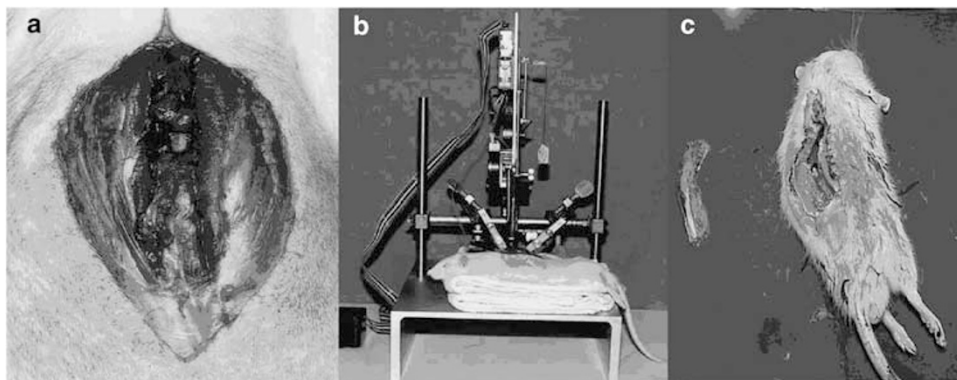


Figure 1 (a) Approach to spinal cord and laminectomy. (b) Impactor. (c) Spine removal.

administered to animals that were subjected to contusional spinal cord injury, in relation to functional performance and histopathological evaluation.

Materials and methods

Forty rats were subjected to injury by means of the NYU Impactor device. The rats were divided into four groups of ten animals each. Group 1 received both vitamins, group 2 received vitamin E, group 3 received vitamin C and group 4 did not receive any vitamins and served as the control group. The first dose of vitamins was administered 30 min after the injury, with daily dosing until the animals were killed.

Rats

Male Wistar rats from a single supplier were used. They were aged 12 weeks, their body weights ranged from 260 to 340 g and they were pathogen-free.

Anesthesia procedures

The rats were anesthetized by means of intraperitoneal pentobarbital. The dose given was 55–75 mg kg⁻¹. This dose was expected to anesthetize the rats for about 2 h.

Laminectomy

The spinal cord was exposed by means of laminectomy to produce the contusion. The dorsal skin was divided at the median line, to expose the T8–T12 vertebrae.

The muscles inserted in the spinal processes from T10 to T11 were cut and retracted, using a bipolar coagulator to stop hemorrhaging as required. T10 and T9 were removed with a cutter. This action started from the caudal border of T10 and small fragments were carefully removed from T10 and up to T9, with the cutter cranially oriented such that a right-handed surgeon would work with the animal's head close to his left hand. The opening thus created was such that it would accommodate the head of the Impactor with a margin of more than 2 mm (Figure 1a).

Contusion (manually operated NYU Impactor, 1993)

The NYU Impactor was used to produce a contusional spinal cord injury and to monitor the contusion.

The rat was positioned in the Impactor frame. The T8 process was clamped and then the caudal clamp was placed on the T11 spinal process.

The Impactor rod was adjusted to the zero position. The basal clip was attached to the border of the surgical wound. The Impactor was then lowered toward the spinal cord, between the upper margin of T9 and the lower margin of T10. When the head came into contact with the spinal cord, the Impactor indicated this by means of audible and visible signals caused by closing the circuit between the basal clip and the Impactor head, passing through the rat.

The Impactor rod was raised to a height of 25 mm. This height was entered into the Impactor program, and it was prepared for data acquisition. The Impactor rod was then released to hit the spinal cord. Immediately after producing the contusion, the rod was lifted off (Figure 1b).

The animal was then removed and the contusion site was inspected and any hemorrhaging was stopped. The para-vertebral muscles and the skin were sutured.

Post-injury procedures

Half an hour after the injury, vitamin C was intraperitoneally administered to group 3, at a dose of 100 mg kg⁻¹; vitamin E was administered orally to group 2, at a dose of 100 mg kg⁻¹; and vitamins C plus E were administered to group 1 (both at a dose of 100 mg kg⁻¹ dose). Group 4 served as the control group. Oral administration was chosen for vitamin E because it is extremely difficult to find an injectable form on the market. The vitamin E oral administration was carried out thru gavage. The doses of both vitamins corresponded to about 10 times the recommended dosage for rats,^{1,12} thus aiming to reach an antioxidant effect that was sufficient for spinal cord protection.

