ORIGINAL ARTICLE

Changes in substance P and NK1 receptor immunohistochemistry following human spinal cord injury

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Study design: An immunohistological assessment of substance P (SP), its NK1 receptor and claudin-5 in human spinal cord injury (SCI) tissue.

Objective: To determine whether SP and NK1 receptor immunoreactivity are altered following human traumatic SCI.

Setting: Australia.

Summary of background data: SP has been implicated in the development of neurogenic inflammation and subsequent edema development following both traumatic brain injury and ischemic stroke. In these conditions, inhibition of its NK1 receptor has been shown to be neuroprotective as reflected in a reduction of edema and improved functional outcome. However, the role of SP following human SCI has not yet been assessed.

Methods: Archived human SCI tissue was grouped according to survival times: control (no injury; $n = 5$); immediate (death within an hour of the incident; $n = 6$); 2–5 h ($n = 3$); 3 days ($n = 5$); 1 week ($n = 3$); and 3–4 weeks ($n = 6$). Sections were assessed for SP, its NK1 receptor and claudin-5 using immunohistochemical techniques.

Results: Following SCI, dorsal horn SP immunoreactivity demonstrated a profound decrease compared with control tissue, indicating the loss of SP with SCI. A marked increase in perivascular NK1 staining was demonstrated after SCI compared with control levels. No obvious change in claudin-5 immunoreactivity was present immediately following injury, however, by 1 week post-SCI, decreased levels were noted.

Conclusion: This study demonstrates that severe acute traumatic human SCI results in decreased SP and an immediate increase in NK1 receptor immunoreactivity, suggesting that there is a neurogenic inflammatory component following human SCI.

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INTRODUCTION

Spinal cord injury (SCI) is an unexpected event that is both devastating and debilitating, commonly resulting in permanent physical disability. The highest incidence of SCI occurs in younger populations, resulting in an enormous financial burden to both individuals and society.[1] Current treatments for SCI are largely ineffective with little functional benefit observed. As such, research into reducing injury and improving neurological function following SCI is of growing importance.

It has been well established that human SCI is characterized by severe hemorrhage and edema. Indeed, the presence of both hemorrhage and edema, as assessed by magnetic resonance imaging and computed tomography scanning, has been associated with poor functional outcome following human SCI.[2] In addition, such studies demonstrated that functional outcome worsened when edema spread rapidly over multiple spinal cord segments,[2] sometimes encompassing an additional vertebral segment each day.[3] Such rostrocaudal spreading of edema results in further tissue damage and worsened functional outcome.[4] Moreover, a study by Koyanagi *et al.*[5] demonstrated that surgical decompression did not affect cord swelling. Thus, edema following SCI presents as a serious complication, with no effective treatment currently available.

Although the mechanisms associated with edema formation are unclear, substance P (SP) is a neuropeptide that has been recently implicated in edema formation through its ability to induce neurogenic inflammation.[6,7] SP illicits such a response through binding and activation of its primary receptor the NK1 receptor,[8] resulting in increased vascular permeability and development of vasogenic edema. Recent experimental studies have demonstrated such a role for SP in both traumatic brain injury (TBI)[9] and ischemic stroke,[10] where increased SP expression was associated with increased blood–brain barrier (BBB) permeability and edema. Furthermore, inhibition of SP with an NK1 receptor antagonist reduced BBB permeability and edema development while improving functional outcome.[9,10] Although similar secondary injury processes occur following TBI, stroke and SCI, including the development of vasogenic edema,[11] the role of SP as a mediator of neurogenic inflammation following SCI has not yet been established.

As such, the current study investigates the expression of SP, NK1 receptor and claudin-5 through immunohistochemical analysis following human SCI. Claudin-5 is an integral tight junction protein of the BBB and blood–spinal cord barrier (BSCB)[12] whose altered expression may reflect a role for tight junction dysfunction in the development of vasogenic edema following SCI.

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MATERIALS AND METHODS

Archived human spinal cord tissue was obtained from the SA brain bank (Adelaide, South Australia, Australia). In total, 23 patients with documented traumatic SCIs and 5 control cases with no trauma evident following post-mortem examination were included. All cases underwent comprehensive neuropathological analysis with macroscopic and microscopic assessment of all spinal cord segments following formalin fixation of the spinal cords. The SCI cases were grouped into survival times post-injury: immediate (death within an hour of the incident; $n=6$); 2–5 h ($n=3$); 3 days ($n=5$); 1 week ($n=3$) and 3–4 weeks ($n=6$).

