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# Effects of Cerebrolysin, a mixture of neurotrophins on spinal cord injury in relation to nanoparticles treatment.

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## **Summary**

Spinal cord injuries (SCI) are of world's most disastrous disease for which no effective treatment has been developed so far. Several studies suggest that nanoparticles from the environment could influence the pathology of SCI. In my study, I have worked with engineered nanoparticles from metals e.g., Ag, Al and Au. These nanoparticles (50-60 nm) were administered in rats daily for 7 days (50 mg/kg, i.p.) prior to the injury that resulted in the exacerbation of cord pathology. The nanoparticles influenced injury leads to the breakdown of the Blood- brain barrier and become permeable to various plasma proteins. The entry of plasma proteins into the cord leads to the formation of edema. It also results in the large number of neuronal damages. Therefore the drug should be designed in such a way that it can be effective even when the injury is influenced by nanoparticles.

There has been extensive research on neurotrophic factors, carried out in lab, and it was proved that these neurotrophic factors are not effective when administered alone. So a new drug, Cerebrolysin, a mixture of different neurotrophic factors such as BDNF, GDNF and NGF was used to treat the normal SCI or following nanoparticles treatment. So far the drug has produced good results. When a higher dose (5.0 ml/kg, i.v.) of this drug was administered to the animals, which were previously treated with nanoparticles, it resulted in the marked reduction of the spinal cord water content, decrease in the leakage of plasma proteins and neuronal damages. The lower dose (2.5 ml/kg, i.v.) was also quite effective in reducing the pathology of the SCI in normal rats. Therefore these results suggest that Cerebrolysin can be a promising drug in the future for treating patients with SCI.

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## List of Abbreviations

SCI	Spinal cord injury
BBB	Blood brain barrier
IHC	Immunohistochemistry
CNS	Central nervous system
NGF	Nerve growth factor
BDNF	Brain derived growth factor
GDNF	Glial cell line derived neurotrophic factor
BSCB	Blood spinal cord barrier
NT-3	Neurotrophin -3
EBA	Evans blue albumin
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin-stimulating hormone
HPLC	High-performance liquid chromatography
MQ	Milli-Q
PBS	Phosphate buffered saline
NGS	Normal goat serum
DAB	Diaminobenzidine
H&E	Haematoxylin and Eosin
nNOS	Neuronal nitric oxide synthase
GFAP	Glial fibrillary acidic protein

## **Introduction**

### **Spinal cord anatomy**

The spinal cord is called “road of communication” as it connects the nervous system to different parts of the body. The cord is very soft and protected by the vertebral column. There are 30 vertebral bones and 31 spinal nerves. It has got different segments such as cervical (C1-C8), thoracic (T1-T12), lumbar (L1-L5), sacral (S1-S5) and single coccygeal nerve. The interior of the spinal cord consists of H-shaped region called grey matter which is made up of neuronal bodies and surrounding the grey matter is the white matter which consists of axons. There are three layers of structures which surround the spinal cord. The first layer is called the spinal pia matter, second layer is called arachnoid matter and the third layer is called dura matter. The space between pia matter and arachnoid matter is known as sub-arachnoid space which has got mesh-like appearance. The cerebrospinal fluid flows through this space. The spinal pia matter has got triangular projections called denticulate ligaments. These ligaments connect the spinal cord to the dura matter.

### **Spinal cord injury**

Spinal cord injury (SCI) is a disastrous disease that can occur following motor vehicle accidents or falls that inflicts damage to the cells that are present within the spinal cord (1). Depending on the magnitude and severity, SCI could result in the quadriplegia/tetraplegia, paraplegia and other lifetime disabilities. Thus, efforts should be made to treat SCI victims in time with suitable drugs to restore functioning of the spinal cord in order to improve the quality of lives of the victims (2).

SCI can either be traumatic or non-traumatic. The traumatic injuries are caused by various factors such as road traffic accidents, domestic work related accidents, sports injuries and gunshot or knife wounds. The non- traumatic injuries can be caused by infection of the nerve cells present in the spinal cord, cysts or tumors (3, 4).

SCI can also be described as complete or incomplete injuries. When there is a complete loss of function below the point of injury it is called complete SCI. However, when there is little movement, which is still evident below the point of injury, it is called incomplete SCI (5, 6 and 7).

## **Vascular events contributing to secondary pathogenesis**

When there is an injury in the spinal cord it first results in the mechanical disruption of the spinal cord structures, known as primary injury that slowly progresses into the secondary injury by damaging the cells in the grey matter and white matter of the cord (8).

The primary injury results in the disruption of the blood vessels. There is a decrease in the blood flow to the damaged tissue and it results in poor delivery of oxygen and nutrients to the neurons (8).

The secondary pathogenesis includes restriction in the blood flow, excessive release of neurotransmitters, inflammation created by the immune cells, free radical production and self-destruction of nerve cells. All of these mechanisms result in the increase of the area of damage in the spinal cord (8). The axons are damaged followed by the formation of glial cell scars. However, there are some mechanisms, which are still unclear like how the nerve cells destroy themselves after the injury and how the neurons are flooded with excitatory neurotransmitters such as glutamate.

## **Blood-brain barrier breakdown**

Under normal conditions the blood-brain barrier (BBB) does not allow the passage of molecules into the central nervous system (CNS). The BBB or blood-spinal cord barrier (BSCB) contains specialized endothelial cells (see figure1) that help in strict regulation of the micro-fluid environment of the CNS. The tight junctions that are present between these adjacent endothelial cells help in blocking the large molecules such as plasma proteins to enter into the neuropil. The glycocalyx, which is rich in glycoprotein, is located on the endothelial cells. It has a negative charge and thus blocks the plasma proteins because of their similar charge. The basement membrane, which is present on the parenchymal side of the endothelial cell, provides structural support to these cell membranes (9). Therefore, the BBB or BSCB structures help in the normal functioning of neurons.