Over the days following the injury, the rats received daily doses of the same vitamins, until the day they were killed. On the first 7 days, the animals received prophylactic antibiotics (cefazolin, 25 mg kg⁻¹). The animals' weights were measured on the 2nd, 7th, 14th, 21st and 28th postoperative days.

The urinary bladder was periodically palpated. The degree of dehydration was reviewed every day, by evaluating skin

turgidity. The need for antibiotics (levofloxacin, 2.5 mg kg⁻¹ for 10 days) was assessed according to the presence of blood in the urine. If after 10 days of treatment with levofloxacin the animal's urine were still to present blood, nonlevofloxacin-sensitive urinary infection would be assumed, and the rat would be killed.

The vitamin therapy was continued for a 1-month period, and then the rats were killed.

Killing and tissue samples

The rats were killed by means of an intraperitoneal lethal dose of pentobarbital. A tissue sample was collected together with the spinal cord, and was fixed in 10% formol. One week later, the spinal cord was dissected so as to select the region from T7 to T12 (thus containing the injury epicenter and two adjacent segments), and samples were sent for histopathological analysis (Figure 1c).

Exclusion criteria

Animals were excluded from the study in the event that they died, developed infection that was refractory to antibiotic treatment, developed significant autophagia and/or had no control over urination.

Analysis of the results

There were two parts to the analysis: histopathological and functional. The functional analysis was performed on the 2nd, 7th, 14th, 21st and 28th postoperative days and consisted of using the Basso, Beattie and Bresnahan (BBB) rating scale.¹³ Scores were given for the movements observed in each rat, and these were subsequently evaluated statistically. These observations (5 min per rat) were made by two different investigators with appropriate training, who came to an agreement regarding the ratings, thereby producing a more objective analysis.

The histopathological analysis was performed in blinded way by skilled personnel from the Laboratory of Pathological Anatomy. Transversal sections were cut from the spinal cord at the injury epicenter and adjacent regions. Hematoxylin–eosin staining was used, and the resultant thin sections were examined with regard to five aspects: necrosis, hyperemia, hemorrhage, nerve substance degeneration and cell infiltration.

Each section was divided in four quadrants and the analysis was carried out in isolation for each aspect. If there is less than 25% of involvement it was classified as mild and graded as (1), if there is 25–50% of involvement it was classified as moderate and graded as (2), if there is more than 50% it was classified as severe and graded as (3) and if there is no involvement it was classified as absent and graded as (0). The sum of the aspects was carried out for each section and this value was used in statistical analysis.

Results

One rat from group 2 and one from group 4 died because of refractory urinary infection on the fourth postoperative day. In addition, one rat from group 1 developed autophagia

on the second postoperative day and died on the sixth postoperative day. On the seventh postoperative day, one rat from group 3 was found dead from an unknown cause.

It was found that one rat from group 3 presented only partial spinal cord injury, on the second postoperative day, which was detected because of the abnormally superior functional performance in relation to the others animals. For this reason, this animal was excluded.

Six rats in group 1 developed urinary lithiasis. In the cases of four rats, most of the stones were eliminated with their urine. However, one animal developed urethral obstruction, forcing the animal to keep their hind legs in constant abduction and severely disturbing their movements. For this reason this animal was killed.

No other interurrences were found in the rats' evolution. All of the remaining rats presented weight gains from the seventh postoperative day onwards.

Results from functional analysis

The results from functional analysis were summarized in Table 1. All rats had functional improvement with the time as shown in Figure 2. The vitamin C group had the best result in the 28 postoperative day followed by the vitamins C and E group. The worst result was the control group.

Results from histopathological analysis

The sum of the values obtained for each variable resulted in scores between 0 and 15. Each rat received three scores: one for the injury site, one for the region cranial to the injury site and one for the caudal region (Table 2). The mean value for each group in the cranial, caudal and injury site was shown in Table 3 and these values were used in the statistical analysis.