Immunohistochemistry

Archived paraffin blocks of the lesion epicenter and adjacent spinal cord segments were used in this study. Five-micrometer-thick sections were cut for histological examination including assessment using hematoxylin and eosin staining to allow for observation of cellular changes within the lesioned area and the adjacent segments. Immunohistochemistry was used to identify the expression of SP (Santa Cruz Biotechnology, Dallas, TX, USA, Sc-9758; 1:2000; EDTA antigen retrieval), NK1 receptor (Thermo Scientific, Waltham, MA, USA, PA3-301; 1:16 000; citrate antigen retrieval) and claudin-5 (Invitrogen, Victoria, Australia, 32–2500; 1:2000; EDTA antigen retrieval). The SP (N-18) is an affinity-purified goat polyclonal antibody raised against a peptide corresponding to full-length mature of SP of human origin. The NK1 receptor antibody is an affinity-purified rabbit polyclonal antibody, which specifically corresponds to residues S(387) S R S D S K T M T E S F S F S S N V L S(407) of human NK1 receptor protein. The claudin-5 antibody is affinity purified from mouse ascites, which reacts specifically with the ~ 22–24 kDa endogenous claudin-5 protein. Generated slides were scanned at high resolution using a Hamamatsu Nanozoomer (Hamamatsu, Hamamatsu, Japan), and viewed using the associated proprietary viewing software (NDP.view v1.1.27, Hamamatsu, Hamamatsu, Japan). Qualitative assessments were made using a ranking system ranging from 0 to 5, where $0=$ no immunoreactivity present, $1=$ very faint, $2=$ faint, $3=$ moderate, $4=$ dark and $5=$ very dark.

Statistical analysis

All data were assessed using a Kruskal–Wallis analysis of variance followed by Dunn's multiple comparisons test.

RESULTS

SCI case data

Case data are summarized in Table 1. There was a higher proportion of males in the study sample than females (males $=21$, females $=3$). The age of individuals was wide ranging (10–96 years) with a mean age of 47 ± 25 years. The most common cause of injury was motor vehicle accidents (vehicle occupant or pedestrian), followed by falls. All causes were traumatic in nature except for one iatrogenic case that involved acute compression of the spinal cord during fusion surgery of the C1 and C2 vertebrae. The predominant type of primary injury was fracture dislocation and the most common level of injury was cervical.

Morphological features of human SCI

Hematoxylin and eosin staining was used to assess general morphological features following human traumatic SCI (Figure 1). Immediately following injury, profound hemorrhage was observed diffusely throughout the swollen injury epicenter of the spinal cord, extending into the adjacent segments. There was extensive microvacuolation of the neuropil consistent with edema. At 3 days post-SCI, severe hemorrhagic necrosis and swelling were present within the injury epicenter and adjacent segments. Axonal swellings were apparent in the white matter regions within the injury epicenter and to a lesser extent within the adjacent segments. At 1 week post-SCI, the extent of hemorrhage and swelling diminished, however, an increase in tissue

Table 1 Case data for human spinal cord injury

Case No.	Age (years)	Gender	Type of injury	Level of injury	Cause of injury	Survival	Time to post-mortem (h)
1	22	M	FD	C2/3	Jet ski accident	1 h	48
2	67	M	FD	C2	MVA—VO	1 h	48
3	65	M	FD	C2/3	Fall	1 h	48
4	28	M	FD	C2/4	Forceful impact	1 h	96
5	50	M	FD	Cervical	Cyclist v car	1 h	96
6	18	M	BF	C/T	MVA—VO	1 h	72
7	24	F	FD	C3/4	Fall	2 h	48
8	60	M	FD	C2	Fall	3–4 h	48
9	73	F	FD	C1/2	Cyclist v car	5 h	48
10	42	M	FD	T3/4	MVA—VO	3 Days	48
11	96	F	FD	C5/6 and T2/3	MVA—VO	3 Days	72
12	90	M	FD	C5	Unknown	3 Days	72
13	30	M	FD	C4/5	MVA—VO	3 Days	24
14	26	M	BF	C7/8	Fall—HV	3 Days	48
15	67	M	FD	T7/8	MVA—VO	1 Week	—
16	54	M	Contusion	C	MVA—pedestrian	1 Week	72
17	—	M	FD	T7	Unknown	1 Week	48
18	87	M	FD	C3/4	Fall	3 Weeks	72
19	26	M	FD	T5/6	MVA—VO	3 Weeks	—
20	37	M	FD	C4/5	MVA—pedestrian	3 Weeks	96
21	73	M	FD	C4	Fall	3 Weeks	24
22	26	M	FD	T5	MVA—VO	3 Weeks	72
23	29	M	FD	C5/6	MVA—VO	4 Weeks	72
24	45	F	No injury	NA	NA	NA	24
25	47	M	No injury	NA	NA	NA	96
26	10	M	No injury	NA	NA	NA	24
27	11	M	No injury	NA	NA	NA	48
28	72	M	No injury	NA	NA	NA	48

