

Original Article

Protective effects of oral creatine supplementation on spinal cord injury in rats

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Study design: To evaluate a potential protective effect of increased creatine levels in spinal cord injury (SCI) in an animal model.

Objectives: Acute SCI initiates a series of cellular and molecular events in the injured tissue leading to further damage in the surrounding area. This secondary damage is partly due to ischemia and a fatal intracellular loss of energy. Phospho-creatine in conjunction with the creatine kinase isoenzyme system acts as a potent intracellular energy buffer. Oral creatine supplementation has been shown to elevate the phospho-creatine content in brain and muscle tissue, leading to neuroprotective effects and increased muscle performance.

Setting: Zurich, Switzerland.

Methods: Twenty adult rats were fed for 4 weeks with or without creatine supplemented nutrition before undergoing a moderate spinal cord contusion.

Results: Following an initial complete hindlimb paralysis, rats of both groups substantially recovered within 1 week. However, creatine fed animals scored 2.8 points better than the controls in the BBB open field locomotor score (11.9 and 9.1 points respectively after 1 week; $P=0.035$, and 13 points compared to 11.4 after 2 weeks). The histological examination 2 weeks after SCI revealed that in all rats a cavity had developed which was comparable in size between the groups. In creatine fed rats, however, a significantly smaller amount of scar tissue surrounding the cavity was found.

Conclusions: Thus creatine treatment seems to reduce the spread of secondary injury. Our results favour a pretreatment of patients with creatine for neuroprotection in cases of elective intramedullary spinal surgery. Further studies are needed to evaluate the benefit of immediate creatine administration in case of acute spinal cord or brain injury.

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Introduction

In acute spinal cord injury the primary damage is caused by the mechanical impact leading to direct contusion, shearing injury, or laceration of the spinal cord. The subsequent secondary damage includes edema formation, local ischemia, and inflammation leading to further cell death and necrosis, thereby exacerbating the initial injury.^{1–3} In contrast to the primary and irreversible trauma, the secondary effects can potentially be influenced, thereby offering possibilities for therapeutic approaches. The secondary injury mechanisms include membrane breakdown due to

phospholipid hydrolysis, formation of free radicals, release of excitotoxic concentrations of glutamate, and very high levels of intracellular Ca^{2+} accumulation.^{2,4–7} The early phase of secondary tissue destruction affects neuronal as well as glial cell populations.^{2,4,8,9} Later, a dense gliotic scar develops, inhibiting axon regeneration in addition to the endogenously present myelin-associated neurite growth inhibitory constituents.^{2,10} Although different glial cells participate in the scar formation, the reactive astrocytes play a predominant role reflected by the very high levels of glial acid fibrillary protein (GFAP).¹¹

A landmark of secondary tissue loss following CNS trauma is a devastating decrease of intracellular energy substrates.¹² This energy loss is due to vascular damage and the subsequent reperfusion induced endothelium

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damage.^{7,13} Hypoxia causes mitochondrial dysfunction leading to ATP depletion and cell death.^{12,14}

In tissues and cells with high and fluctuating energy requirements, such as in muscles and the CNS, the creatine kinase (CK)/phospho-creatine (PCr) circuit acts as a temporal and spatial energy buffering system.¹⁵ CK isoenzymes, located subcellularly at strategic sites where energy is produced or consumed, are involved in the regulation of mitochondrial energy production¹⁶ and energy consumption, eg by Ca^{2+} -ATPases.¹⁷

Supplementation of muscle cells or neurons with the high-energy phosphate precursor creatine is known to improve cellular energy levels, eg PCr/ATP ratio, and to exert a protective effect against a variety of stressors.^{18–21} In animal models of several neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS)²² and Huntington's disease,²³ creatine has proven to be a remarkably potent neuroprotective agent. Orally supplemented creatine is transported by a specific creatine transporter into muscle and neural tissues²⁴ as measured non-invasively by ³¹P-NMR spectroscopy, giving rise to a significantly increased PCr/ATP ratio in the rat brain²⁵ or by ¹H-NMR spectroscopy in either creatine-deficient patients²⁶ or healthy subjects,²⁷ showing that total creatine concentration is elevated in the brain after creatine supplementation. In a recent study with several animal species the elevation by creatine supplementation of total creatine into various organs including the brain has been confirmed by chemical analysis.²⁸