The group of vitamins C and E had the best results in all three sites of analysis because the lowest value indicated that it had a less inflammatory response. It can be demonstrated in Figure 3 that shows a decrease in the inflammatory response using the criteria above, in the group of vitamins C and E when compared with control group.

Results from statistical analysis

The one-sided, nonpaired, nonparametric Mann–Whitney test was used, comparing the four groups, two by two. Events with $P < 0.05$ were considered statistically significant.

In evaluating the results from the functional analysis, the tests were performed for each of the postoperative days on which the analysis was carried out. The BBB scale values were used as the elements from each group, taking together the data relating to the left and right hind legs (Table 4).

The analysis of the histopathological data was also performed by means of the Mann–Whitney test, and the data used were the ratings obtained for each criterion considered, as described above (Table 5).

Six rats were excluded asymmetrically between groups. If those rats that were excluded were considered as the worst

Table 1 Performance results of the functional analysis; the scale ranges from 0 to 21

Treatment	Number	Days after injury									
		2 PO R	2 PO L	7 PO R	7 PO L	14 PO R	14 PO L	21 PO R	21 PO L	28 PO R	28 PO L
<i>Group 1</i>											
Vitamins C and E	Rat 1	0	0	7	7	9	9	12	12	14	14
Vitamins C and E	Rat 2	0	0	7	7	9	9	10	10	13	13
Vitamins C and E	Rat 3	1	1	8	8	13	13	20	20	20	20
Vitamins C and E	Rat 4	0	0					Killing			
Vitamins C and E	Rat 5	0	0	7	7	12	12	16	16	16	16
Vitamins C and E	Rat 6	0	0					Death			
Vitamins C and E	Rat 7	0	0	7	7	9	9	12	12	14	14
Vitamins C and E	Rat 8	0	0	1	1	9	9	9	13	10	14
Vitamins C and E	Rat 9	1	1	7	7	10	10	12	12	14	14
Vitamins C and E	Rat 10	0	0	7	7	10	10	12	12	13	13
<i>Group 2</i>											
Vitamin E	Rat 11	0	0	7	7	8	8	8	8	9	10
Vitamin E	Rat 12	0	0	8	8	10	10	13	13	16	16
Vitamin E	Rat 13	0	0	7	7	9	9	9	9	14	14
Vitamin E	Rat 14	0	0	7	7	10	10	13	13	14	14
Vitamin E	Rat 15	0	0	7	7	10	10	13	13	16	16
Vitamin E	Rat 16	0	0	7	7	8	9	13	13	14	14
Vitamin E	Rat 17	0	0					Death			
Vitamin E	Rat 18	2	2	10	10	19	19	21	21	21	21
Vitamin E	Rat 19	0	0	7	7	9	9	13	13	19	19
Vitamin E	Rat 20	1	0	7	7	11	11	16	16	19	19
<i>Group 3</i>											
Vitamin C	Rat 21	0	0	7	7	9	9	13	13	14	14
Vitamin C	Rat 22	1	0	7	7	12	12	18	18	21	21
Vitamin C	Rat 23	0	0	7	7	8	8	8	8	13	13
Vitamin C	Rat 24	0	0					Death			
Vitamin C	Rat 25	0	0	1	8	8	8	13	10	14	14
Vitamin C	Rat 26							Excluded			
Vitamin C	Rat 27	2	2	8	8	14	14	19	19	21	21
Vitamin C	Rat 28	0	0	8	8	10	10	19	19	19	19
Vitamin C	Rat 29	1	0	7	7	10	10	13	13	18	18
Vitamin C	Rat 30	0	0	7	7	10	10	16	16	17	17
<i>Group 4</i>											
None	Rat 31	0	0	7	7	10	10	13	13	14	14
None	Rat 32	0	0	8	1	8	8	12	12	13	13
None	Rat 33	0	0	7	7	12	12	13	13	15	15
None	Rat 34	0	0	0	3	8	8	12	12	12	12
None	Rat 35	0	0	7	7	8	11	13	13	16	16
None	Rat 36	0	0	7	7	10	10	15	14	16	16
None	Rat 37	0	0	7	7	8	8	9	10	14	14
None	Rat 38	0	0					Death			
None	Rat 39	1	0	7	7	8	8	12	12	14	14
None	Rat 40	0	0	7	7	10	10	13	13	14	14

Abbreviations: PO R, postoperative right; PO L, postoperative left.

result possible, it still had no statistical difference when groups were compared regarding function.