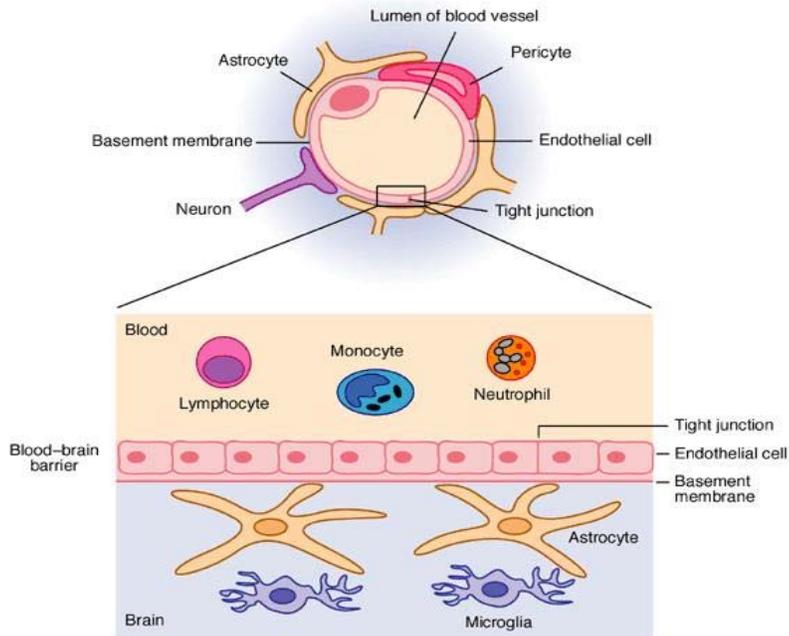


Figure 1 Structure of the blood brain barrier under normal conditions.

But when there is an injury in the brain or the spinal cord structure, the BBB or BSCB gets disturbed and there is a rapid entry of albumin into the brain or spinal cord compartment. Neurons or the non-neuronal cells can be identified using albumin immunohistochemistry after albumin was entered into the neurophil. The brain or spinal cord is then exposed to various immune system cells such as neutrophils, T-cells, macrophages and monocytes. These immune cells produce an inflammatory response that results in the damage of nerve cells. But in certain types of injury these immune cells also prove to be protective. Therefore it is still controversial weather the immune system is protective or destructive (9, 10). The entry of the immune cells into the CNS also results in the increased production of free radicals. Free radicals are a highly reactive form of oxygen molecules that cause destruction of neurons. So once the BBB or BSCB is damaged it results in neuronal damage.

## Brain edema formation

Once the BBB gets disrupted and serum proteins enter into the extra cellular environment of the CNS there is a formation of edema inside the brain or the spinal cord. Edema is caused due to an increase in the water content of the spinal cord or the brain. There are two main types of brain edema formation e.g., vasogenic and cytotoxic edema formation. Vasogenic edema is formed

when the plasma proteins and water leak into the extracellular space of the brain. Cytotoxic edema is formed when the water gets accumulated inside the intracellular brain compartment (9). During the cytotoxic edema there is a swelling of neuronal cells, non-neuronal cells and endothelial cells. The vasogenic edema formation occurs due to an increased rate of capillary filtration and reduced rate of removal of fluids from the tissues.

There are several neurochemicals that can cause an alteration in the BBB permeability and influence edema formation inside the brain, thereby resulting in the damage of cells and tissues. These neurochemicals are serotonin, prostaglandins, histamine etc (10). In many experimental cases it has been proven that if these neurochemicals are inhibited before the SCI occur then it will help to attenuate the BSCB disruption, edema formation and/or cell injury.

In the clinical or experimental situations it has been observed that the plasma protein leakage and spread of edema fluid increases with the advancement of time. The severity of brain damage influences the intensity of the edematous swelling.

### **Neurotrophic factors in spinal cord injury**

There are various neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) present within the CNS. These growth factors are required for the survival and proper functioning of the neurons (11). When there is an injury in the cord the immune cells release cytokines, which in turn upregulates the neurotrophic factors but their production is not sufficient and as a result it neither prevents the degeneration of neurons nor promotes their regeneration.

In many experimental cases these growth factors has been administered exogenously over the injured cord and it helped in reducing the pathological conditions of the spinal cord (12). When the BDNF or NGF was administered alone in the early phase of injury they improved the conditions of the injured cord by minimizing the BSCB disruption and edema formation. However, if they were given at 60-90 minutes after the injury then there was no sign of improvement.

When the BDNF or GDNF was administered in combination over the injured cord at 60-90 minutes after the injury it attenuated the pathological conditions of the cord by reducing the BSCB disruption and edema formation (13). There was some combination of neurotrophic

factors which when administered exogenously at 30-90 minutes after injury did not prove to be effective such as Brain-derived neurotrophic factor (BDNF) with Nerve growth factor (NGF) or Neurotrophin-3 (NT-3). Therefore, the combinations of neurotrophic factors should be made in such a way that it produces useful biological effects (14).

Through research work it was found that the applying time and dose of different neurotrophic factors depends on their expression level which changes depending on the different phases after injury (15). High concentration of neurotrophic factors given in different combinations improve the motor function and help in the regeneration of neurons.

