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The Neuroprotective Effects of Rituximab in Rat Spinal Cord Injury Model: An Immunohistochemical Study

Rat Spinal Kord Hasarında Rituksimabın Nöroprotektif Etkileri: Bir İmmünohistokimyasal Çalışma

Guner MENEKSE[1], Ergun DAGLIOGLU[2], Osman Arikan NACAR[2], Ersin POLAT[2], Cagatay OZDOL[2], Ali DALGIC[2], Gulnur TAKE[3], Ali Ihsan OKTEN[1], Ahmet Deniz BELEN[2]

[1]Adana Numune Education and Research Hospital, Department of Neurosurgery, Adana, Turkey
[2]Ankara Numune Education and Research Hospital, Department of Neurosurgery, Ankara, Turkey
[3]Gazi University, Faculty of Medicine, Department of Histology and Embryology, Ankara, Turkey

Corresponding Author: Ergun DAGLIOGLU / E-mail: edaglioglu@gmail.com

ABSTRACT

AIM: In the present study, we investigate the neuroprotective effects of rituximab, a monoclonal antibody directed towards B cell mediated humoral immunity, on a rat spinal cord injury (SCI) model with immunohistochemical methods.

MATERIAL and METHODS: Twenty-four rats were used for the study. Rats were divided as control, SCI, and rituximab-treated SCI groups. Intraperitoneal rituximab administration was performed on days 0, 3 and 5 in the third group. Rats were sacrificed 7 days after trauma. Antibodies against IL-1β, IL-6, TNF-α and CD20 were studied with the ELISA method together with electron microscopic analysis.

RESULTS: It was found that rituximab suppressed oligodendrocytes at the phagocytic stage but was still inefficient for the regenerative phase. TNF-α expression was markedly increased in rats subjected to SCI and suppressed after rituximab treatment. Decreased CD20 expression was another prominent finding in rats under rituximab therapy. However, expressions of IL-1β and IL-6 were both increased in glial cells without significant change after rituximab administration.

CONCLUSION: TNF-α expression was augmented at the level of SCI both in neuronal and glial cells, particularly in oligodendrocytes. All were suppressed after rituximab administration and rituximab reduced CD20 expression both in neuronal and supportive glial cells which may be related to neural healing.

KEYWORDS: B-lymphocyte, CD20, IL1β, Rats, Rituximab, Spinal cord injury, TNF-α

ÖZ

AMAÇ: Bu çalışmanın amacı spinal kord hasarı (SKH) modeli oluşturulan ratlarda bir B hücresi monoclonal antikoru olan rituksimabın nöroprotektif etkisinin immünhistokimyasal olarak araştırılmasıdır.

YÖNTEM ve GEREÇLER: Bu çalışmada, 24 adet rat kullanıldı. Ratlar kontrol, yalnızca SKH ve SKH+ Rituksimab tedavisi alanlar olmak üzere 3 gruba ayrıldı. Tedavi grubuna 0,3 ve 5. gün intraperitoneal olarak rituksimab verildi. Ratlar travmadan 7 gün sonra sakrifiye edildi. ELISA yöntemi ile IL-1β, IL-6, TNF-α ve CD20 antikorları ile immünhistokimyasal ve elektron mikroskopik olarak incelendi.

BULGULAR: Bulgular rituksimab uygulamasının oligodentrositlerde fagositik süreci baskıladığını, ancak rejenerasyon fazında yetersiz kaldığını göstermiştir. SKH sonrasında Rituksimab tedavisi ile TNF-α ekspresyonunun tamamen baskılandığı saptanmıştır. Yine rituksimab tedavisi alan ratlarda azalmış CD20 ekspresyonu belirgin bir bulguydu. Glial hücrelerde IL-1β ve IL-6 ekspresyonunun arttığı ancak rituksimab tedavisiyle herhangi bir değişiklik oluşmadığı gözlendi.

SONUÇ: SKH'da özellikle oligodendrositlerde belirgin olmak üzere tüm nöronal ve glia hücrelerinde TNF-α ekspresyonunun arttığı görüldü. Bu bulguların rituksimab uygulaması ile tamamen baskılandığı saptandı. Ayrıca rituksimab tedavisi ile nöronal hücrelerle birlikte destekleyici glial hücrelerde görülen azalmış olan CD20 ekspresyonu saptandı. Bu bulgu da nöral iyileşme ile ilişkili olabilir.

ANAHTAR SÖZCÜKLER: B-lenfosit, CD20, IL1β, Ratlar, Rituksimab, Spinal kord hasarı, TNF-α

INTRODUCTION

Traffic accident is the most frequent reason of spinal cord injury (SCI) (4). Morbidity of SCI is a major social and health care problem (19). Studies against treatment of SCI mainly focus on prevention of secondary mechanisms after SCI and many pharmacological agents were still under trial (7). Despite all these advancements, a definitive agent for treatment of SCI was not provided yet.