Abbreviations: BF, burst fracture; C, cervical; F, female; FD, fracture dislocation; HV, high velocity; M, male; MVA, motor vehicle accident; NA, not applicable; T, thoracic; —, unavailable/unknown; VO, vehicle occupant .

loss was observed with marked axonal swelling in the white matter. Within the adjacent segments, minimal hemorrhage was observed with localized areas of axonal swelling. By 3–4 weeks post-SCI, a substantial loss of tissue, because of reabsorption of necrotic tissue, was apparent with hemorrhage no longer present. Within the adjacent segments at this time, greater tissue loss within the white matter was observed when compared with the 1 week time point and cyst formation was present in some cases.

SP response following human SCI

SP immunoreactivity following human traumatic SCI is summarized in Figure 2 and Table 2. In control cases, a high level of SP immunoreactivity was observed within lamina II and III of the dorsal horn region, which was expected given that SP stores are known to exist within this area. No difference in immunoreactivity was observed along the length of the spinal cord. Immediately following injury there was little change in SP immunoreactivity within the injury epicenter or along the adjacent segments when compared with control. Within 2–5 h of injury, the level of SP immunoreactivity began to decrease in the injury epicenter and also in the segments caudal and rostral to the injury. At 3 days post-injury, a decrease in SP
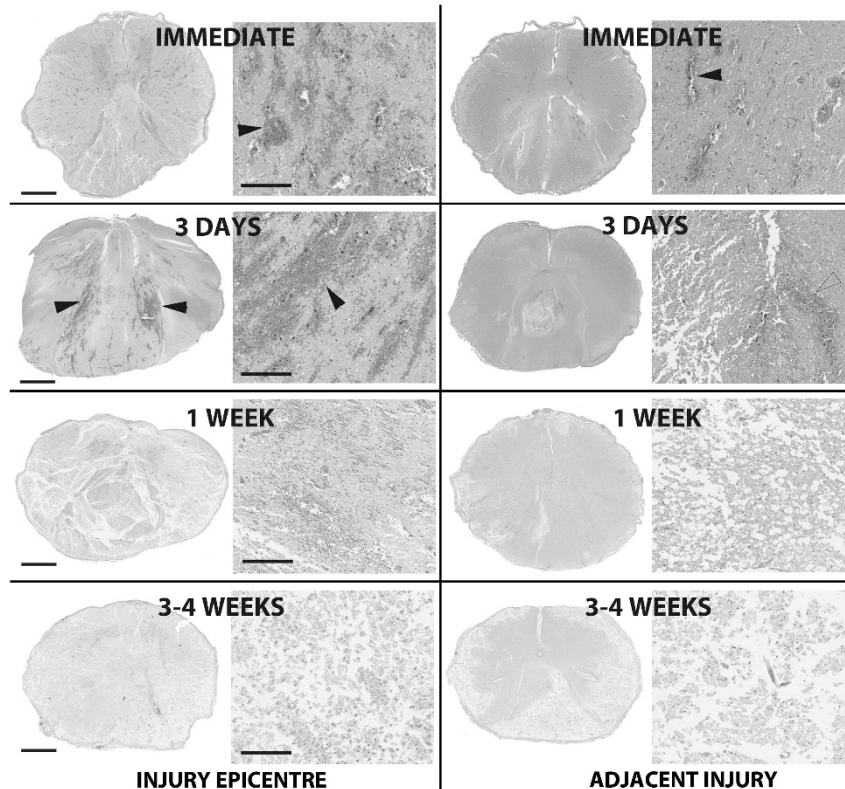
Figure 1 Hematoxylin and eosin stain—general morphological features. Immediately post-SCI severe hemorrhage was observed within the injury epicenter, which extended into the adjacent segments (black arrowheads indicate areas of hemorrhage). At 3 days post-SCI, severe hemorrhage was still evident within the injury epicenter and to a lesser extent within the adjacent segments. At 1 week post-SCI, moderate hemorrhage was still apparent with significant loss of tissue architecture. However, the adjacent segments demonstrated conserved tissue architecture with minimal hemorrhage and vacuolation of white matter. At 3–4 weeks post-SCI, significant loss of tissue architecture was observed within the injury epicenter, with extensive white matter changes within the adjacent segments. Whole cross-sections: scale bar = 2 mm; higher magnification: scale bar = 200 μm.

immunoreactivity was still evident within the injury epicenter, however, the distal segments both rostral and caudal showed staining comparable to control levels. At 1 week post-injury, there was again a decrease in all segments of the spinal cord when compared with control immunoreactivity. Finally, at 3–4 weeks post-injury, a significant decrease in SP immunoreactivity was observed within the injury epicenter ($P < 0.01$). Segments both caudal and rostral to the injury epicenter showed greater intensity of immunoreactivity than that of the injury epicenter most evident in the most distal segments.