Sometimes injury in the spinal cord also decreases the level of growth hormones therefore they are administered in high concentrations exogenously to provide some neuroprotective effects.

### **Nanoparticles influencing the pathology of spinal cord injury**

In the environment we have different types of nanoparticles such as silica dust particles, copper, silver and aluminum. If these nanoparticles are more in number then it can exacerbate the extent of neural injury for example the soldiers who fight during the war are exposed to different kinds of these nanoparticles so when they get injured in the spinal cord their pathophysiology is different from the persons who receive SCI from a clean and healthy environment (16). But details of these conditions are still not well known.

These nanoparticles when inhaled from the environment could enter into the body fluid system and then through endocytosis they will enter into various non-neuronal cells and induce cellular toxicity. When the cells or tissues are exposed to these small particles in the size range of 10-100 nm, they exert higher inflammation.

Nanoparticles, which are considered to be a recent research study in the field of modern science, can prove to be either constructive or destructive. When they are sized in the range of 50-200 nm they can be used for various biomedical applications such as for diagnostic and treatment purposes but sometimes they can produce some toxic effects, which can be harmful to living organisms (16). However, it is still not clear how nanoparticles produce neurotoxicity.

### *Exposure of Nanoparticles results in the damage of BSCB*

When nanoparticles such as silver, copper or aluminum in the size range of 50-60 nm were administered systematically, it induces the breakdown of BSCB making it permeable to protein tracers such as Evans blue albumin and radioiodine (17). Intravenous or intracerebral administration of these nanoparticles can result in total damage of BSCB whereas intraperitoneal administration results in less damage. Further investigations are required in order to study how the dose of nanoparticles and their route of administration result in the damage of BSCB.

### *Nanoparticles results in the formation of edema*

The nanoparticles also resulted in the exacerbation of edema formation. The edema formation in terms of an increase in the brain water content with more than 1% was observed when the nanoparticles were administered intravenously. The water content inside the brain is measured by taking the differences between the dry weight and wet weight of the samples (17).

Obviously, when the BSCB is disrupted and becomes permeable to various protein tracers it leads to the formation of vasogenic edema. The BSCB disruption and edema formation results in various morphological changes inside the brain (18). This indicates that the nanoparticles also result in the damage of cells and tissues.

## **Different treatments for spinal cord injury**

When there is an injury to the cord the first response is instant swelling. Therefore the injured patient requires immediate treatment within 8 hours of primary injury to increase the chances of recovery. Normally, the patient is treated with three different types of drugs, e.g., anti-inflammatory compounds that help to fight against the immune responses, anti-oxidants drugs that will reduce the free radical formation and anti-excitotoxins that prevent the effects of excessive release of excitatory neurotransmitters such as glutamate (19).

Methylprednisolone is an anti-inflammatory drug that is effective only if administered within the 8 hours of injury. This drug responds to the injury by reducing the immune system responses. But there are various limitations of this drug, which cannot be ignored (20). In the case when the SCI is severe enough to induce complete paralysis a higher dose of drug is given. However, if methylprednisolone is given in higher doses it can result in several complications such as muscle weakness, blood clot in veins and/or no improvement of the injury (19).

Lazaroids, 21-aminosteroids are anti-oxidant drugs and they respond to the injury by preventing the excessive production of free radicals. They are modified from the methylprednisolone drug. But FDA has not approved these drugs as they are created from the existing unapproved methylprednisolone drug (19). The efficacy of these drugs in SCI is still under investigations.

Thyrotropin-releasing hormone is an anti-excitotoxin drug and it minimizes the excessive production of neurotransmitters such as glutamate by releasing the thyroid-stimulating hormones. However, further research is required for the drug to be used for SCI (19).

These drugs work in the normal SCI patients but they do not seem to be effective in the cases where the injury in the spinal cord is exacerbated due to the exposure of nanoparticles (20). Therefore the drug should be designed in such a way that it enhances neuroprotection in both normal SCI and nanoparticles influenced injury.

### **Cerebrolysin: Promising drug for the treatment of spinal cord injury**

Cerebrolysin is the only drug available for clinical use containing active fragments of some important Neurotrophic factors. This drug is used to induce neuroprotection when there is an injury in the CNS and also help in the regeneration of neurons in neurodegenerative diseases like multiple sclerosis, Parkinson's disease, Alzheimer's disease, dementia, acute and chronic stroke victims (21).

It is the only drug whose action is similar to the various neurotrophic factors such as BDNF, GDNF and NGF etc. if a higher dose of this drug is administered systematically it is proved to be effective in the situation where the injury in the spinal cord is influenced by the exposure of the nanoparticles, but still further research is required (22).

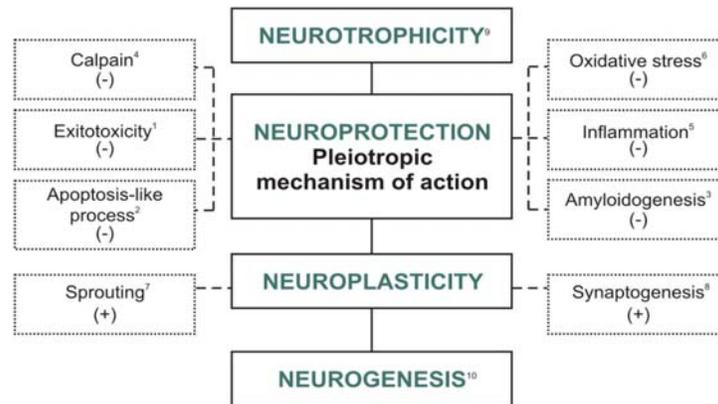


Figure 2 : Neuroprotective effects of Cerebrolysin (Refer 24, 25).