In this study, creatine was used as a substrate to increase the cellular energy status with the goal to reduce secondary tissue loss by buffering the intracellular energy levels following an SCI. To test this hypothesis, adult rats were fed with a creatine-supplemented diet for 4 weeks before undergoing a moderate contusion of the spinal cord. Locomotor capacity was assessed 1 and 2 weeks post-traumatically. Volumes of the caverns and the lesion size (including scar thickness) were measured and a scar/cavity ratio was calculated and correlated to the locomotor capacity. Spared white matter, where the main motor tracts are running, was quantified and also correlated to the post-traumatic motor behavior.

Materials and methods

Animals

Adult female Lewis rats ($n=26$) were operated on at 10 weeks of age (174–220 g body weight (bw)). The experimental procedure was approved by the Veterinary Department of the Kanton, Zürich, Switzerland.

Nutrition

To eliminate external creatine intake, control rats ($n=13$) were fed *ad libitum* for 4 weeks before and also after SCI with a purely vegetarian food (Provimi-

Kliba, SODI), free of fish and meat products or any other animal-derived proteins. In the 'creatine group' ($n=13$), the same vegetarian diet was mixed with creatine (5 g creatine (CREAPURE®)/100 g dry food) gifted from SKW (Trostberg, Germany), and subsequently prepared to rat food pellets by Provimi-Kliba Co. Kaiseraugst (Basel, Switzerland). This food was provided *ad libitum*, for 4 weeks before and 4 days after SCI. From the fifth day after the injury all the animals were fed with standard rodent nutrition. From human studies it is known that with a relatively high dosage of creatine (20 grams of creatine monohydrate per day for an adult person of approximately 70 kg, given in intervals of 4×5 grams) the maximal level of creatine is increased by 10–15% depending on the individual, with the pool being saturated after 7–10 days.²⁹ Following this loading phase, the maximal pool size can be maintained by a lower maintenance dosage of 2–5 grams per day. It has been shown in rat and other animal species that a creatine supplementation period of 2–4 weeks at the dosage used in this study is necessary and sufficient to significantly augment the creatine pool to its maximal size.²⁸

Surgical procedures and postoperative care

Rats were anesthetized using a combination of Dormicum (0.5 mg midazolam/100 g bw, Roche) and Hypnorm (0.025 mg fentanyl and 0.8 mg fluanosine/100 g bw; Janssen). As antibiotic prophylaxis Baytril (0.005 mg enrofloxacinum/100 g bw; Bayer) was given. The SCI was performed using the NYU impactor weight-drop device.^{30–33} The rats underwent a laminectomy of the eighth thoracic vertebra (T8) under microscopic vision. The vertebral column was stabilized using clamps on T7 and T9 spinous processes, and a 10 g weight was dropped from 12.5 mm height onto the exposed, intact dura overlying the dorsal aspect of the spinal cord. The rod was immediately removed, and the muscles and the skin were closed. The animals were kept on a heating pad (38°C) until they were awake. Following administration of 2 ml lactated Ringer solution intraperitoneally and Rimadyl (5 mg Carprofen/100 g bw; Pfizer) subcutaneously for analgesia, the rats were returned to their cages. Impact velocity and compression depth were recorded and analyzed (NYU spinal cord contusion system, Impactor software version 7.0) as described previously.^{30,31}

For postoperative analgesia the animals received Rimadyl (5 mg Carprofen/100 g bw) subcutaneously for the following 2 days. Their bladders were manually expressed three times a day until the voiding reflex was established.