NK1 receptor immunoreactivity following human SCI
NK1 receptor immunoreactivity following human SCI is summarized in Figure 3 and Table 3. The control human spinal cord sections showed a low basal level of staining prominent in the perivascular region. Immediately following SCI there was a profound increase in perivascular NK1 receptor immunoreactivity both within the injury epicenter and in the adjacent segments when compared with controls. At 2–5 h post-SCI, there was a slight decrease within the injury epicenter, and a further decrease observed at 3 days post-SCI for all levels of spinal cord. At 1 week post-SCI, a slight increase in NK1 receptor immunoreactivity within the injury epicenter was observed. In addition, a slight increase was observed in both rostral segments when compared with control levels. By 3–4 weeks post-SCI, all segments demonstrated immunoreactivity comparable to control, except the injury epicenter, which was slightly decreased.

No significant differences were observed at any time point or at any level of the spinal cord tissue.

Claudin-5 expression following human SCI
As an integral member of the BSCB protein family, claudin-5 immunoreactivity was assessed to determine if its expression is altered following traumatic SCI (Figure 4 and Table 4). The control sections demonstrated pronounced intense perivascular immunoreactivity. Immediately post-injury and at 3 days post-injury all segments of spinal cord remained comparable to control immunoreactivity. However, by 1 week post-injury a decrease in claudin-5 immunoreactivity was noted within the injury epicenter and adjacent segments. This decrease remained apparent at 3–4 weeks with a significant decrease observed within the injury epicenter ($P < 0.05$).

DISCUSSION
Given that human SCI occurs in an uncontrolled environment, the results of the present study must be interpreted in light of the varying severity, post-mortem times and demographics of obtained cases. In an attempt to reduce such variability, cases were grouped according to survival time points and it was assumed that most cases represent the severe end of the injury spectrum given that all tissue was post-mortem. The assessed human cases demonstrated significant primary tissue damage, which manifests as severe hemorrhage and necrosis with loss of tissue architecture. Such tissue damage was greatest within the injury epicenter with extension in a rostrocaudal manner
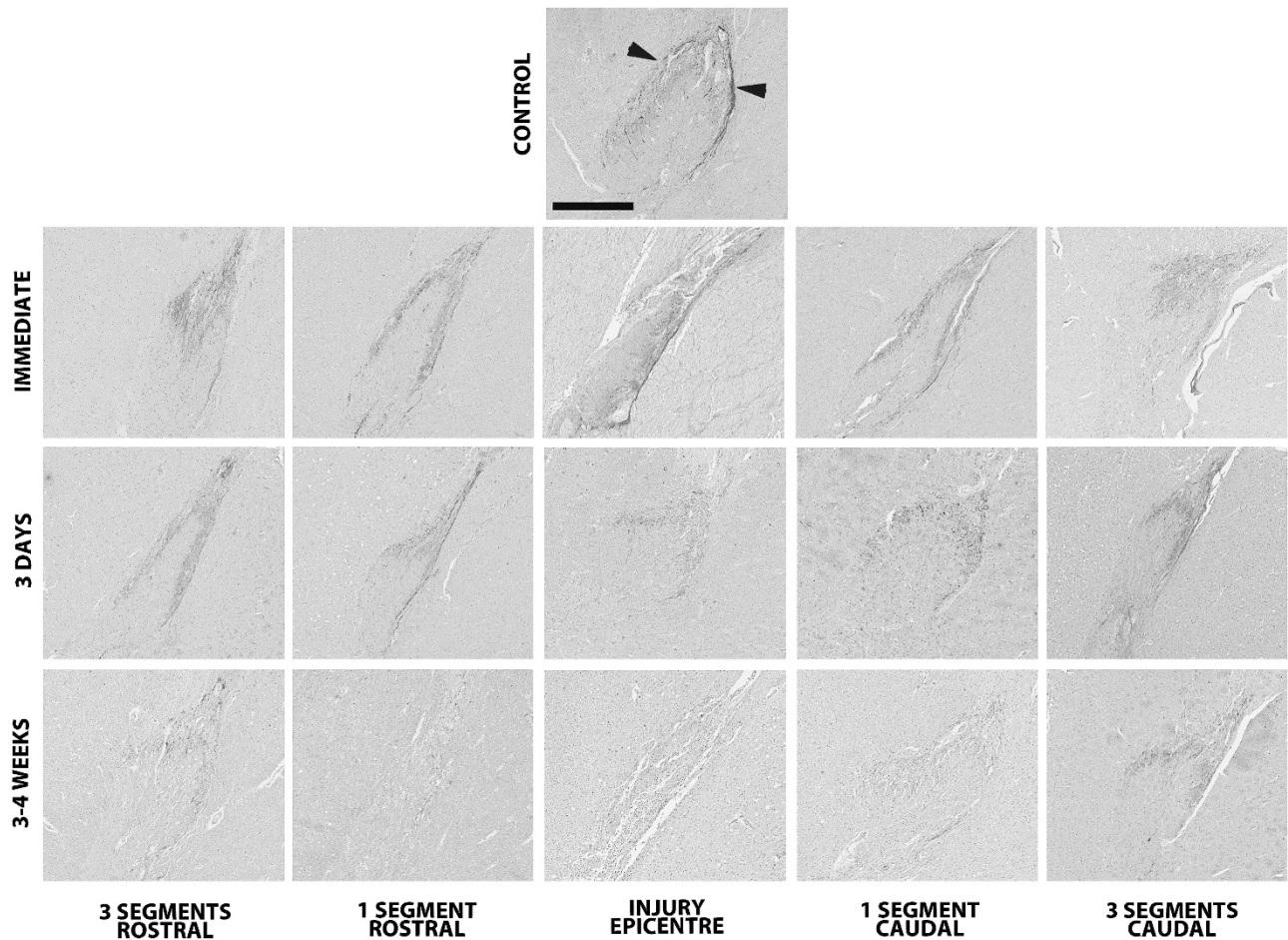
Figure 2 Human SP immunoreactivity following SCI within the dorsal horn region. High immunoreactivity was observed within the dorsal horn of control human sections. No change in SP immunoreactivity was observed immediately following injury. However, by 3 days post-injury, a decrease was apparent within the dorsal horn region. The greatest decrease in SP immunoreactivity was observed at 3–4 weeks post-injury, most notably within the injury epicenter, although apparent along all segments of the spinal cord. Rostral and Caudal images are both 1 segment away from the injury epicenter. Arrows indicate region of immunoreactivity within the dorsal horn. Scale bar = 250 μm.