There are various mechanisms of the Cerebrolysin drug as neuronal survival, neuroprotection, neuroplasticity and neurogenesis (23). Due to these mechanisms Cerebrolysin is considered as a modulating drug which has an immediate neurotrophic activity.

Neuronal survival: the neurotrophic factors that are present in this drug help in the survival of neurons and prevent cell death.

Neuroprotection: it induces neuroprotection inside the brain even in detrimental conditions by maintaining the cellular interactions.

Neuroplasticity –it helps the process of re-wiring and thereby making the brain adapt to the various changes in the environment. It also helps in the sprouting of axons or dendrites.

Neurogenesis- it allows the formation of neurons by differentiation of progenitor cells.

The treatment with Cerebrolysin should not be used in combination with anti depressant drugs (24). So far the Cerebrolysin has been an effective therapeutic treatment for CNS injuries. However, its role in SCI and related disorders is still under investigation.

## **Aims and Objectives of the present Investigation**

In my investigations, the first aim was to observe how the nanoparticles influence the pathophysiology of SCI. The second aim was to see how effective the Cerebrolysin is when used in the normal or nanoparticles treated SCI in a rat model.

## **Materials and methods**

### Animals

Experiments were carried out on male wistar rats (body weight 250-300 g) housed at a controlled room temperature with 12 hours of light and 12 hours of dark schedule. Standard laboratory diet and tap water was supplied *ad libitum*. All experiments were approved by the local institutional ethics committee and conducted according to the National Institute of Health (NIH, USA) guidelines for the care of experimental animals.

### Nanoparticles Administration

Engineered nanoparticles from copper, silver, aluminum in the size range of 50-60 nm were first suspended in 0.05 % Tween 80 solution. The solution was then administered intraperitoneally (50 mg/kg once) for seven days.

### Spinal cord injury

Under Equithesin anesthesia (3 ml/kg, i.p.), the injury was performed as a longitudinal incision (1.5 mm deep and 4 mm long) into the dorsal horn of the T10-11 segment of the spinal cord.

### Treatment with Cerebrolysin

Cerebrolysin in a dose of either 2.5 ml/kg or 5.0 ml/kg was administered intravenously to rats 30 min before SCI in saline or nanoparticles treated animals. The animals were allowed to survive 5 h after injury.

### Control group

Normal rats were used as controls.

### BSCB permeability

The BSCB permeability was quantified with Evans blue albumin leakage (2% of 3 ml/kg, Evans blue albumin, EBA) and serum albumin immunohistochemistry. The EBA was administered intravenously and its leakage was measured in the tissue using calorimetrically.

## Albumin Staining Protocol

1. Take 4-micron thick paraffin-embedded tissue sections and apply to super frost plus slides.
2. Deparaffinize in xylene solution using three changes for 5 minutes each.
3. Hydrate sections gradually through graded alcohols; twice in 100% ethanol for 5 minutes each, twice in 95% ethanol for 5 minutes each and once in 70 % ethanol for 5 minutes.
4. Wash in milli q water for 2 minutes.
5. Incubate for 30 minutes in 3% H<sub>2</sub>O<sub>2</sub> in the dark.
6. Wash the tissues with PBS for three times and allow it to stay for 5 minutes.
7. Apply 10% normal goat serum on the slides and keep it for incubation for 60 minutes.
8. Remove blocking serum from the slides.
9. Wipe away and carefully blot excess liquid around tissue with filter paper.
10. Apply primary antibody albumin of concentration 1/4000 on to the slides.
11. Keep it for overnight incubation at +4° C in a humidified chamber.
12. Wash in PBS three times and allow it to stay for 5 minutes.
13. Apply biotin conjugated secondary antibody goat  $\alpha$  rabbit of concentration 1/200 on to the slides.
14. Keep it for incubation for 30 minutes.
15. Wash it in PBS three times and keep it for 5 minutes.
16. Apply ABC reagent on the slides and incubate it for 30 minutes.
17. Wash it in PBS three times and allow it to stay for 10 minutes.
18. Take DAB, apply it on the slides and keep it for incubation for 5 minutes.
19. Rinse sections in tap water ten times and allow it to stay for 10 minutes.
20. Dehydrate sections by washing the slides twice with 70% alcohol, twice with 95% alcohol, thrice with 100% ethanol and then it let it stay for 5 minutes.
21. Now wash the slides thrice with xylene solution and let it stay for 5 minutes.
22. Cover slip in mounting medium.
23. Now the slides can be viewed under the microscope after it is dried.

### Spinal cord edema formation

The spinal cord water content was calculated from the differences between the wet and dry weight of the tissues.

### Morphological changes inside the cord

The morphological changes such as neuronal or glial cell damage in control, injured and Cerebrolysin treated animal tissues were observed by various staining techniques such as Nissl stain, Haematoxylin & Eosin (H&E) stain, nNOS and GFAP immunostaining.

