Neuroprotective effects of hyaluronic acid hydrogel on organotypic spinal cord cultures

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Abstract

**Study Design.** Experimental study of neuroprotective effects of hyaluronic acid hydrogel on organotypic spinal cord cultures. Organotypic spinal cord slice cultures positioned on a hyaluronic acid hydrogel were compared with spinal cords slice on plain cultures with respect to neuronal survival and glial activation.

**Objective.** The aim of this study was to investigate the effects of hyaluronic acid hydrogel on organotypic spinal cord cultures after four days in vitro.

**Background.** This is part of a larger project with the aim to investigate the potential of Interleukin-1 receptor antagonist and Mycophenolate Mofetil for their capacity to promote neuronal survival and enhance axonal sprouting. Neuroprotective properties of the hyaluronic acid hydrogel that was meant to be a drug carrier were discovered and have to be further characterized.

**Methods.** Organotypic spinal cord cultures were prepared. Two ages of p6 and p9 (p=postnatal) mice were used making a total of four groups (p6 HA, p6 ctrl, p9 HA & p9 ctrl), each group containing five cultures.

Hyaluronic acid hydrogel was placed directly on the culture membrane and in the experimental group the slice culture was lying on top of the gel, while in the control groups the slice culture was positioned directly on the membrane. Three types of cells were investigated: Motorneurons, astrocytes and microglia.

To visualize and photograph the immunoreactive cells, a camera was attached to an epifluorescence microscope (U Inverted Microscope) and connected to a computer. For quantification, the software Image-J was used.

**Results.** The cultures that were positioned on the hyaluronic acid hydrogel showed less motorneuron loss and an increased number of active astrocytes. We also observed an increase in the number of microglial cells and activation as well as tissue anatomy disruption in both p6 and p9 animals.

**Conclusions.** It seems that the hyaluronic acid hydrogel which is meant to be used as a vehicle for drug delivery is by itself neuroprotective and might have a slight immunosuppressive action as well.
Introduction

2.1 Spinal cord injury

Spinal cord injury (SCI) is today an irreparable condition that often occurs in young adults after severe trauma.

Two steps will follow after a lesion in the spinal cord:

Primary injury leads to the formation of a region of haemorrhagic necrosis which in turn results in cell apoptosis immediately after spinal cord injury. “Secondary damage” follows and basically represents an inflammatory reaction that is activated immediately after the primary injury. Secondary damage also leads to cell apoptosis, proportional to the size of the initial injury, thus affecting the surrounding tissue that survived the initial trauma [3].

It is now considered that pharmacological agents that interrupt the secondary damage cascade could be used for the treatment of this condition

2.2 Adjuvant therapy

Steroids are today the only approved drug to be administered after spinal cord injury. However, side effects such as myopathy, pulmonary emboli, wound infections and ultimately death are correlated with high doses of steroids. Yet, no evidence of functional recovery due to steroid treatment after SCI exists and it has thus been suggested that high doses of steroids after brain and spinal cord injuries should not be administered. [6]

2.3 Secondary damage

Microglial cells are resident CNS macrophages and populate the entire neuroaxis. After SCI they become activated by signals from the damaged surrounding neurons. Microglial activation is characterized by transformation to a tissue macrophage that releases pro-inflammatory cytokines and neurotoxic factors. These soluble factors aggravate the initial damage by inducing neuronal apoptosis. Interleukin (IL)-1 is considered to be among the most prominent. In the chronic phase after SCI, microglial cells along with astrocytes prevent axonal regeneration by forming a glial scar tissue. [1]

Astroglial cells (astrocytes) are star-shaped glial cells that support the endothelial cell in the blood brain barrier and that one major compound of the glial scaffold supporting neurons and axons in the CNS. Recent research shows that they take part in the regulation of signaling between neurons. [12]. Astroglial cells also become activated when injury to the spinal cord occurs, until the tissue is so destroyed that glial cells can no longer survive.