Neuroinflammation is a major secondary mechanism after SCI. Studies within the last decade focus on anti-inflammatory agents and these agents were accepted to be beneficial for neural healing (16). B-lymphocytes were noted at the injury site within the first week after SCI and subsequent secretion of cytokines like IL1, IL6 and TNF-α play a major role in cytotoxic cell damage. It is suggested that increased cytokine production at the injury site is correlated with the augmentation of neural injury (3). On the other hand, production of autoantibodies and activation of several inflammatory pathways induces activated B-cell accumulation at the SCI region and undoubtedly correlated with the pathological events after SCI (15,17). This mechanism was similar to neuroinflammatory cell damage which is a significant mechanism for autoimmune neurological diseases (1).

In this study, we investigate anti-inflammatory potential of rituximab, a new generation monoclonal antibody agent, on rat SCI with clip compression. Rituximab was presumed to be neuroprotective while preventing B cell functions and specific antibodies (ELISA) against CD20, a marker for B-lymphocytes, were studied with electron microscope and immunohistochemistry. Moreover, specific antibodies against cytokines like TNF-α, IL-1β and IL-6 were also studied.

MATERIAL and METHODS

Animals and General Experimental Procedure

The study was approved by Ethic Committee of Ankara Education and Research Hospital in 2010. All experimental procedures were performed at Ankara Education and Research Hospital (Ankara, Turkey) and histological studies were done at Histology and Embryology Department of Gazi University Medical School (Ankara, Turkey).

24 male Wistar albino rats weighing between 240 and 260 gram were used for the study. Ketamin (Ketalar®, Pfizer, Istanbul, Türkiye) 50 mg/kg and Xylazine (Rompun®, Bayer, Istanbul, Türkiye) 10 mg/kg were mixed and injected intraperitoneally for general anesthesia. General rules for antisepsis were applied during the experimental protocol and cardiac rate, rectal temperature and arterial saturation were all monitorized.

Experimental Groups and Spinal Cord Injury

Rats were divided into 3 groups at random. Group 1 includes sham operated animals. Rats in this group were only subjected to midthoracic incision and thoracal laminectomy without any experimental procedure. Group 2 includes spinal cord injury group. Rats in this group were subjected to spinal trauma by compression of spinal cord with a modified Walsh-Tator clip that inserts a force of 35 gram/cm^2 for one minute. The site of injury was marked with a prolene suture at the neighbouring tissue and all rats were noted to be paraplegic with typical tail reflex after experimental procedure. Rats in group 3 were subjected to same experimental procedures in group 2 and rats in this group received and three separate intraperitoneal rituximab injection (375 mg/m^2, SC) on days 0, 3 and 5 in the third group.

Tissue Preparation

Rats in all 3 groups were sacrificed on day 7 after perfusion. The chest was opened and left ventricle was cannulated to perform intracardiac perfusion. Perfusion was done with 100-300 ml phosphate-buffered saline (PBS; 0.1 M, pH 7.4) and 100 ml paraformaldehyde (4% in 0.1MPBS) (116). All hemorrhagic components of spinal cord tissue was removed with NaCl 0,9 % solution. Spinal cord samples were excised with micro scissors from the injured segment under microscope from cranial and caudal points of injury level so that 1 cm length of spinal cord tissue was reserved for light and electron microscopic study. Fixation of tissues were done with paraformaldehyde for light microscopic and gluteraldehyde for electron microscopic studies.

Light and Immunohistochemical Procedures

Tissue samples from all three groups were fixated under formaldehyde 10% solution for 72 hours. All tissue samples were embedded in paraffin blocks. For immunohistochemical studies, sections of 4 to 5 micron thickness were collected from paraffin blocks over polylysin lamel. After dehydration and deparaffinization of lamella with xylol and alcohol, endogeneous peroxidase activity was blocked with hydrogen peroxide 3% (Lab Vision, USA). During the procedure, lamella was washed with PBS (phosphate buffer saline, pH=7.4). For studies with IL-1ß (polyclonal anti-rabbit Ig, sc-7884, Lot: G2110, Santa Cruz) and TNF-a (monoclonal anti-mouse Ig, sc-130349, Lot: B0310, Santa Cruz), Invitrogen Universal Kit (Histostain plus, Lot: 724944A) was used. For studies with CD-20 (polyclonal anti-goat Ig, sc-7735, Lot: E0410, Santa Cruz) and IL-6 (polyclonal anti-goat Ig, sc-1265, Lot: 10310, Santa Cruz), Santa Cruz ABC Staining System Goat Kit (sc-2023) was used. Non-specific ligands were eliminated by blocking serum. Primary antibodies were overlaid onto sections and were incubated at +4^0 C overnight. On the following day, sections were rinsed with PBS and secondary antibodies with biotin were added which allows binding to primary antibodies. Sections rinsed with PBS were subjected to enzyme complex and this allows combination of enzyme and biotin. Lastly, DAB chromogen (Lot: F2909, Santa Cruz) for IL-1ß and TNF-a and DAB chromogen (Lot: 720221A, Invitrogen) for CD-20 and IL-6 were added to provide significant yield to appear. Harris' Hematoxylin was used for baseline color. Negative staining was done at primary antibody phase. These sections were closed with entellan and studied under computerized photolight microscope (DCM 4000, Leica).