Open field locomotion

Open field locomotor capacity at days 7 and 14 was assessed by using the 21-point Basso, Beattie, Bresnahan (BBB) locomotor rating scale.^{30,31} The rats were

observed for 4 min in a transparent Plexiglas box with a non-slippery floor. A score of 0 indicates no spontaneous movement, 21 points represent normal gait. Both observers were blind regarding the nutritional status of the animals.

Histology

Two weeks after SCI the animals were anesthetized with an overdose of Nembutal (25 mg pentobarbital/100 mg bw; Abbott Laboratories), and perfused transcardially with Ringer solution containing heparin (50 000 IU/l) and 0.25% NaNO₂, followed by 4% formalin in 0.1 M phosphate buffer containing 5% sucrose. The spinal cords were dissected and postfixed for 2 days in the same fixative. After immersion in 30% sucrose solution the spinal cords were embedded in Tissue-Tek (O.C.T. compound; Sakura) and frozen in isopentane at -40°C. Serial sagittal sections of 25 µm thickness and 25 mm in length were taken through the full dimension of the cord.

Immunohistochemistry

The whole procedure was carried out at room temperature. All sections were blocked in 0.1 M phosphate buffer containing 0.9% NaCl, 0.3% Triton X-100 and 10% normal goat serum for 30 min before applying the first polyclonal rabbit antibody against glial fibrillary acidic protein (GFAP (Dakopatts, Switzerland), diluted 1/2000) for 1 h to visualize astrocytes. The corresponding secondary antibody (biotinylated anti-Rabbit IgG (H+L) BA-1000 (Vector, Burlingham)) and the elite avidin-biotin-peroxidase complex (Vector, Burlingham) were each applied for 45 min to localize the primary antibodies. In between these two steps the sections were quenched with hydrogen peroxide in methanol (30% H₂O₂/100 ml methanol) for 20 min. Finally, the slides were placed in 0.05% DAB solution (3,3'-Diaminobenzidine (Sigma, St. Louis, MO, USA)) for up to 3 min. The reaction was stopped in 0.1 M phosphate buffer. Sections were dehydrated and mounted in Eukitt (Kindler, Freiburg, Germany) before evaluation.

In addition to the immunohistochemical staining, a standard cresyl violet staining was performed on every fourth section on top of the GFAP staining. This allowed judging of the overall histology of the lesion and to visualize the border of gray and white matter, which was important for the evaluation of spared white matter.

Quantification of lesion volumes

To assess the size of the cavern and the surrounding scar tissue, the volume of the cavern as well as the volume of the whole lesion (including the scar tissue) was calculated using the Neurolucida (Version 2.1; MicroBrightField, Inc.) program. Measurements were performed using a 40× magnification and volumes

were given in mm³. The contour of the cavern and of the total lesion (cavern and scar) area was highlighted in eight sections spaced 200 µm apart, and the corresponding volumes were calculated. Scar tissue was defined as areas with substantially increased GFAP expression of activated astrocytes in the gray and white matter compared to the normal background staining of astrocytes. In high magnification (40×), the changed morphology of activated astrocytes with a consecutively upregulated GFAP expression enabled a distinction between scar and preserved tissue. The volume of the scar tissue was calculated by subtraction of the cavern volume from the total lesion volume.

Quantification of spared white matter

Histological examinations were performed with a light microscope (Olympus BX50, Japan). The extent of the lesion was reconstructed from a series of sagittal sections of each animal. Thus the maximal lesion depth (as percentage of the size of the uninjured cord rostral to the lesion) of every second sagittal section was marked at the appropriate location on a schematic cross-section. To calculate the percentage of spared white matter, a grid was projected onto the reconstructed cross sections dividing them into 120 squares (10 in the dorso-ventral and 12 in the horizontal plane). The ratio between destroyed and total white matter was calculated according to the number of squares with destroyed or spared white matter, respectively.

All measurements of lesion volumes and percentage of SWM were performed on coded section series by one investigator to assure a consistent evaluation.