Microglial cells show a quick response already 24h after an injury and their proliferation peaks 2-4 days later. Afterward their numbers quickly decline, as opposed to astrocytes that have a delayed and long-lasting activation period. [1]

Microglial cells along with astrocytes therefore represent potential targets for pharmacological intervention.
2.4 Immunosuppressive substances for pharmacological intervention

Mycophenolate mofetil (MMF) induces a forceful inhibition of microglial and astrocyte activation [4]. When comparing MMF treatment with methylprednisolone (MP) in spinal cord injured rats, the apoptotic cells were significantly reduced after 24 h in both treatment groups compared to control. Regarding histological and functional recovery MMF appeared to have the same effect as MP while treatment with the two compounds did not yield any further improvement. [14]

Interleukin (IL) -1 receptor antagonist (IL-1ra) shows immunosuppressive and neuroprotective effects by reducing the number of activated microglial cells as well as by promoting neuronal survival in lesioned organotypic hippocampal sliced cultures [5]. According to latest research about spinal cord injury, treatment with IL-1ra leads to increased expression of NF (neurofilament protein) which in turn leads to reduced expression of the inflammatory cells in the spinal cord and that seems to have a promising neuroprotective effect by reducing secondary damage after SCI. [13]

2.5 Hyaluronic acid

A special hyaluronic acid gel will be used as a carrier for the above immunosuppressants in order to ensure continuous and controlled release.

When preparing a drug carrier for drugs, it is important that it is free from undesirable effects [7].

Hyaluronic acid has been chosen because of its natural functions in the human body, such as cell adhesion regulation, morphogenesis and modulation of inflammation [8,9]. One problem though is the difficulties to control the hyaluronic acid when they prepare the hydrogel [10].

By showing that the human dermal fibroblast is still viable after 3 days in this type of gel without any signs of cytotoxicity it was indicated that hyaluronic acid hydrogel is a suitable candidate as a carrier for growth factors into different tissues [10].

Aim

The aim of this study is to investigate the effects of hyaluronic acid hydrogel on organotypic spinal cord cultures after four days in vitro. Pilot experiments indicated a neuroprotective role of the gel. The study includes organotypic spinal cord from mice, placed on hyaluronic acid hydrogel and control cultures placed on plain culture inserts.

This is a part of a larger project of which the aim is to investigate the potential of Interleukin 1 receptor antagonist and Mycophenolate Mofetil for their capacity to promote neuronal survival and enhance axonal sprouting.
Materials and Methods

4.1 Preparation of spinal cord slice cultures

Mice were raised and kept etc. according to the local legislation for animal welfare, and the experimental management is performed under antiseptic conditions with sterile instruments. Ethical approval from the Uppsala animal ethics committee, C5/9

Newborn animals postnatal 6 and 9 (p6 and p9) are assessed as described below.

The mice are decapitated with a scissor and the skin from the lower back is removed to expose the lumbar spine which is then detached from the sacrum. An injection with ice cold preparation medium is pushed into the spinal canal through the lumbar spine and the spinal cord is “flushed out” through the cervical spine. This method of removing the spinal cord minimizes injuries to the tissue.

After extraction of the spinal cord, it is sliced in a “McIlwain” tissue chopper” to 500µm thick slices. About 10-20 pieces are purchased from each spinal cord and transferred in ice cold preparation medium [Minimal essential medium (MEM) + 1% glutamine]. Subsequently, the slices are transferred to culture insert-membranes (0,4µm pore size, Millipore) positioned in culture wells (Multiwell 6) containing 1ml culture medium [(MEM, HBSS, NHS (normal horse serum), glutamine, insulin, vitamin C, glucose, penicillin and streptomycin]. This is called the “sandwich technique” and according to that method the culture slice gets nutrition through the membrane.

Hyaluronic acid hydrogel is placed directly on the culture membrane. In the experimental group the slice culture is lying on top of the gel, while in the control groups the slice culture is kept directly on the membrane.