Discussion

The results from the functional analysis showed that all four groups improved with time (Figure 2), but the *P*-values <0.05 were only found in the comparisons between groups 3 and 4, on the 21st and 28th postoperative days. This demonstrated that the group that received vitamin C performed better than did the control group. No statistically

significant values were found in the other comparisons (Table 4).

The histopathological evaluation produced statistically significant values (*P*<0.05) in several comparisons, which indicated the reduced inflammatory response obtained through combined use of vitamins C and E. This was observed from the evaluation of the following groups: combination of vitamins C and E at the injury site versus surrounding regions; combination of vitamins C and E versus vitamin C at the injury site; combination of vitamins C and E versus control in the region caudal to the injury site (Table 5).

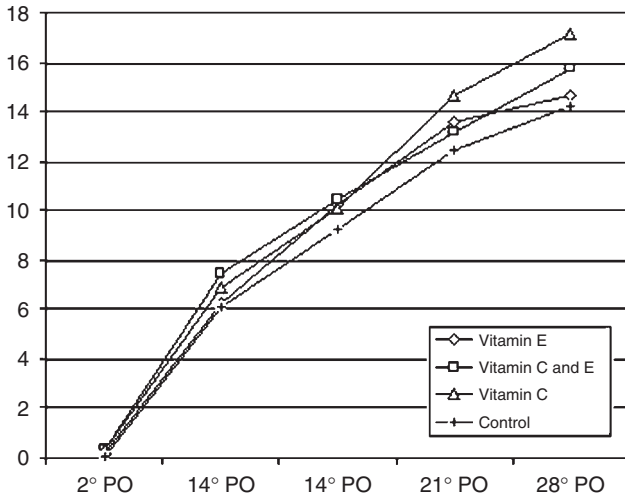


Figure 2 Graph showing results of the functional analysis of the 33 rats divided by groups: vitamin E represented by circles, vitamin C with E by squares, vitamin C by triangles and control by crosses.

From the above, some hypotheses may be raised to explain these differences in the results. Firstly, it must be remembered that the group of animals receiving the combination of vitamins had a higher incidence of urinary lithiasis. Although two animals had to be killed at an early stage of the experiment because their functional performance was severely impaired, the other four rats in this group with this condition were able to eliminate the stones through their urine, and were therefore not excluded from the study because they did not present any noticeably different movements. However, it is possible that small changes were detected by means of the functional performance scale (BBB), and this would explain why this group did not present statistically significant improvement in motor performance.

Another possible reason for this discrepancy was the duration of the study, considering that the histopathological evaluation gave information on the status of the rats at the end of the experiment, whereas the functional analysis described the evolutive behavior of the rats. At the end of this experiment, the rats in the vitamin combination group were starting to show a certain improvement in relation to the other groups and, if the study had been continued, a statistically significant functional improvement might have been achieved in this group at a later stage.

Finally, however, the possibility that there might not be any really important correlation between the rats' motor performance and the degree of inflammatory response in the spinal cord cannot be dismissed.

Conclusion

From this study, it was concluded that vitamins C and E do not had an impact on experimental contusional injury to the spinal cord in rats, with regard to functional and histopathological aspects, and the combination of this vitamins improves only the histopathological aspects. It