Statistical analysis

All values were expressed as means ± standard error of the mean (SEM). For statistical analysis the Mann-Whitney *U*-test was used (Prism, Graph Pad Software). The level of statistical significance was set at *P* < 0.05. Regression lines and Pearson's correlation coefficient (*r*) were calculated using MS Excel.

Results

Spinal cord contusion

In 10 out of 13 animals of both treatment groups, the computer analysis of the impact data revealed a comparable spinal cord contusion. These 20 rats were included in the analysis. All animals manifested significant bilateral hindlimb paralysis immediately following the surgery.

Locomotor capacity

Locomotor capacity in an open field was quantified according to the BBB rating system.^{25,26} In control rats the mean BBB score 7 days after SCI was 9.1 (±0.81).

In creatine supplemented animals this value was 11.9 ± 0.8 ($P=0.03$, Figure 1a). At this time-point creatine fed rats frequently showed weight-supported stepping with no or occasional coordination of the fore- and hindlimbs. In contrast, controls were able to support their weight with a plantar placement in stance only.

Two weeks after SCI the mean BBB value for the creatine fed rats was 13.0 ± 0.73 , for the control rats 11.4 ± 0.7 ($P<0.05$) (Figure 1b). Both groups were able to perform weight-supported stepping, and in the creatine-supplemented animals only, fore- and hindlimb coordination was found.

Cavern and lesion size, volume of scar tissue

The volume of the cystic cavern and the total lesion, defined as cavern volume and the scar tissue, were measured. In the controls, the mean cavern volume was $1.95 \text{ mm}^3 \pm 0.23$, in creatine fed rats the value was $2.23 \text{ mm}^3 \pm 0.23$; the difference between the groups was not significant. The total lesion volume in the controls was $4.56 \text{ mm}^3 \pm 0.41$ as compared to that in creatine treated rats of $4.43 \text{ mm}^3 \pm 0.38$ ($P>0.05$).

The scar tissue volume amounted to $2.61 \text{ mm}^3 \pm 0.21$ in the controls and $2.2 \text{ mm}^3 \pm 0.2$ in the creatine fed animals. The scar/cavern ratio was significantly smaller in the creatine supplemented rats (1.03 ± 0.07) than in the controls (1.38 ± 0.09 ; $P=0.016$; Figure 2). Representative histological sections of both groups are shown in Figure 3, showing the difference in the area of GFAP positive scar tissue surrounding the central cavern.

Locomotor capacity and cavern volume

To investigate the degree of correlation between cavern or total lesion size and functional outcome the Pearson correlation coefficient was calculated at the time-point of the sacrifice 2 weeks after the injury. There was a moderate correlation of the locomotor capacity and the cavern size in creatine fed animals ($r=0.642$). However, in controls the locomotor capacity was not correlated with the cavern volume ($r=0.181$). Comparing single animals with caverns of similar volume showed that creatine fed rats displayed distinctly higher BBB scores than the control group (Figure 4).

Spared white matter (SWM)

To correlate the amount of neural tissue destruction, especially of the motor tracts with the locomotor capacity, a SWM percentage of the different funiculi was determined (Table 1). The average amount of SWM in creatine fed rats was not different from the control group. However, Figure 5 demonstrates the significant differences in the correlation of SWM and the locomotor capacity of control and creatine fed rats 2 weeks after SCI (controls $r=0.874$; creatine $r=0.457$). Correlating the SWM values of the lateral funiculi, where the reticulospinal tract is located, with