Table 2 Ranking of SP immunoreactivity intensity

Survival time	Level of spinal cord				
	3	1	0	−1	−3
Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Immediate	+ + +	+ + +	+ + +	+ + +	+ + +
2–5 h	+ +	+ +	+ +	+ +	+ + +
3 Days	+ + +	+	+	+	+ + +
1 Week	+ +	+	+	+	+ +
3–4 Weeks	+ +	−	−	−	+ +

3 = 3 segments rostral, 1 = 1 segment rostral, 0 = injury epicenter, −1 = 1 segment caudal, −3 = 3 segments caudal.

to the adjacent segments. The observed pathological features demonstrated time-dependent differences with the greatest hemorrhage seen early following SCI. Maximal tissue loss was seen at 3–4 weeks following injury. These pathological features correlate with the clinical findings of acute severe SCI.[13]

Although a number of research studies have investigated the role of SP in a variety of different conditions,[7] no research has investigated the role of SP as a mediator of neurogenic inflammation following human SCI. The current study demonstrates that SP is found predominantly within the Rexed lamina I and II (substantiagelatinosa) of the human dorsal horn, consistent with previous reports of SP expression within the human spinal cord.[14] Assessment of the injured spinal cord segments revealed a significant decrease in SP immunoreactivity over time within the injury epicenter and the adjacent segments of the spinal cord. Such diminished SP immunoreactivity is likely due in part to the tissue loss observed at this time. Nonetheless, reduced SP persisted in areas of conserved tissue and as such suggests the release and subsequent depletion of the SP stores within the dorsal horn. Changes in the expression of SP have similarly been observed following disorders such as pain sensitivity.[15] A depletion of SP stores from the substantia gelatinosa was observed in people affected with diminished pain sensitivity.[15] Such a finding demonstrates that peripheral injury can alter the stores of SP within the spinal cord. Our present results are the first to demonstrate depletion of SP from the dorsal horn because of a direct injury to the spinal cord in humans.