The cultures are incubated for four days in 35°C, 5% CO2 and 95% O2. New medium will be added every second day.

4.2 Tissue fixation

After four days in vitro the tissue will be fixed with Zambonis fixative, 4% paraformaldehyde, 0,2% picric acid in 0,2M Sorensen phosphate buffer for 24h. Washing with sucrose solution (Sorensen phosphate buffer 0,1M, 20 % sucrose, 0,1 % sodium azid ) is followed until the yellow color is disappeared.

4.3 Immunohistochemistry

To visualize the neuronal and glial population, immunohistochemistry is used. Primary antibodies, CHAT and GFAP and one lectin, IB4 are used to visualize the different cell-populations.

-CHAT (choline acetyltransferase) Chat Novus (NB100-89724) made in rabbit. Concentration 1:2500. Diluted in 2%BSA in PBS + 0,3% Tritin (50:50). Chat is used to characterize motor neurons.


-IB4 (griffona simplicifolia isolectin B4) Lectin from Bandeiraea simplicifolia (Griffonia simplicifolia) Prod.nr.L2895, Sigma-Aldrich. IB4 stains active and inactive microglia cells.

Immunohistochemistry is performed as described below using different primary antibodies or lectin, blocking solutions and secondary antibodies, depending on which cells are to be visualized.
Take the samples directly from the freeze and hydrate with washing buffer for 5 min. Block with normal serum (the same as the host, the secondary antibody is made in). Add primary antibody/lectin and incubate in 4°C or room temperature over night. Rinse off fast, wash for 3x5 min in washing buffer. Add the secondary antibody (directed to the host the primary antibody is made in). Incubate for 30 min in room temperature. Rinse and wash in washing buffer 3x5 min. Incubate with dyLIGHT streptavdin (vector sa-5488 green 1mg/ml). Rinse and wash 3x5 min. Rinse in MilliQ-water to wash off the PBS-Triton solution as the mounting medium is water soluble. Mount with vectashield. Keep in refrigerator.

### 4.4 Microscopy

To visualize and photograph the immunoreactive cells, a camera (Nikon Eclipse TE2000) was attached to an epifluorescence microscope (-U Inverted Microscope) and connected to a computer.

Chat positive motorneurons were captured in two pictures within the dorsal horn. IB4 immunoreactive cells showing active and inactive microglial cells were captured within the ventral and dorsal median fissure and the grey substance. GFAP positive astrocytes were photographed within the ventral and dorsal median fissure of the culture

#### 1. Basic anatomy of the culture slice

All images were taken with 20x magnification.

For quantification, the software Image-J was used.
4.5. Hyaluronic acid hydrogel

Hyaluronic acid has been transformed to a hyaluronic aldehyde with a mild cleavage of a disulfide bond followed by elimination of the 2-thioethoxycarbonyl, giving free amine-type functionality. That change made it easier to insert different nucleophile counterparts into this new HA-aldehyde and make a specific hydrogel out of it.

Synthesis of Hydrogel

2. Hyaluronic acid -aldehyde component

![Reaction scheme for the synthesis of hyaluronic acid aldehyde](image)

Fig. 1 Hyaluronic acid-aldehyde synthesis is performed at pH = 6.0 by carbodiimide (EDC) coupling of Hyaluronic acid-Na salt with β-aminoglycerol using HOBt as a catalyst. After reaction and purification by dialysis the vicinal diol was reacted with sodium periodate in order to obtain the final product with a degree of substitution between 8 and 12%.