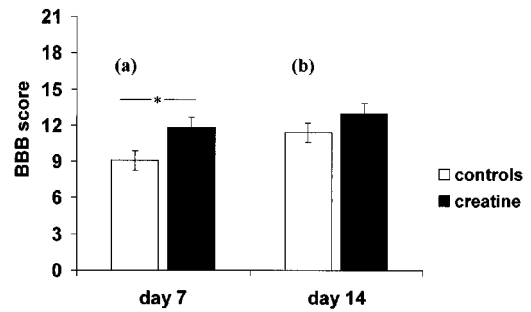


Figure 1 In creatine supplemented rats the BBB locomotor score at day 7 (BBB 11.8 ± 0.8) is significantly ($P=0.035$) better than in controls (BBB 9.1 ± 0.8). At day 14 the locomotor capacity in the creatine group is still higher (BBB 13.0 ± 0.7) than in controls (BBB 11.4 ± 0.8), $P>0.05$

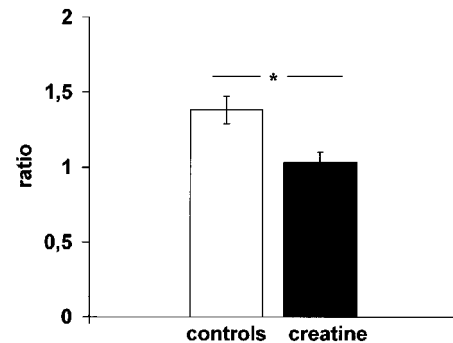


Figure 2 Creatine supplemented rats had significantly ($P=0.016$) less scar tissue in respect to the reduced ratio of the scar/total lesion volume

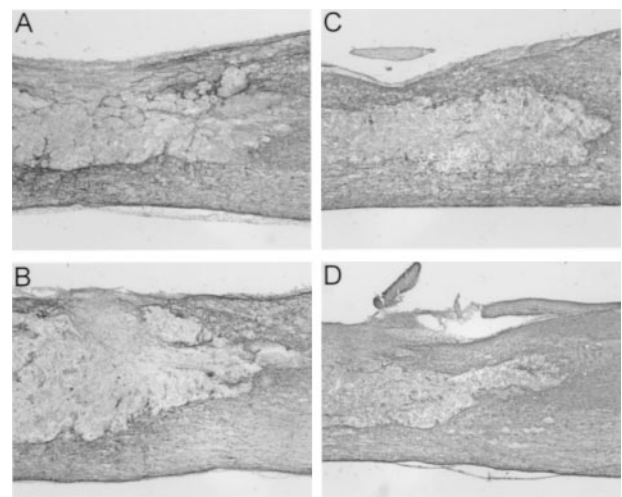


Figure 3 Photomicrograph of sagittal $25 \mu\text{m}$ GFAP-stained representative sections of the spinal cord showing the lesion cavity with surrounding scar tissue. The dark area with stained reactive astrocytes representing scar tissue is bigger in controls (A,B) than in creatine supplemented rats (C,D). Original magnification $\times 25$

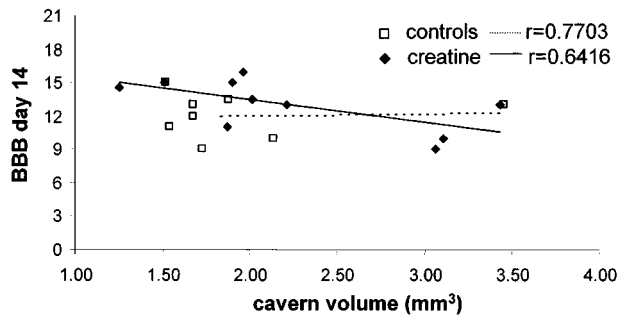


Figure 4 Correlation between cavern volume and locomotor function of individual animals 14 days after injury

Table 1

	Controls	Creatine treated group
Lateral funiculus		
(reticulospinal tract)	45.5% (± 4.2)	44.8% (± 3.3)
Dorso-lateral funiculus		
(rubrospinal tract)	35.2% (± 4.3)	37.0% (± 2.9)
Dorsal funiculus		
(corticospinal tract)	5.2% (± 1.4)	9.8% (± 2.9)
Ventral funiculus		
(vestibulospinal tract)	22.7% (± 3.5)	28.2% (± 2.9)
Total white matter	27.0%	29.9%