Electron microscopic Study: Tissue samples from the site of injury (just at the midpoint of suture mark from groups) were divided into blocks at 1 mm^3 in volume. They were subjected to 0.1M phosphate buffer 2.5 % gluteraldehyde (pH 7.4) for 2 hours. The blocks were rinsed 3 times with buffered solution and postfixed in 2% osmium tetroxide and dehydrated in serially diluted alcohol solutions for dehydration. After treating with propylene oxide, they were embedded in Araldite CY 212 kit. Semi-thin sections were prepared from the blocks that were incubated at 56^0 C at 48 hours and stained with toluidine blue. These sections were examined by light microscopy. The ultrathin sections were taken at a thickness of 60 to 90 nm and prepared with LKB NOVA ultratome. After double staining with uranyl acetate and lead citrate, these ultrathin sections were examined by transmission electron microscopy (EVO LS10 transmission EM). All the samples from the regarding group (Control, SCI injury, SCI+Rituximab) were taken simultaneously after 7 days for cellular analysis. Antibodies against IL-1β, IL-6, TNF-α and CD20 were studied with ELISA method together with electron microscopic analysis.

RESULTS

A. Immunohistochemical Findings

1. Studies for TNF-α

Control group

Spinal cord tissue was normal in general structure. TNF-α staining was minimal in multipolar motor neurons and astrocytes (Figure 1A). Myelinated axons and sheath were normal however oligodendrocytes were stained with this stain and this was assumed to be a response to stress (Figure 1B). Vascular endothelial cells were also stained with TNF-α.

Trauma group

TNF-α staining density was not uniform in the trauma group. Marked tissue staining for TNF-α was observed in apical cytoplasm and cellular layer of multipolar motor neurons. This may be a clue of induction apoptosis by fas ligands in this group. Many glial cells in gray matter were noted to be reactive (Figure 1C). Myelinated nerve cells were found to be normal or enlarged in diameter in white matter. Oligodendrocytes were more reactive and enlarged in this group assumed to be a reaction to stress. Some areas within the endothelium were spared with a normal tissue staining of endothelial cells. Vascularization was marked in areas subjected to trauma as well as increased staining for TNF-α (Figure 1D).

Trauma+Rituximab Group

TNF-α staining was not noted in multipolar motor neurons, glial cells and blood vessels of gray matter (Figure 1E). Similarly, no immunoreactivity was noted in white matter. However few areas were noted to be prominent for enlarged