### GFAP Staining Protocol

1. Take 4-micron thick paraffin-embedded tissue sections and apply to super frost plus slides.
2. Deparaffinize in xylene solution using three changes for 5 minutes each.
3. Hydrate sections gradually through graded alcohols; twice in 100% ethanol for 5 minutes each, twice in 95% ethanol for 5 minutes each, once in 70% ethanol for 5 minutes.
4. Wash in milli q water for 2 minutes.
5. Incubate for 30 minutes in 3% H<sub>2</sub>O<sub>2</sub> in the dark.
6. Wash the tissues with PBS three times and allow it to stay for 5 minutes.
7. Apply 10% normal goat serum on the slides and keep it for incubation for 60 minutes.
8. Remove blocking serum from the slides.
9. Wipe away and carefully blot excess liquid around tissue with filter paper.
10. Apply primary antibody glial fibrillary acidic protein (GFAP) of concentration 1/800 on to the slides.
11. Keep it for overnight incubation at +4° C in a humidified chamber.
12. Wash in PBS three times and allow it to stay for 5 minutes.
13. Apply biotin conjugated secondary antibody goat α rabbit of concentration 1/200 on to the slides.
14. Keep it for incubation for 30 minutes.
15. Wash it in PBS for three times and keep it for 5 minutes.
16. Apply ABC reagent on the slides and incubate it for 30 minutes.
17. Wash it in PBS three times and allow it to stay for 10 minutes.
18. Take DAB, apply it on the slides and keep it for incubation for 5 minutes.

19. Rinse sections in tap water ten times and allow it to stay for 10 minutes.
20. Dehydrate sections by washing the slides twice with 70% alcohol, twice with 95% alcohol, thrice with 100% ethanol and then let it stay for 5 minutes.
21. Now wash the slides thrice with xylene and let it stay for 5 minutes.
22. Cover slip in mounting medium.
23. Now the slides can be viewed under the microscope after it gets dried.

#### nNOS Staining Protocol

1. Take 4-micron thick paraffin-embedded tissue sections and apply to super frost plus slides.
2. Deparaffinize in xylene solution using three changes for 5 minutes each.
3. Hydrate sections gradually through graded alcohols; twice in 100% ethanol for 5 minutes each, twice in 95% ethanol for 5 minutes each, once in 70% ethanol for 5 minutes.
4. Wash in milli q water for 1 minute
5. Wash in tap water for 2 minutes.
6. Keep the sections inside a staining jar containing 10 mM citrate buffer and then microwave at 750 W for 10 minutes and 350 W for 5 minutes.
7. Remove the staining jar from the microwave and allow it to cool for 20 minutes at room temperature.
8. Wash in PBS for three times and allow it to stay for 5 minutes.
9. Incubate for 30 minutes in 3% H<sub>2</sub>O<sub>2</sub> in the dark.
10. Wash the tissues with PBS for three times and allow it to stay for 5 minutes.
11. Apply 2.5% normal horse serum on the slides and keep it for incubation for 60 minutes.
12. Remove blocking serum from the slides.
13. Wipe away and carefully blot excess liquid around tissue with filter paper.
14. Apply primary antibody nNOS of concentration 1/500 on to the slides.
15. Keep it for overnight incubation at room temperature in a humidified chamber.
16. Wash in PBS three times and allow it to stay for 5 minutes.
17. Apply Immpress detection rabbit reagent on to the sections.
18. Keep it for incubation for 30 minutes.
19. Wash it in PBS three times and keep it for 5 minutes.
20. Take DAB, apply it on the slides and keep it for incubation for 5 minutes.

21. Rinse sections in tap water ten times and allow it to stay for 10 minutes.
22. Dehydrate sections by washing the slides twice with 70% alcohol, twice with 95% alcohol, thrice with 100% ethanol and then it let it stay for 5 minutes.
23. Now wash the slides thrice with xylene and let it stay for 5 minutes.
24. Cover slip in mounting medium.
25. Now the slides can be viewed under the microscope after it gets dried.

### H&E Staining Protocol

1. Take 4-micron thick paraffin-embedded tissue sections and apply to super frost plus slides.
2. Deparaffinize in xylene solution using three changes for 5 minutes each.
3. Hydrate sections twice in 100% ethanol for 5 minutes each,
4. Twice in 95% ethanol for 5 minutes each,
5. Once in 70% ethanol for 5 minutes.
6. Wash in milli q water for 1 minute
7. Dip the sections in Mayers HTX for 3-4 minutes.
8. Now rinse the sections in tap water for 10 minutes.
9. Rinse in milli Q water for 1 minute.
10. Dip the sections in eosin for 90 seconds
11. The sections are then dipped in 95% alcohol so that the eosin gets dissolved in it.
12. Let stand the sections twice in absolute alcohol for 5 minutes.
13. Let stand the sections thrice in xylene for 5 minutes.
14. Cover slip in mounting medium.
15. The slides can be viewed under the microscope after it gets dried.

### Nissl Staining Protocol

1. Take 4-micron thick paraffin-embedded tissue sections and apply to super frost plus slides.
2. Deparaffinize in xylene using three changes for 5 minutes each.
3. Hydrate sections twice in 100% ethanol for 5 minutes each,
4. Twice in 95% ethanol for 5 minutes each,
5. Once in 70% ethanol for 5 minutes.
6. Wash in milli q water for 1 minute

7. Dip the sections in cresyl violet solution for 3 - 5 minutes.
8. Now rinse the sections in distilled water.
9. The sections are then dipped in 95% alcohol for 5-10 minutes and check microscopically.
10. Let stand the sections twice in absolute alcohol for 5 minutes.
11. Let stand the sections thrice in xylene for 5 minutes.
12. Cover slip in mounting medium.
13. The slides can be viewed under the microscope after it gets dried.

## Results

### Nanoparticles effect on BSCB permeability

Various nanoparticles such as silver, copper and aluminum (50-60nm) were administered to the animals intraperitoneally for seven days. This was taken as control. Then the same sets of animals were subjected to SCI to observe how the pathology of injury is worsened when the animals are exposed to nanoparticles. This resulted in the breakdown of BSCB. The control group showed a mild but significant increase in the BSCB permeability to Evans blue albumin (figure 3), whereas the nanoparticles treated animals subjected to SCI showed a much larger effect (table 1).