Mean percentage ($\pm$ SEM) of spared white matter (SMW) of the different funiculi and of the total white matter in control and creatine fed animals. None of the differences between the groups is significant ($P > 0.05$)

the BBB score of the given animals, a high correlation was found for the control group animals ($r = 0.927$), but not for creatine fed animals ($r = 0.306$). For the dorso-lateral funiculus (location of the rubrospinal and partly the reticulospinal tract) similar findings were found (controls $r = 0.851$; creatine supplemented $r = 0.199$). These values are due to the interesting fact that several of the creatine treated animals with a relatively low amount of SWM in the lateral funiculus displayed a high locomotor capacity, a phenomenon not observed in the controls (Figure 6A,B).

No correlation between behavioral outcome and relative amount of tract sparing was found for the dorsal or ventral funiculi (Figure 6C,D).

Discussion

In the present study, creatine was administered orally to adult female rats 4 weeks prior to a moderate spinal cord contusion injury, and continued for 4 days after the surgery. Rats fed with creatine supplemented diet showed a significantly better posttraumatic locomotor capacity compared to controls. The improved hindlimb function was most marked 1 week after SCI, and less pronounced after 2 weeks. However, the fore- and hindlimb coupling was much more consistent in the

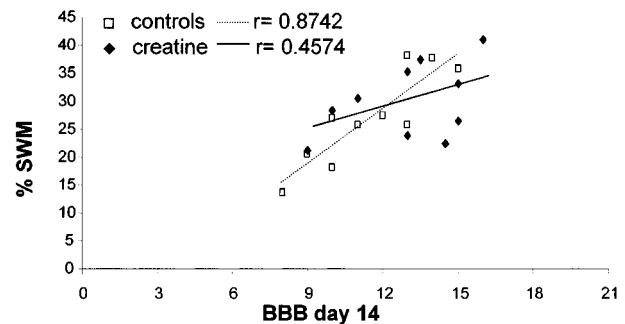


Figure 5 Correlation between the percentage of spared white matter (SWM) and the locomotor function of individual 14 days after injury. Note that the creatine fed animals reached higher locomotor scores with less SMW as compared to the controls

creatine supplemented animals. Histological analysis of the lesion site revealed a slightly smaller scar tissue in creatine treated animals. Creatine fed rats with similar amounts of SWM in the lateral funiculus had higher locomotor capabilities than the corresponding control animals. Our results indicate that creatine supplementation reduced the effect of secondary neurotrauma, reflected by a significant reduction of the scar/cavern ratio. The mechanism how increased creatine levels could dampen astrocyte activation and GFAP expression is unclear at the moment. One possibility is the reduction of diffuse cell death in the penumbra zone of the lesion. Apoptotic cells, including oligodendrocytes were found from 6 h to 3 weeks after injury, especially in the white matter of the spinal cord.⁴ These cells could be responsible for axonal demyelination and further functional impairment.^{3,4,9} Considering the protective effects of the energy precursor creatine on metabolically stressed neural cells *in vitro*,¹⁸ it is likely that higher intracellular energy levels reached after creatine supplementation *in vivo* may have saved some of these cells in the penumbra zone around the primary lesion. Rescuing oligodendrocytes and preservation of myelin is expected to have large effects on the functional outcome after SCI.³ From our data it is also clear that the significant reduction of the scar/cavern ratio in the creatine treated group (1.03 in creatine fed animals *versus* 1.38 in controls, $P = 0.016$) is not due to the difference of the cavern volume, since this parameter was similar in both groups, but it is rather due to a reduction by creatine of the extent of scar tissue formation.

An interesting finding of our study is the poor correlation of the descending SWM and locomotor capacity in creatine treated animals. It indicates that creatine fed animals performed significantly better with proportions of white matter comparable to the controls. Again, intactness of myelin, which would have to be assessed by ultrastructural or physiological techniques could play a crucial role.