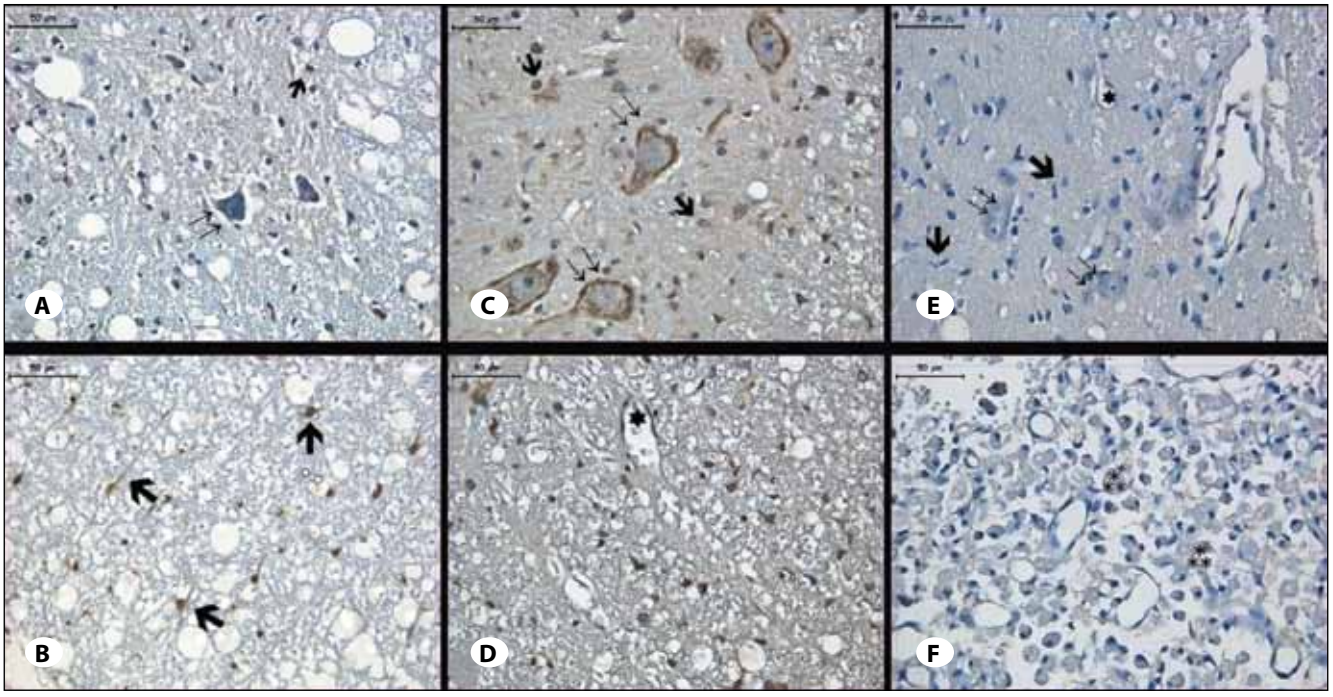

Figure 1: TNF-α immunohistochemical stain. A) control group, gray matter, ↑↑: neurons with minimal reactivity, ↟: glial cells with minimal reactivity; **B)** control group, white matter, ↟: glial cells with TNF-α involvement (oligodendrocytes); **C)** trauma group, gray matter, ↑↑: neurons with cytoplasmic and membranous involvement, ↟: glial cells with positive stain for TNF-α; **D)** trauma group, ✸: TNF-α immunoreactivity in vascular endothelial cells; **E)** multipolar motor neurons in trauma+rituximab group (↑↑), glial cells (↟) and negative TNF-α staining in blood vessels (✸); **F)** trauma+rituximab group, ✳✳: Negative staining for TNF-α in areas subjected to trauma (Immune peroxidase-hematoxylin X400).

myelinated nerve fibers. Vascularity was also prominent in areas subjected to trauma however TNF-α staining was still negative (Figure 1F) excluding few neighbouring glial cells with minimal reactivity.

Trauma was noted to increase expression of TNF-α, a known inductor of apoptosis, in neuronal and glial cells of spinal cord particularly in oligodendrocytes, Rituximab was noted to suppress expression of TNF-α with its regulatory role on immune system however no proven benefit was noted in terms of neural healing.

2. Studies for IL-6

Control group

Minimal reactivity was noted in multipolar motor nerve cells of gray and white matter (Figure 2A). IL-6 staining was not noted in vascular endothelial cells in this group.

Trauma group

There was no difference when neuronal involvement was of trauma group was concerned. Reactivity was more prominent in cytoplasmic extensions of glial cells. IL-6 involvement was also prominent in white matter similar to findings of gray matter. There was no involvement of blood vessels. In areas subjected to trauma, increased immunoreactivity was prominent in glial cells (Figure 2B).

Trauma+Rituximab group

Multipolar motor neurons of gray matter as well as vascular endothelial cells of both gray and white matter were noted to have prominent IL-6 reactivity (Figure 2C). Involvement in areas of trauma was quite similar to the trauma group (Figure 2D).

In conclusion, trauma was noted to increase IL-6 expression in glial cells in a limited manner and rituximab treatment does not seem to have an effect on this expression.