It has been well established that SP exerts its effects on target cells via binding to the NK1 receptor.[8] Furthermore, it has been demonstrated that the process of neurogenic inflammation is
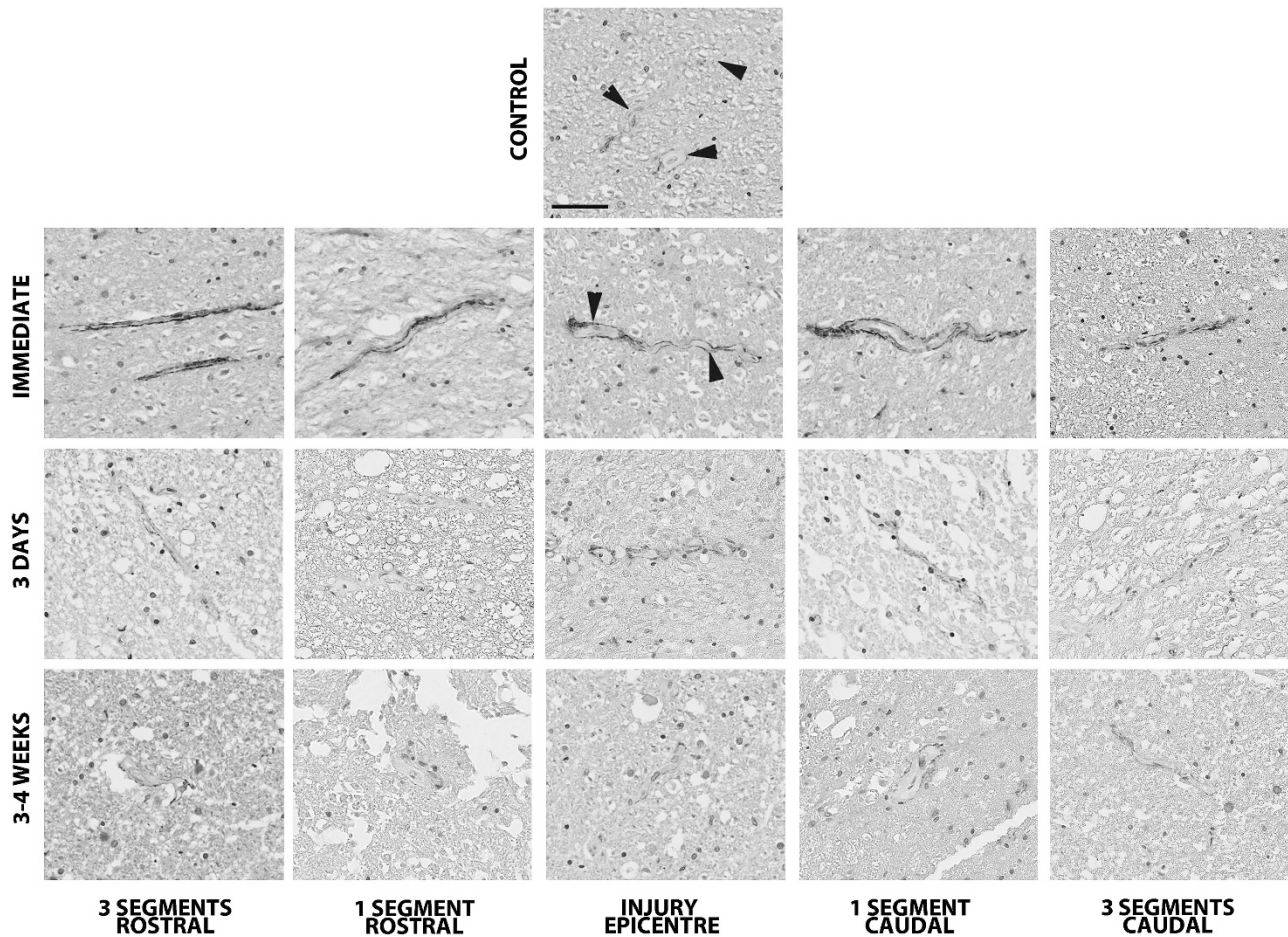
Figure 3 NK1 receptor immunoreactivity following human SCI—perivascular. All images are within the injury epicenter of the spinal cord. Faint NK1 receptor immunoreactivity was present within the perivascular region in control cases. A marked increase in immunoreactivity was observed immediately following SCI when compared with control. NK1 receptor immunoreactivity then decreased slightly at 3 days post-SCI, although remained above control levels. At 3–4 weeks post-SCI, NK1 receptor immunoreactivity was slightly decreased when compared with control. Arrows indicate perivascular immunoreactivity. Scale bar = 50 µm.

Table 3 Ranking of NK1 perivascular immunoreactivity intensity Abbreviation: NA, not applicable.