There are a number of documented effects of creatine on cell survival. Creatine is taken up into the CNS via a

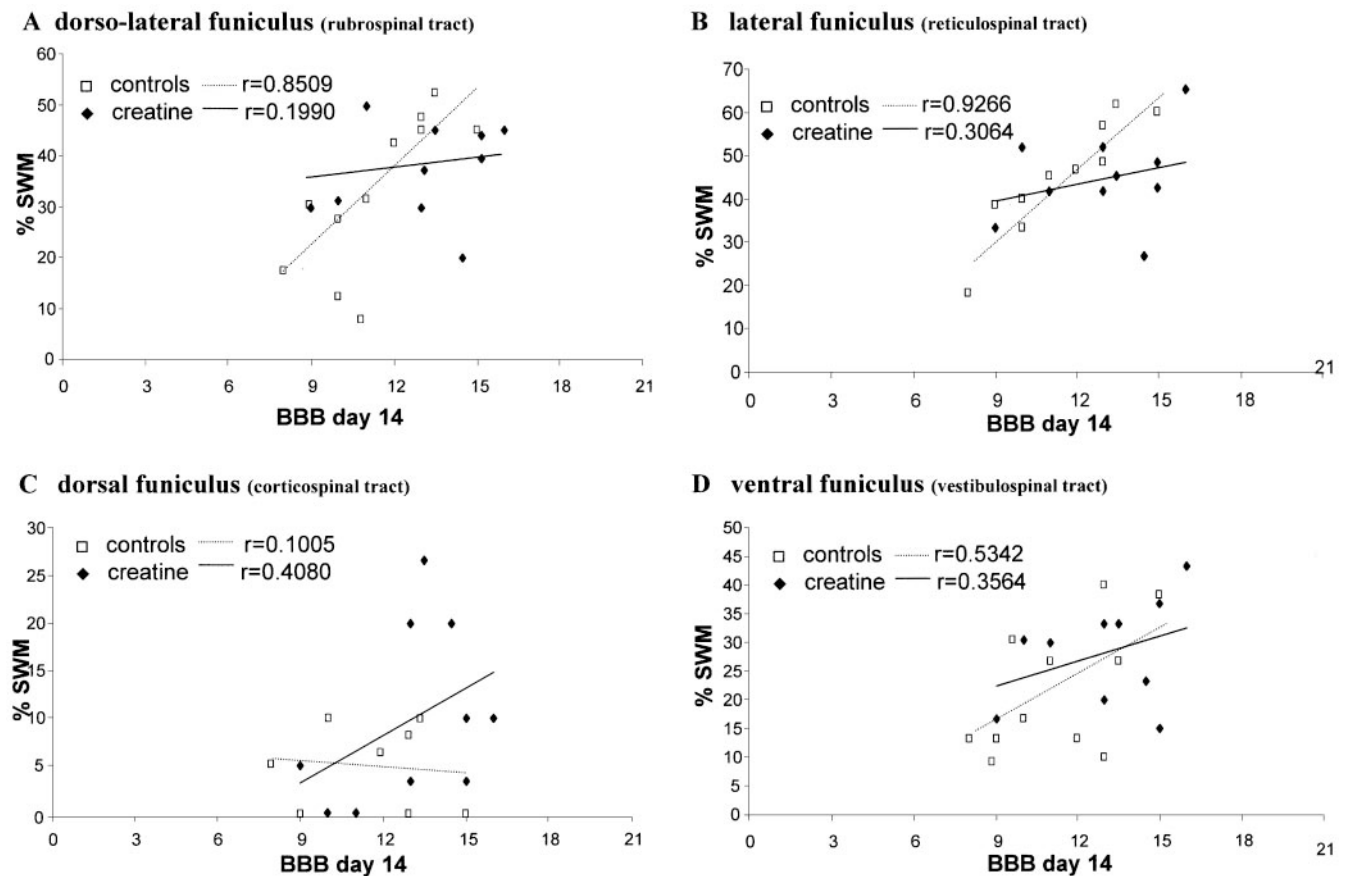


Figure 6 Correlation between the percentage of spared funiculi representing the localization of major descending tracts and the locomotor function of individual animals