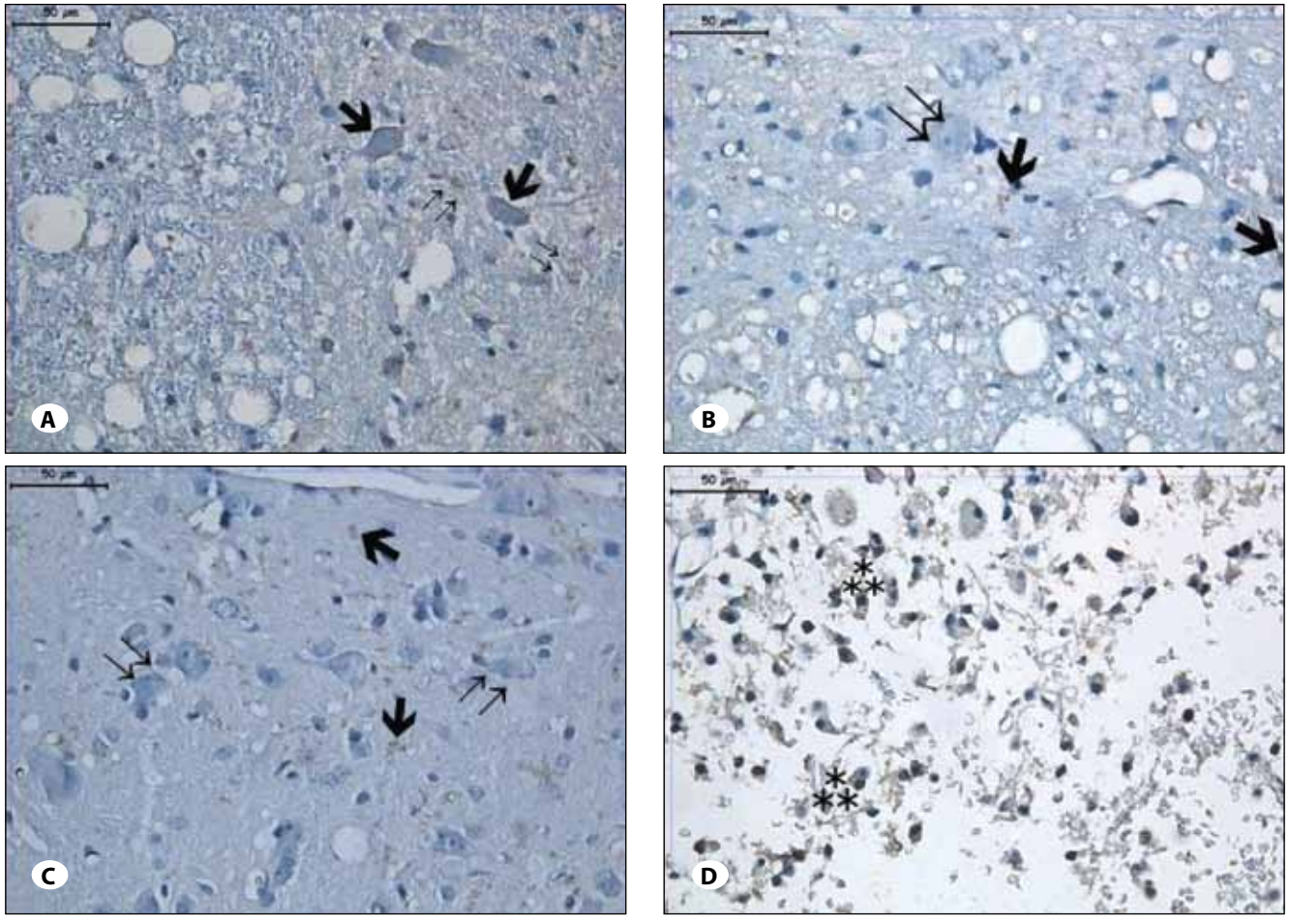

Figure 2: IL-6 immunohistochemical stain. A) control group, gray and white matter, minimal reactivity in multipolar motor neurons of gray matter (↑↑) and glial cells (↟); **B)** trauma group, gray matter, ↑↑: minimal staining in localized areas of neurons, ↟: intense involvement of glial cells; **C)** trauma+rituximab group, ↑↑: minimal staining in localized areas of neurons, ↟: intense involvement of glial cells; **D)**trauma+rituximab group, ⁂: intense staining for IL-6 in areas subjected to trauma (Immune peroxidase-hematoxylin X400).

3. Studies for IL-1β

Control group

IL-1β expression was marked in control group particularly in the cell bodies of multipolar motor neurons. Involvement of glial cells was less marked (Figure 3A). In areas of white matter, immunoreactivity of glial cells was more dense and prominent. Minimal reactivity was noted multipolar motor nerve cells of gray and white matter. IL-1β expression was also marked in axoneme. Prominent reactivity was noted in vascular endothelial cells of control group (Figure 3B).

Trauma group

The most prominent finding of trauma group was decreased expression of IL-1β in multipolar motor neurons. The involvement was weak in the soma and moderate in some other parts. No reactivity was noted in glial cells of gray matter (Figure 3C). Reactivity and density of glial cells of white matter was similar to control group however there was no reactivity in vascular endothelial cells of vessels (Figure 3D). IL-1β expression was augmented in regions subjected to trauma when compared to areas without trauma.

Trauma+Rituximab group

The findings in multipolar motor neurons and glial cells of gray matter of this group were similar to control group (Figure 3E). Wide distribution of IL-1β was prominent in gray matter. Vascular endothelial cells were negative in this group similar to trauma group. The involvement in areas of trauma was intense and widely distributed (Figure 3F).