3. Protected nucleophile component

![Reaction scheme for the synthesis of protected nucleophile](image)

Fig. 2 Trimethylene carbonate and hydrazine are reacted under reflux to obtain 1. The ring-opened hydroxy-propane carbazate (1) is reacted with carbonyldiimidazole-activated dithiodiethanol (2) to obtain 3. The symmetric di-ol is activated with CDI and reacted with hydrazine to give the protected nucleophile.
4. Hyaluronic acid-carbazate component

\[ \text{HY} \xrightarrow{\text{pH } 8.0-8.5} \text{HY} \xrightarrow{\text{DTT}} \text{HY} \xrightarrow{\Delta} \text{HY} + \text{CO}_2 \]

Fig. 3 DTT deprotection of hyaluronic acid-carbazate pre-component yielding the final product with a degree of substitution between 5 and 8%, episulfide and carbon dioxide.

5. Synthesis of hydrogels

\[ \text{HY} \xrightarrow{\text{amine}} \text{HY} \xrightarrow{\text{amine}} \text{HY} \xrightarrow{\text{amine}} \text{HY} \]

Fig. 4 Schematic of bonds formed in hyaluronan-based hydrogel in PBS at pH = 7.4.

Experimental Design

Organotypic spinal cord slice cultures lying on a hyaluronic acid hydrogel were compared with spinal cords on plain culture inserts with respect to neuronal survival and glial activation. Three types of cells were investigated: Motorneurons, astrocytes and microglia.

Two ages of p6 and p9 (p=postnatal) mice were used making a total of four groups, each group containing five cultures.
Results

5.1 postnatal 9

The quantitative data concerning Chat, GFAP and IB4 positive cells types in p9 are presented in table 1. After four days in vitro the control slice cultures underwent degenerative processes that were characterized by early motorneuron loss, an increase of the number of astroglial- and microglia cells and activation of all glial cells, tissue anatomy disruption finally lead to a decrease in the number of all glial cells (fig 6, 7, 8). The slice cultures lying on hyaluronic acid hydrogel also showed signs of degeneration after four days in vitro, however to a lesser extent compared to controls. Motorneurons were significantly increased in the hyaluronic acid hydrogel group. Astrocytes showed signs of degeneration with loss of their processes and disruption in the control group compared to hyaluronic acid hydrogel group. Their number within the median fissures was significantly increased in the hyaluronic acid hydrogel group compared to controls. Microglial cells did not show any significant difference at p9.
Table 1. Quantitative evaluation of the number of cells positive for choline acetyl transferase (chat), glial fibrillary acidic protein (GFAP) and simplicifolia isolectin B4 in organotypic spinal cord cultures lying on hyaluronic acid hydrogel or controls lying on plain culture inserts. P6 and P9 is age of animals at time of tissue explantation. Mean numbers ± standard errors of mean are indicated. P-value was calculated in Mann-Whitney test and Kruskal-Wallis test, p < 0.05 shows significance.