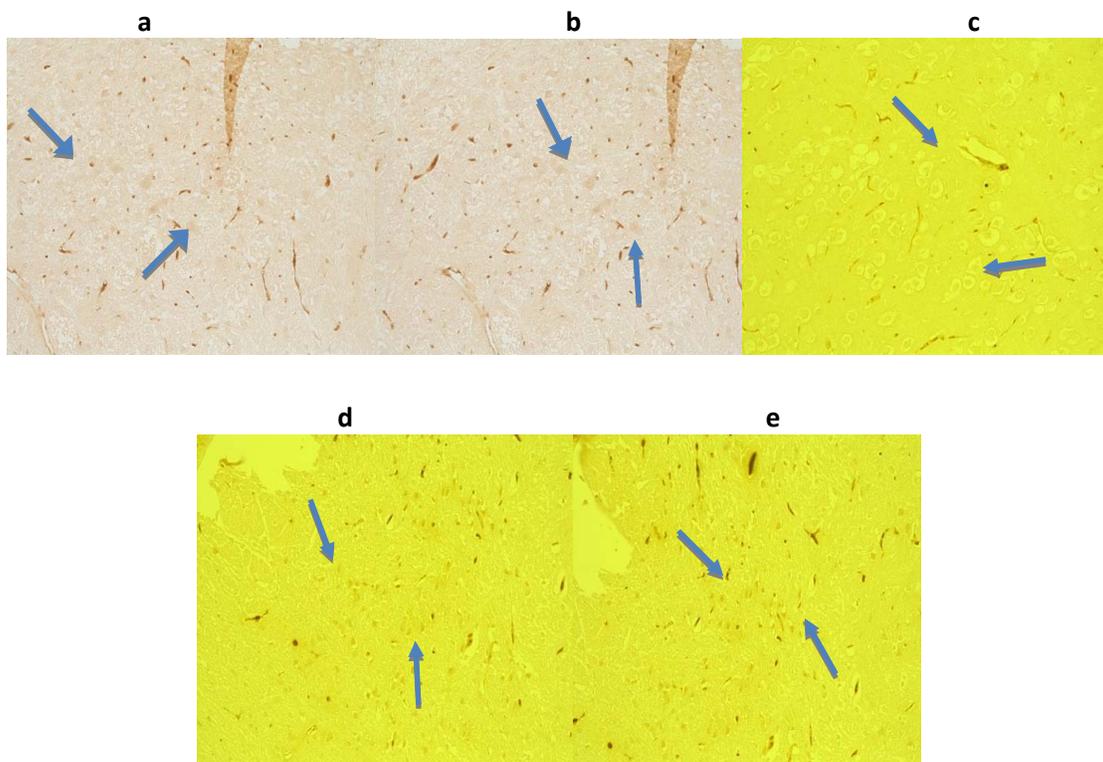


Figure 3. Representative examples of the leakage of EBA (a/c) show the leakage of plasma proteins and edema formation. (b/d/e) shows damage to the neurons present in the dorsal horn of the spinal cord. Blue arrows indicates completely damaged neurons.

## Nanoparticles effect on brain water content

These proteins when entered inside the cord results in the formation of edema. Thus the water content was measured in the nanoparticles-treated animals after injury and without the injury. The water content was calculated from the differences between the dry and wet weight. It was found that the nanoparticles treated animals without the injury showed a mild increase in the water content whereas in nanoparticles treated rats there was a significant increase in the water content after SCI (table 1).

Table 1: Effect of various nanoparticles on pathology of spinal cord injury in rats.

Expt.Type	EBA (mg%)		Spinal cord water (%)		Neuronal damage (nr)	
	T10	T11	T10	T11	T10	T11
Control	0.23±0.04	0.25±0.06	64.23±0.12	64.56±0.08	2±1	1±2
Ag	0.42±0.08	0.44±0.06	65.89±0.21	65.84±0.21	8±2	8±4
Cu	0.46±0.06	0.48±0.05	65.94±0.13	65.89±0.10	10±4	9±3
Al Treated	0.40±0.08	0.42±0.06	65.13±0.07	65.23±0.10	8±2	7±2
SCI	0.94±0.12	0.98±0.14	66.23±0.12	66.54±0.21	15±3	18±6
Ag	1.89±0.14	1.98±0.23	68.34±0.43	68.89±0.28	36 ± 8	43±6
Cu	1.98±0.18	2.04±0.21	68.89±0.34	68.94±0.33	44±7	48±9
Al Treated	1.24±0.08	1.43±0.10	67.56±0.12	67.78±0.23	28±8	24±6

## Nanoparticles induces neuronal damage

To observe the neuronal changes inside the spinal cord, 4  $\mu\text{m}$  thick sections were cut and stained with GFAP and H&E. The nanoparticles treated animals before and after the injury both showed neuronal damage (figure 4). But the nanoparticles treated animals after the injury showed more number of damaged neurons whereas before the injury there was only a mild neuronal damage (Table 1).