IL-1β was expressed to activate microglia and the expression of IL-1β was decreased with trauma. Rituximab was found to have no effect on this expression.

4. Studies for CD20

Control group

No immunoreactivity was noted in neuronal and non neuronal cells (multipolar motor neurons, glial cells and vascular endothelial cells) of spinal cord.

Trauma group

Immunoreactivity for CD20 was negative in nontraumatic areas similar to areas subjected to trauma (Figure 4A).

Trauma+Rituximab group

Staining for CD20 was negative or less marked in most areas (Figure 4B).

CD20 was not expressed in neuronal cells and its synthesis was associated with immune system induction due to local stress and trauma. Rituximab was noted to suppress this expression in the present study.

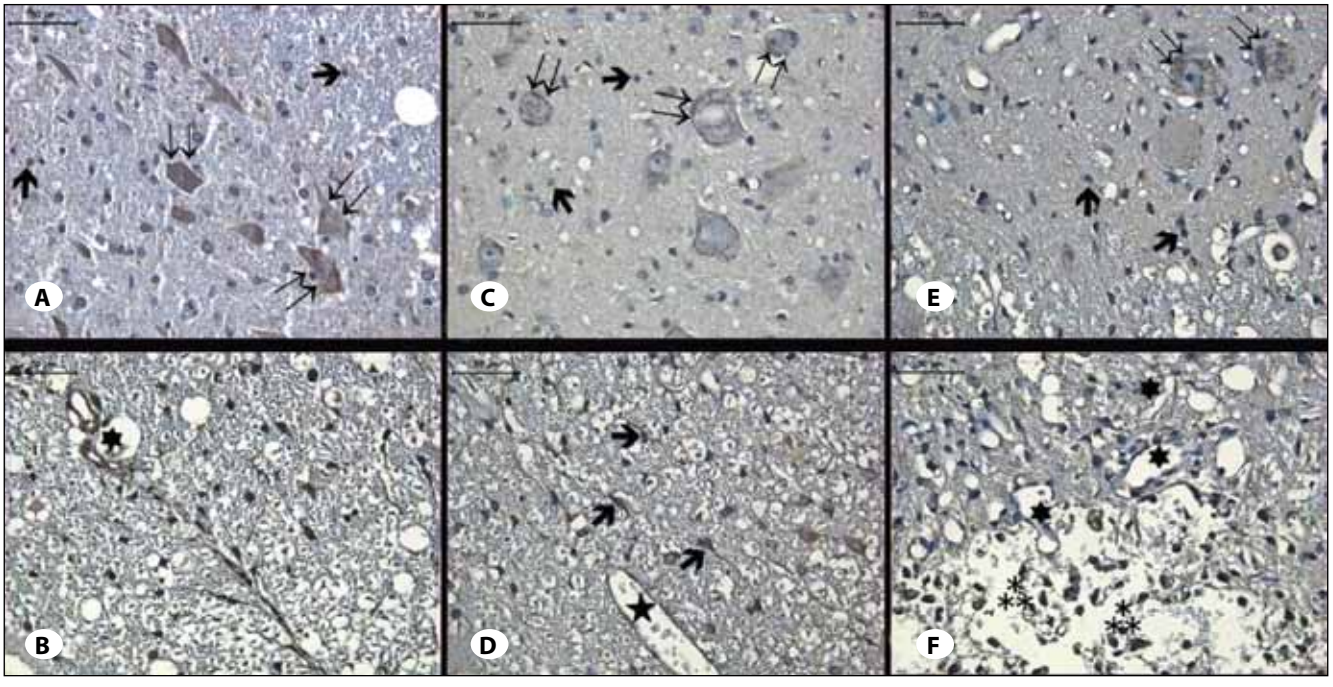

Figure 3: IL1β immunohistochemical stain. **A)** control group, ↑↑: neurons with intense staining for IL1β, ↟: a few glial cells with weak immunoreactivity; **B)** control group, ✸: Intense immunoreactivity for IL1β in vascular endothelial cells; **C)** trauma group, ↑↑: neurons with weak cytoplasmic and moderate membranous involvement, ↟: glial cells with negative staining; **D)** trauma group, ↟: positive and intense staining for IL1β in glial cells, ✸: no immunoreactivity in vascular endothelial cells; **E)** trauma+rituximab group, ↑↑: neurons with weak cytoplasmic and moderate membranous involvement, ↟: negative staining for IL1β among glial cells; **F)** trauma+rituximab group, ✳✳: intense involvement for IL1β in areas subjected to trauma, ✸: No immunoreactivity in vascular endothelial cells (Immune peroxidase-hematoxylin X400).