Survival time	Level of spinal cord				
	3	1	0	−1	−3
Control	+ +	+ +	+ +	+ +	+ +
Immediate	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
2–5 h	NA	+ + +	+ + +	+ + +	+ + +
3 Days	+ +	+ + +	+	+ +	+ +
1 Week	+ + +	+ + +	+ +	+ +	+ +
3–4 Weeks	+ +	+ +	+	+ +	+ +

3 = 3 segments rostral, 1 = 1 segment rostral, 0 = injury epicenter, −1 = 1 segment caudal, −3 = 3 segments caudal.

mediated by such binding.[16] Indeed, in experimental models of TBI[9] and ischemic stroke,[10] inhibition of the NK1 receptor abated neurogenic inflammation thereby reducing BBB disruption and edema formation. Furthermore, depletion of SP via administration of 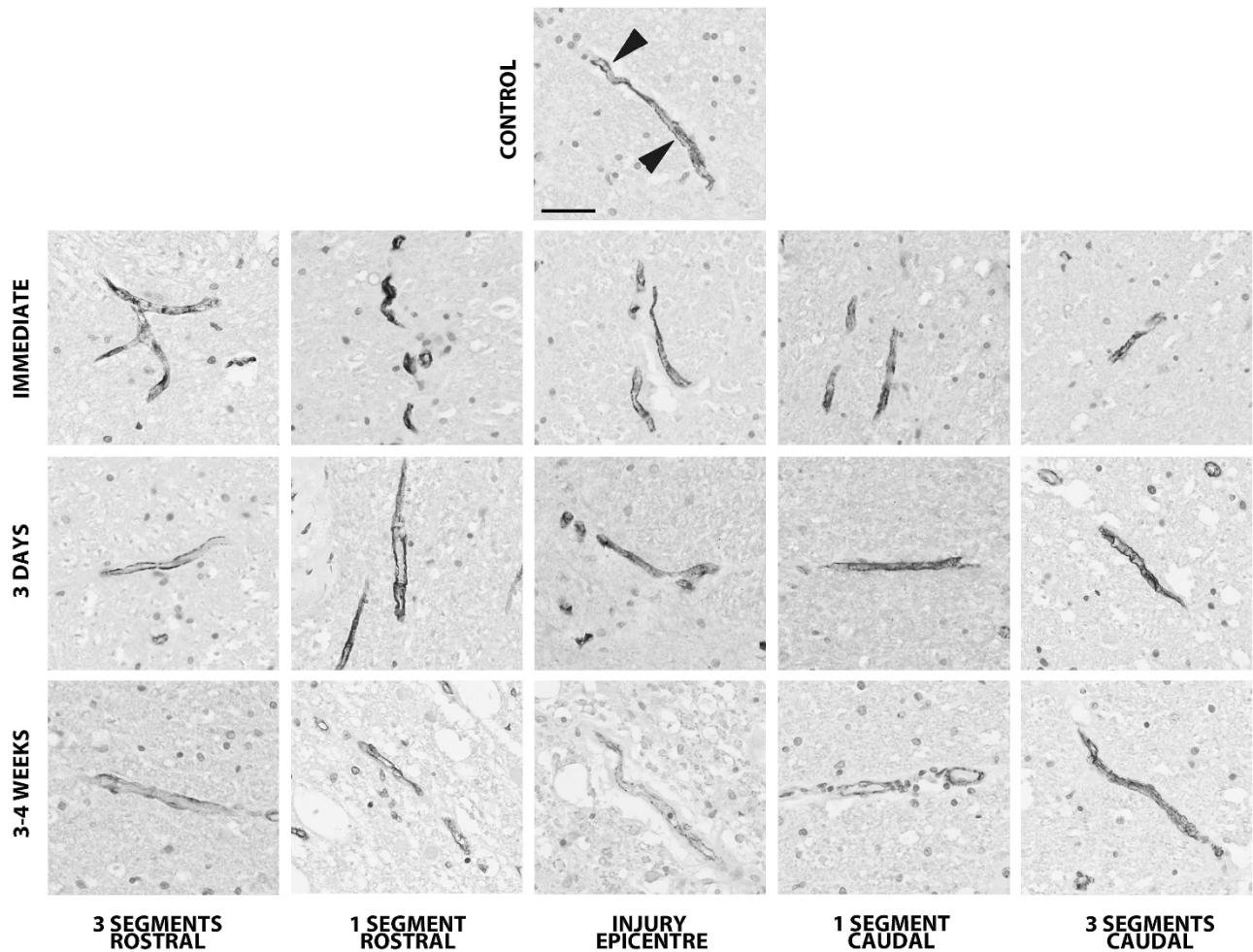capsaicin similarly demonstrated reduced BBB disruption and edema formation following TBI.[16] Localization of the NK1 receptor within the spinal cord has been extensively investigated in rats using *in situ* hybridization[17] and immunocytochemistry techniques.[18] These studies demonstrated the highest density of NK1 binding sites in Laminae I-II of the dorsal horn, intermediolateral cell column and lamina X. Also noted was moderate labelling of the ventral horn regions. Until now, no research has been performed to confirm such localization within the human spinal cord.