<table>
<thead>
<tr>
<th></th>
<th>P6</th>
<th>P9</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>CHAT</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>12.7±5.3</td>
<td>10.2±3.1</td>
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<tr>
<td>H.A-gel</td>
<td>44.7±9.2</td>
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<tr>
<td><strong>GFAP white matter</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50.2±8.7</td>
<td>85.5±11.5</td>
<td>0.09</td>
</tr>
<tr>
<td>H.A-gel</td>
<td>98.5±18.7</td>
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<tr>
<td>p-value</td>
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<td><strong>GFAP grey matter</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>28.8±7.7</td>
<td>44.2±11</td>
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<tr>
<td>H.A-gel</td>
<td>32.2±2.5</td>
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<tr>
<td>p-value</td>
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<td><strong>IB4 inactive white matter</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>13.3±5.4</td>
<td>14.2±5.3</td>
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<tr>
<td>H.A-gel</td>
<td>26.6±4.3</td>
<td>29.2±6.8</td>
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<tr>
<td>p-value</td>
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<td>0.18*</td>
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<tr>
<td><strong>IB4 active white matter</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>21.3±4.1</td>
<td>15.2±5.1</td>
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<tr>
<td>H.A-gel</td>
<td>28.4±8.4</td>
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<td>p-value</td>
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<td></td>
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<tr>
<td><strong>IB4 inactive grey matter</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>26.5±4.7</td>
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<td><strong>IB4 active grey matter</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>29±9.3</td>
<td>7.8±3</td>
<td>0.03</td>
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<tr>
<td>H.A-gel</td>
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<tr>
<td>p-value</td>
<td>0.05</td>
<td>0.93</td>
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Image 6. Micrographs of transverse slices through spinal cord slice cultures lying on culture inserts (6:1, 6:2) and of cultures lying on hyaluronic acid hydrogel (6:3, 6:4) after staining against Chat. Chat-positive motor neurons are noted in 6:4 (x 320 objective) as well as in 6:3 (x80 objective) while in the respective controls they have disappeared.
Image 7 Micrographs of transverse slices through spinal cord slice cultures lying on culture inserts (7:1, 7:2, 7:3) and of cultures lying on hyaluronic acid hydrogel (7:4, 7:5, 7:6) after staining against GFAP showing astrocytes. Overview pictures (7:1, 7:4, x80 objective) show astrocyte localization within the ventral and dorsal median fissures. Higher magnification images (x32 objective) taken within the median fissures show typical astrocyte morphology in the hyaluronic acid hydrogel group (7:5) in contrast to controls (7:2). The images 7:3 and 7:6 show astrocytes localized within the grey matter.
Image 8  Micrographs of transverse slices through spinal cord slice cultures lying on culture inserts (8:1, 8:2, 8:3) and of cultures lying on hyaluronic acid hydrogel (8:4, 8:5, 8:6) after staining with IB4 showing microglia. Note microglial cells in their active state (8:2, 8:5) as well as typical inactive ones (8:3, 8:6).
Chat positive motoneurons show significance for cultures laying on hyaluronic acid hydrogel with a p-value of 0.008.

GFAP positive astroglia cells show significance for cultures laying on hyaluronic acid hydrogel with a p-value of 0.03 in white substances and 0.84 for grey substances.

Active IB4 positive microglia cells show no significance either in white or grey substances.

Inactive IB4 positive microglia cells show no significance either in white or grey substances.
5.2 postnatal 6.

Quantitative data for the three different cell types in p6 are shown in table 1. Structures for p6 are shown in figures 9, 10 and 11. The pattern was similar to p9 concerning motor neurons and astrocytes. The tissue anatomy of the cultures seemed to be disrupted to a higher extent in p6 compared to p9. Larger animal defects indicating degeneration were visible within the culture slices at p6 and they were not as homogenous as at p9. Concerning cell populations, astrocytes at p9 were found to be significantly increased at p9 both in the control and hyaluronic acid hydrogel groups compared to p6. No other differences in the cell populations were observed between p6 and p9 after four days in vitro. At p6 inactive microglial cells within the white matter were significantly reduced in the control group compared to the hyaluronic acid hydrogel group. Accordingly, activated microglial cells within the grey matter were significantly increased in the control group compared to hyaluronic acid hydrogel group.
Image 9. Micrographs of transverse slices through spinal cord slice cultures lying on culture inserts (9:1, 9:2) and of cultures lying on hyaluronic acid hydrogel (9:3, 9:4) after staining against Chat. The slices are much more disrupted than in p9. Still, positive motor neurons are visible in the ventral horn on hyaluronic acid hydrogel (9:4) compared to control (9:2).
Image 10  Micrographs of transverse slices through spinal cord slice cultures lying on culture inserts (10:1, 10:2, 10:3) and of cultures lying on hyaluronic acid hydrogel (10:4, 10:5, 10:6) after staining against GFAP showing astrocytes. Overview pictures (x80 objective, 10:1, 10:4) show astrocyte localization within the ventral and dorsal median fissures. Higher magnification images (x320 objective) show typical astrocyte morphology in the hyaluronic acid hydrogel group (10:5) in contrast to controls (10:2). The images 10:3 and 10:6 show astrocytes localized within the grey matter.
Image 11  Micrographs of transverse slices through spinal cord slice cultures lying on culture inserts (11:1, 11:2, 11:3) and of cultures lying on hyaluronic acid hydrogel (11:4, 11:5, 11:6) after staining against IB4 showing microglia. Significant increase of activated microglial cells was shown in the grey matter of the control group (11:2) compared to hyaluronic acid hydrogel (11:5). Accordingly, significant decrease of inactive microglial cells was shown within the white matter of the control group (11:3) compared to hyaluronic acid hydrogel (11:6).
Quantitative analysis in p6.