B. Electron Microscopic Findings

Trauma group

Oligodendrocytes at different stages of activity and degenerated myelinated nerve fibers were prominent at areas of trauma. A group of oligodendrocytes were characterized with normal thin appearance however some demonstrated typical autophagic vacuoles (Figure 5A). Studies at high magnification showed normal oligodendrocytes with normal granular endoplasmic reticulum and mitochondria. Granular endoplasmic reticulum was markedly dilated in oligodendroctes rich in vacuoles.

Trauma +Rituximab group

Degenerative appearance of ultrastructural morphology was not recognized and oligodendrocytes with normal granular endoplasmic reticulum and mitochondria. However myelinated nerve fibers still protect the degenerated structure (Figure 5B).

DISCUSSION

B cells have a key role in the pathogenesis of several autoimmune neurological disorders. B-cell depletion is an effective therapy in autoimmune disorders such as rheumatoid arthritis and this finding explains the reason of newly conducted studies of B cells functions on neurological

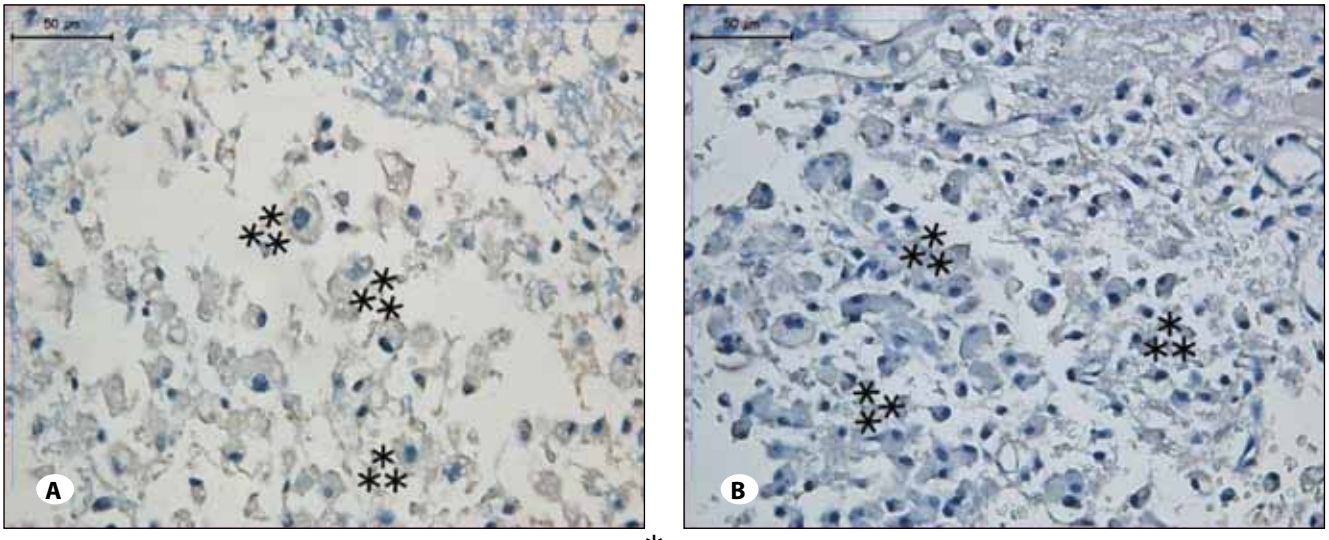

Figure 4: CD20 immunohistochemical stain. **A)** trauma group, ⁂: cells with positive staining; **B)** CD20 staining in trauma+rituximab group, ⁂: weak or negative staining for CD20 (Immune peroxidase-hematoxylene X400).