In contrast to experimental research, our results demonstrate little NK1 immunoreactivity within the dorsal horn region of the human spinal cord, with moderate immunoreactivity observed within the ventral horn neurons and the intermediolateral cell column. The vasculature of the white matter was comprehensively investigated to determine the possible role of SP as a mediator of neurogenic information. NK1 receptor immunoreactivity markedly increased immediately following injury within the injury epicenter and the adjacent segments. Thereafter, a gradual decrease was observed in all segments with immunoreactivity in the longer surviving cases comparable to control sections. These findings suggest that increased NK1 receptor immunoreactivity occurs in response to injury. Indeed, previous studies have demonstrated that exposure to a SP releasing stimulus

Figure 4 Claudin-5 immunoreactivity following human SCI. Control spinal cord sections demonstrated intense claudin-5 immunoreactivity. Immediately, and at 3 days post-injury, no difference in claudin-5 immunoreactivity was observed when compared with control intensity. At 3–4 weeks post-SCI, a marked reduction in immunoreactivity was apparent. Arrows indicate perivascular immunoreactivity. Scale bar = 50 μm.

Table 4 Ranking of claudin-5 immunoreactivity intensity

Survival time	Level of spinal cord				
	3	1	0	−1	−3
Control	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Immediate	NA	+ + + +	+ + + +	+ + + +	+ + + +
2–5 h	NA	+ + + +	+ + + +	+ + + +	+ + + +
3 Days	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1 Week	+ + +	+ + +	+ + +	+ + +	+ + +
3–4 Weeks	+ + +	+ + +	+ +	+ + +	+ + + +

Abbreviation: NA, not applicable. 3 = 3 segments rostral, 1 = 1 segment rostral, 0 = injury epicenter, −1 = 1 segment caudal, −3 = 3 segments caudal.

increases expression of the NK1 receptor.[19] This provides further support for the notion that the SP loss observed was in part due to SP release from the dorsal horn region. The binding of SP to the NK1 receptor mediates rapid endocytosis and internalization of the receptor.[8] Therefore, the decreased NK1 receptor immunoreactivity in the longer surviving cases is likely due to increased SP binding given the observed depletion of SP immunostaining over time.

The genesis of edema following human SCI is thought to be vasogenic in nature given the reported presence of protein extravasation within the spinal cord tissue.[11] However, this could not be accurately assessed in our post-mortem material. Claudin-5 is a major constituent of CNS endothelial cell tight junctions[12] and as such was assessed to visualize the integrity of the BSCB within the human tissue. Claudin-5 immunoreactivity remained unchanged along the entire length of the spinal cord in the first 3 days following human SCI. However, a decrease was observed at 1 week and 3–4 weeks post-SCI, particularly, within the injury epicenter and immediate adjacent segments. This decrease may reflect a decline in tight junction integrity at these time points, or alternatively may be a reflection of continued tissue loss. No changes in claudin-5 were seen in the early phases post-SCI when BSCB has been found to be most disrupted, indicating that tight junction dysfunction is not responsible for the early large-molecule extravasation following SCI. Mechanical disruption of the BSCB or increased protein transcytosis across the endothelial cells may account for this early protein extravasation. Consistent with this view, claudin-5-deficient mice died within 1 day of birth with no

macroscopic abnormalities or edema apparent, despite increased permeability to small molecules being evident.[20] As such, although claudin-5 is an integral tight junction protein of the BSCB, tight junction dysfunction is unlikely to significantly contribute to early BSCB disruption following SCI. Nonetheless, decreased claudin-5, and thus tight junction integrity, is apparent after 1 week.

CONCLUSION

The current study demonstrates decreased SP immunoreactivity associated with an increase in NK1 receptor immunoreactivity, suggesting that there is a neurogenic inflammatory component following human SCI. These events may be associated with an increase in permeability of the BSCB in the absence of tight junction failure. Whether there is potential for an NK1 receptor antagonist to reduce acute BSCB permeability and edema formation after SCI warrants further investigation.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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