specific creatine transporter (for review see²⁴); thus leading to an elevation of the total creatine concentration, ie, creatine as well as phospho-creatine.^{34–36} Creatine may decrease the effects of trauma-induced hypoxia especially by buffering intracellular ATP.^{15,37} Due to improved cellular energetics, the cells are likely to be more resistant against calcium overload and noxious acidic pH values.³⁷ Additionally, neurons can be protected by creatine by inhibition of mitochondrial permeability transition pore opening via the stabilizing action of mitochondrial CK octamers.³⁸ Furthermore, creatine's neuroprotective activity may also be related to its influence on the synthesis and release of neurotransmitters.³⁹ Additionally, creatine is also enhancing synthesis and accumulation of CK isoenzymes in nerve cells,¹⁸ thus again improving cellular energetics. Several reports have shown positive effects of creatine and its analogs on neuronal survival in culture following stress with glutamate or beta-amyloid,¹⁸ as well as *in vivo* in the brains of newborn mammals under conditions of anoxia.^{20,25,36} In transgenic mice models of ALS²² and Huntington's disease²³ creatine was shown to increase life span and improve motor behavior. Indeed, the recent study by Sullivan and coworkers shows that creatine brings about a

significant neuroprotection in traumatic brain injury by decreasing the extent of cortical damage by as much as 50% in rats.⁴⁰ This neuroprotection seems to be related to creatine induced maintenance of mitochondrial bioenergetics; cellular ATP levels were maintained, mitochondrial permeability transition inhibited, and mitochondrial Ca^{2+} concentration was lowered in the creatine group. Finally, clinical trials showed a beneficial effect of creatine supplementation in neuromuscular diseases and posttraumatic rehabilitation by improving muscle strength and counteracting atrophy.^{41–43} In the present study, a direct effect of creatine on the muscles appears unlikely as the BBB scores are based on specific qualitative criteria eg trunk and limb coordination or specific foot placement. Therefore, it seems highly unlikely that the distinct improved locomotor behavior depends mainly on the increased muscular strength, although the latter could be a synergistic factor for better recovery.

Taken together, our findings provide evidence for positive effects of dietary creatine on spinal cord tissue preservation and functional outcome after SCI, reflected by a proportionally smaller lesion scar and increased locomotor capacities. Considering the proven safety and efficacy of creatine supplementation in

sports at the present time (for review⁴⁴), creatine may be considered as an adjuvant therapy for neuroprotection in the clinic in the future, eg in elective surgery of the spinal cord or brain, where oral creatine could be given prior to the operation. With respect to acute SCI, beneficial effects might still be expected if creatine would be given immediately after the injury, because creatine could be added to neuronal cell cultures as late as 2 h after a glutamate insult and was still protective against glutamate excitotoxicity.¹⁸ Future studies will be aimed to determine the therapeutic window, the optimal dosage, and the effects on long-term behavior of creatine administration in animal models of SCI.

Conclusions

Oral creatine supplementation elevates the phosphocreatine content in the nervous tissue and acts therefore as a potent intracellular energy buffer. Following a spinal cord contusion, locomotor capacity was increased in creatine supplemented animals 1 and 2 weeks after the injury. Histological examination revealed a significant reduction of the scar tissue surrounding the lesion cavity in creatine fed rats. Thus creatine treatment seems to reduce the spread of secondary injury, although effects of creatine on increased muscle strength, and therefore, on the improved locomotor performance may also be a contributing factor. Our results favor a pretreatment of patients with creatine for neuroprotection in cases of elective surgery within the spinal cord. Further studies have to evaluate the benefit of immediate creatine administration in case of acute spinal cord or brain injury.

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