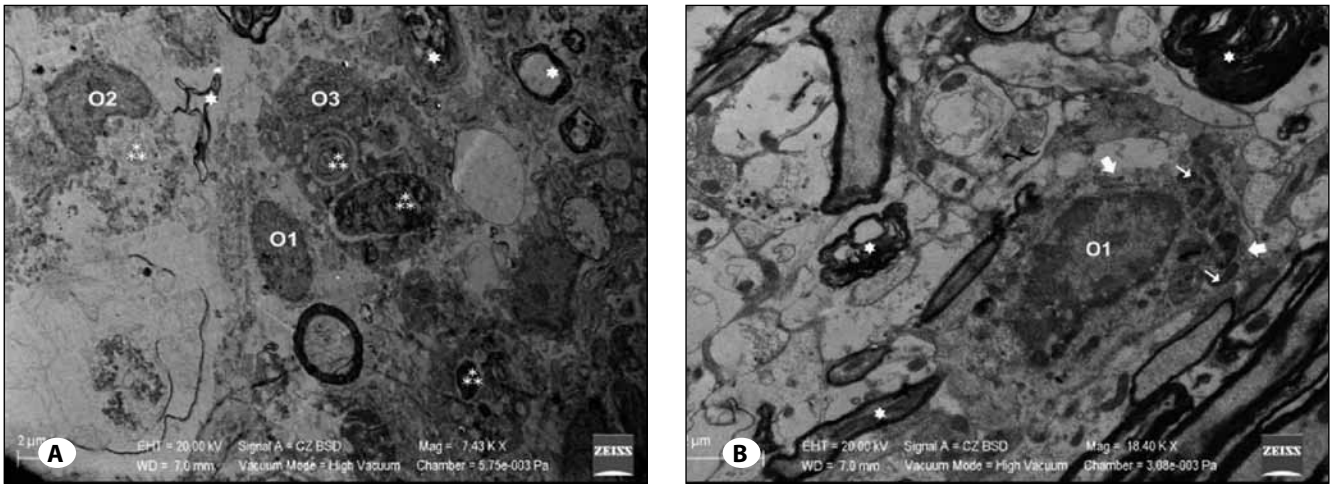

Figure 5: Electron microscopic studies. A) Electron microscopic sections of spinal cord in trauma group, ✸: myelinated nerve fibers at different stages of degeneration, O1: normal oligodendrocyte, O2: oligodendrocytes with a few autophagic vacuoles, O3: oligodendrocytes that phagocyted myelinated nerve fibers and scarce autophagosomes, ⁂: autophagosomes with different shapes and sizes; **B)** trauma+rituximab group, section of spinal cord, O1: normal oligodendrocyte, ✸: myelinated nerve fibers at different stages of degeneration, ←: tubuli of granular endoplasmic reticulum in oligodendrocytes, ↓: normal shaped mitochondria (Uranyl acetate-Lead citrate).

diseases (6). Drugs directed against the CD20 on B-cell surface glycoproteins can coat B cells and cause their depletion. Rituximab, a monoclonal antibody directed against CD20, is currently used in several clinical trials. This anti-CD20 monoclonal antibody has been newly introduced in the treatment of relapsing–remitting multiple sclerosis, autoimmune neuropathies, neuromyelitis optica, myasthenia gravis and inflammatory myopathies with satisfactory results (11, 13).

In this study, we investigate anti-inflammatory effects of rituximab on rat SCI with clip compression. Rituximab was presumed to be neuroprotective and specific antibodies (ELISA) against CD20 were studied with electron microscope and immunohistochemistry. Specific antibodies against cytokines like TNF-α, IL-1β and IL-6 were also studied.

Secondary mechanisms after spinal cord injury include neurogenic shock, excitotoxicity and free radical scavengers, fluid and electrolyte imbalance, vascular changes and apoptosis (15, 17). There is no doubt that the immune system is affected or significant changes were observed in neuroendocrine system after SCI but nevertheless neuroinflammation is the main secondary mechanism after SCI (5). Several pioneer studies within the last decade focus on anti-inflammatory agents or agents against these secondary mechanisms and these agents were accepted to be beneficial for neural healing (8, 10, 14). B-lymphocytes are noted to appear at the injury site within the first week after SCI and subsequent secretion of cytokines like IL1, IL6 and TNF-α through conversion into plasma cells and T-cell activation play a major role in cytotoxic cell damage (3, 14, 16, 18). Accordingly, cytokines like IL-1b, 1L-6, TNF-α or adhesion molecules like p-selectin may well be targets for treatment. Long term activation of B cell response results in increased IgG and IgM levels as well as increased lymphoproliferation in bone marrow and spleen after SCI (1, 2). The evolution of these autoantibodies after SCI and their binding to CNS proteins is an unknown issue but it is not impossible that these events stimulate a neurodegenerative process in the spinal cord (2, 9, 12). This mechanism of events shows similarity with the neuroinflammatory cell damage, a significant mechanism for autoimmune neurological diseases. With regard to electron microscopic studies, degenerative findings of myelin after SCI were markedly improved and oligodendrocytes were almost normal in appearance in electron microscopic studies of group 3 (trauma+rituximab treatment). This finding showed rituximab to suppress phagocytic stage but may still be inefficient for a positive contribution on regenerative phase.

In summary, TNF-α expression was augmented at the level of SCI both in neuronal and glial cells particularly in oligodendrocytes. These findings were suppressed after rituximab administration. Decreased CD20 expression both in neuronal and supportive glial cells was another prominent finding in rats under rituximab therapy and this finding showed that the drug was effective in suppression of B-lymphocyte invasion. On the other hand, expression of IL-1β and IL-6 were increased in glial cells without a significant change after rituximab administration. The decrease of CD20 expression after rituximab treatment may be correlated with neural healing.

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