Table 2 Pathoanatomy analysis

Treatment	Number	Pathoanatomy analysis		
		Cranial	Injury	Caudal
Vitamins C and E	Rat 1	5	11	5
Vitamins C and E	Rat 2	5	6	3
Vitamins C and E	Rat 3	5	9	5
Vitamins C and E	Rat 4		Killing	
Vitamins C and E	Rat 5	3	3	2
Vitamins C and E	Rat 6		Death	
Vitamins C and E	Rat 7	2	3	2
Vitamins C and E	Rat 8	2	5	2
Vitamins C and E	Rat 9	6	6	10
Vitamins C and E	Rat 10	4	10	4
Vitamin E	Rat 11	6	12	12
Vitamin E	Rat 12	11	11	11
Vitamin E	Rat 13	4	12	4
Vitamin E	Rat 14	2	6	2
Vitamin E	Rat 15	7	7	7
Vitamin E	Rat 16	7	12	12
Vitamin E	Rat 17		Death	
Vitamin E	Rat 18	7	10	10
Vitamin E	Rat 19	6	6	6
Vitamin E	Rat 20	8	12	12
Vitamin C	Rat 21	4	11	4
Vitamin C	Rat 22	8	14	14
Vitamin C	Rat 23	6	14	14
Vitamin C	Rat 24		Death	
Vitamin C	Rat 25	5	11	5
Vitamin C	Rat 26		Excluded	
Vitamin C	Rat 27	5	7	5
Vitamin C	Rat 28	6	8	6
Vitamin C	Rat 29	3	7	3
Vitamin C	Rat 30	9	13	9
None	Rat 31	4	8	4
None	Rat 32	7	13	7
None	Rat 33	4	8	8
None	Rat 34	5	5	5
None	Rat 35	3	3	8
None	Rat 36	6	6	6
None	Rat 37	11	11	11
None	Rat 38		Death	
None	Rat 39	6	6	11
None	Rat 40	6	10	10

Table 3 Mean of anatomopathology analysis

	Cranial (s.d.)	Injury (s.d.)	Caudal (s.d.)
Vitamins C and E	4 (1.51)	6.6 (3.06)	4.1 (2.69)
Vitamin E	6.4 (2.5)	9.8 (2.68)	8.4 (3.8)
Vitamin C	5.75 (1.98)	10.6 (2.97)	7.5 (4.37)
None	5.8 (2.3)	7.8 (3.15)	7.8 (2.53)

Table 4 Results of the Mann-Whitney test (functional analysis)

Data	2nd PO (P)	7th PO (P)	14th PO (P)	21st PO (P)	28th PO (P)
Groups 1 and 2	0.881	0.333	0.582	0.459	0.210
Groups 1 and 3	0.826	0.328	0.755	0.212	0.059
Groups 1 and 4	0.425	0.531	0.084	0.969	0.894
Groups 2 and 3	0.736	0.986	0.931	0.262	0.325
Groups 2 and 4	0.579	0.079	0.268	0.334	0.069
Groups 3 and 4	0.357	0.088	0.248	0.035	0.011

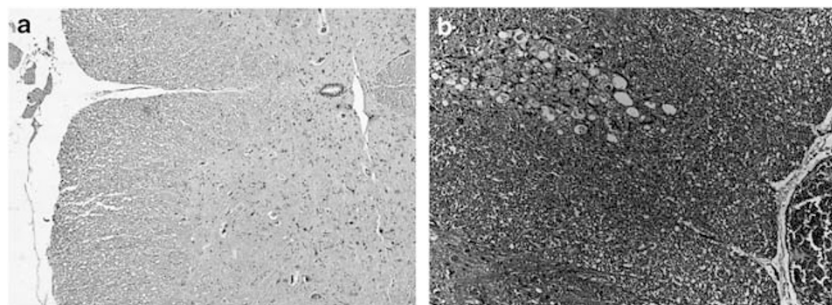


Figure 3 Transversal section of injury site of spine (H&E). (a) Combination of vitamins C and E (mild inflammatory response). (b) Control (severe inflammatory response).

Table 5 Analysis of the pathoanatomical results were also performed by the Mann–Whitney test

	Cranial (P)	Injury (P)	Caudal (P)
Groups 1 and 2	0.03	0.204	0.039
Groups 1 and 3	0.093	0.043	0.105
Groups 1 and 4	0.09	0.56	0.026
Groups 2 and 3	0.47	0.47	0.665
Groups 2 and 4	0.331	0.185	0.536
Groups 3 and 4	1.0	0.083	0.596

would be useful to perform new studies, with longer duration and larger numbers of animals, to determine the real influence of these treatments on experimental contusional spinal cord injury.

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