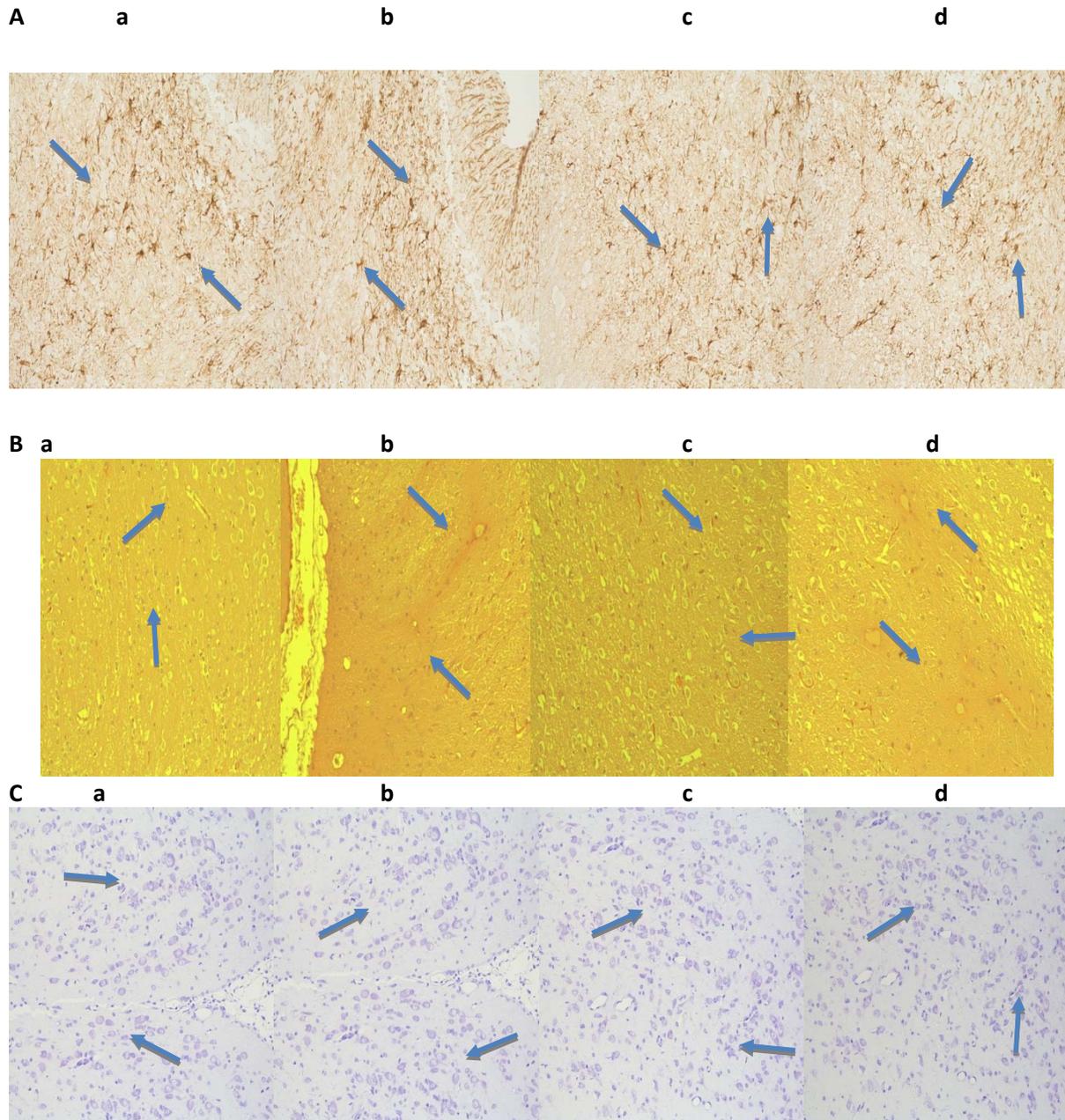


Figure 4. Representative examples of the neuronal damages (A) Represents the GFAP staining. (a/b/c/d) shows the damage of astrocytes. (B) Represents the H&E staining. (a/b/d) shows the neuronal damages. (c) Shows the partial damage of neurons. (C)

Represents the Nissl Staining. (a/b/c) shows the partial damage of neurons and (d) shows totally damaged neurons. Blue arrows (A/B/C) indicate completely damaged neurons.

### Effect of Cerebrolysin on Brain edema and BBB permeability

The Cerebrolysin was given to three sets of groups. The first was the control group where there was neither any nanoparticles administration nor were the animals subjected to any kind of SCI. The second group was the normal SCI in animals and the third group was the nanoparticles affected rats subjected to SCI. Two doses of Cerebrolysin 2.5 ml/kg and 5.0 ml/kg were administered intravenously 30 minutes before the injury. The lower dose of Cerebrolysin had moderate effects in reducing the brain water content whereas the higher dose had greater effects in reducing the water content in all the three cases (table 2). There was a moderate decrease in the BSCB permeability when lower dose of cerebrolysin was given whereas the larger doses of cerebrolysin attenuated the EBA leakage, edema formation and neuronal damage in nanoparticles treated group (table 2).

Table 2: Effects of Cerebrolysin on pathology of spinal cord injury in rats.