Chat positive motorneurons show increase with significance for cultures laying on hyaluronic acid hydrogel.

GFAP positive astroglia cells show increase with significance for cultures laying on hyaluronic acid hydrogel in white substances, but not in grey substances.

Active IB4 positive microglia cells show a significant increase of the active microglia in the control group compared to the hyaluronic acid hydrogel group in grey substance. In white substance a decrease with significance in the hyaluronic acid hydrogel group.

Inactive IB4 positive microglia cells show a significant decrease of the inactive microglia in the white matter of the control group.
Discussion

This study was performed in order to investigate the effects of hyaluronic acid hydrogel on neuronal survival in organotypic spinal cord slice cultures.

The results of our quantitative analysis, both at p6 and p9, show that survival of motorneurons is improved on hyaluronic acid hydrogel compared with controls after four days in vitro.

It was shown in previous experiments on organotypic spinal cord slice culture derived from rats that early motorneuron death occurs after three days in vitro [3]. In this line of experiments the number of motorneurons after four days is significantly increased in cultures positioned on hyaluronic acid hydrogel.

With respect to astrocytes the analyses do not show any significant difference in the number of astrocytes located in the grey matter. However, a significant increase in the hyaluronic acid hydrogel group as compared to controls is noted both at p6 and p9 in white matter, showing that the astroglial cells are not activated to the same extent if the preparations are cultured on gel. The astrocyte structure is preserved on the hyaluronic acid hydrogel group, with their cell bodies and processes indicative of an inactive state.

In the control group, astrocyte fragments and processes are most prominent.

We believe that the reason for the significant increase in number of astrocytes in the hyaluronic acid hydrogel group is the tremendous tissue disruption and subsequent glial death in the control group.

Astrocytes are normally inactivated and located in the white matter however after four days in vitro they proliferate and begin to migrate into the grey matter [3]. In our studies quantitative analysis indicates that the number of astrocytes decreases in the control group. Higher number of astrocytes in the hyaluronic acid hydrogel group than in the control would suggest that the cultures on gel are not well preserved. However, in this case the problem is that the tissue in the control group is so destroyed after four days in vitro that the glial cells vanish[4][5].

Microglial cells are normally inactive, in a so called “resting state”, but after four days in vitro they show signs of activation as in previous studies [11]. At p6 we observed a significant decrease in the number of inactive microglia in the white matter of the control group, accompanied by a significant increase in the number of active microglia in control group compared to hyaluronic acid hydrogel group. This could be attributed to an immunosuppressive role of the gel although no significant differences were observed at p9.

Without quantitative analysis one can easily see that p9 animal tissue holds together better than the tissue derived from p6 and also that the hyaluronic acid hydrogel seems to keep the tissue together better than control conditions. The histological differences between cultures at p6 and p9 could be attributed to the biomechanical properties of the tissue taken from younger mice. Nevertheless, concerning cell populations, only differences in the astrocytes could be shown which might reflect the general image of tissue disruption at p6 which was more obvious compared to p9.

In conclusion, it seems that the hyaluronic acid hydrogel which is meant to be used as a vehicle for
drug delivery is by itself neuroprotective and might have a slight immunosuppressive action as well. The reason for that is for the moment unknown, the answer however lies between the hyaluronic acid and the biomechanical properties of the gel which probably holds the tissue together. Further experiments with soluble hyaluronic acid and gels with different biomechanical properties would be of great interest.
References