Expt.Type	EBA (mg %)		Spinal cord water (%)		Neuronal damage (nr)	
	T10	T11	T10	T11	T10	T11
A. Control						
2.5 ml/kg Cerebrolysin	0.24±0.04	0.25±0.041	65.13±0.12	65.10±0.03	1±1	nil
5.0 ml/kg Cerebrolysin	0.20±0.03	0.18±0.08	65.08±0.10	65.10±0.02	nil	nil
B. Normal SCI						
2.5 ml/kg Cerebrolysin	0.54±0.06	0.58±0.10	65.34±0.12	65.44±0.14	3±2	2±1
5.0 ml/kg Cerebrolysin	0.34±0.08	0.36±0.04	65.10±0.07	65.06±0.04	2±2	1±2
C. Nanoparticles+ SCI						
2.5 ml/kg Cerebrolysin						
Ag+SCI	1.04±0.11	1.14±0.17	67.89±0.12	67.67±0.32	23±6	22±8
Cu+SCI	1.21±0.12	1.34±0.21	67.76±0.34	67.88±0.23	20±6	19±5
Al+SCI	0.89±0.21	0.94±0.22	67.06±0.10	66.89±0.21	18±6	15±3
5.0 ml/kg Cerebrolysin						
Ag+SCI	0.67±0.10	0.65±0.12	65.34±0.12	65.04±0.14	8±3	7±4
Cu+SCI	0.74±0.14	0.68±0.14	65.05±0.08	65.34±0.18	7±6	8±2
Al+SCI	0.68±0.08	0.72±0.10	65.13±0.21	65.06±0.21	4±6	3±4

## Effect of Cerebrolysin on neuronal damage

The higher and lower doses of Cerebrolysin both resulted in the reduction of neuronal damages following SCI in normal rats but the higher dose of Cerebrolysin was needed for significant reduction in edema formation, BSCB breakdown and neuronal injuries after SCI in nanoparticles treated animals as compared to the lower doses in this group (table 2).

## **Discussion**

These results suggests that the nanoparticles (50-60nm) when administered intraperitoneally to the animals and then subjected to the SCI can result in the exacerbation of the breakdown of BSCB. The leakage of plasma proteins into the spinal cord can result in the formation of edema thereby the water content inside the cord is increased and leads to severe neuronal and non-neuronal cell reactions.

Our results further show that cerebrolysin in low or high doses resulted in marked reduction in neural injury, glial cell activation, reduction in BSCB breakdown and edema formation in normal rats after SCI. However, in nanoparticles treated rats after SCI, high doses of cerebrolysin are needed to achieve neuroprotection. This means that nanoparticles treatment by itself could induce marked depletion of neurotrophic factors in the cord. Thus, additional SCI in these nanoparticles treated animals need greater amount of neurotrophins replacement. As a result, low doses of cerebrolysin were ineffective in reducing cell injuries in nanoparticles treated injured rats. Obviously, high dose of cerebrolysin could supply required amount of neurotrophins to counteract cell injuries.

There are many neurotrophic factors such as BDNF, GDNF or NGF, which can be used to treat normal injuries, but in the case of SCI where it is influenced by nanoparticles these neurotrophic factors are not so effective (results not shown). Results from the investigation show that Cerebrolysin, a combination of various neurotrophic factors, amino acids, vitamins, macronutrients, micronutrients and anti-oxidant enzymes could definitely induce neuroprotection. Thus, as mentioned above, higher dose of this drug was effective in the case where the nanoparticles were administered to the animals prior to trauma. On the other hand, lower dose of this drug worked effectively in the normal SCI. Cerebrolysin resulted in significant decrease in the BSCB permeability, reduction in the water content and there were reduced number of neuronal damages inside the cord. If this drug is administered after 1- 2 hr of injury it

does not result in any significant reduction of cord pathology (results not shown). Therefore Cerebrolysin induces neuroprotection inside the cord depending on its dose and time. In my work I have observed dose- related effects of Cerebrolysin in inducing the neuroprotection within the cord. So it is clear from the data that the higher dose of Cerebrolysin has shown good results when compared to the lower dose of this drug especially in the nanoparticles treated injured rats. Further investigations are needed with the time- related effects of this drug.

The probable mechanisms of nanoparticles induced BSCB disruption; edema formation and cellular injuries are unclear. It appears that nNOS upregulation could contribute to such injuries caused by nanoparticles. Since SCI in itself induces nNOS expression, a combination of nanoparticles and SCI will exacerbate nNOS expression and cell injuries. It appears that cerebrolysin could reduce nNOS expression in the cord in dose dependent manner. However, this is a subject that requires further investigation.

## **Conclusion**

In conclusion, my results show that nanoparticles aggravate SCI induced BSCB breakdown, edema formation and neuronal injuries. Cerebrolysin appears to be very effective in reducing pathophysiology of SCI in normal or nanoparticles treated injured animals in comparison to other neurotrophic factors. Thus, Cerebrolysin has proved to be a good tool for treating SCI. If a higher dose of this drug is administered it proves to be very effective in reducing the pathology of SCI and morphological changes inside the cord in nanoparticles treated animals. However, further research is needed to find a suitable dose and time combination to reduce cord damage if Cerebrolysin is given after SCI.

## **Annexure**

### **1. 3 % H<sub>2</sub>O<sub>2</sub> Solution**

Take 5 ml of H<sub>2</sub>O<sub>2</sub> and dissolve it in 45 ml of PBS. Mix it well.

### **2. PBS Solution (1000 ml)**

Take 100ml of PBS (x10 dilution) and 900 ml of MQ- H<sub>2</sub>O.

### **3. ABC Elite**

Take 20 µl A, 1000 µl PBS and 20 µl B. Mix it well.

### **4. DAB Stain**

Take 2500 µl MQ-H<sub>2</sub>O, 1 drop of buffer, 2 drops of DAB and 1 drop of H<sub>2</sub>O<sub>2</sub>. Mix it well.

### **5. Citrate Buffer**

Dissolve 2.1 gm citric acid in 1 liter of distilled water. Adjust the pH to 6.0 with approximately 13 ml of 2 M NaOH.

### **6. 0.5 % Cresyl Violet**

Dissolve 0.5 gm of Cresyl Echt Violet Acetate in 100.0 ml of distilled water. Mix it well.

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