

Encouraging Remyelination: Rejuvenating Aged Macrophages through the Use of Vascular Endothelial Growth Factor

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Multiple Sclerosis (MS) is a complex disease which has both an autoimmune and a neurological component (Huang et al., 2011). Affecting over 2.5 million people worldwide, MS is a progressive disease with debilitating physical and cognitive symptoms, such as: loss of vision, weakness, depression, both acute and chronic pain, and paralysis (Jadasz, Aigner, Rivera, & Kury, 2012). Each patient varies in regards to how their disease progresses, and what symptoms they present, however for the research being conducted in this study, MS will be discussed in terms of a type known as relapsing-remitting.

Relapsing-remitting MS is termed as such due to periods of demyelination (relapsing) followed by cessation of the inflammatory response and remyelination of the damaged axons (remitting). It begins with an acute phase, in which periods of inflammation occur as an immune response and cause areas of demyelination on axons. In this form of MS the demyelinated lesions undergo remyelination naturally, as oligodendrocyte precursor cells (OPCs) migrate to the lesion and differentiate into oligodendrocytes, the cells of the CNS which form new myelin sheath (Penderis, Shields, & Franklin, 2003). However, this natural remyelination eventually begins to slow or stop entirely, leading to a chronic phase referred to as secondary progressive. It is not fully understood why, but at this chronic phase, the rate and efficiency of remyelination begins to fail; however, several key factors have been implicated, including age and failure of OPCs to differentiate.

When considering treatment for MS, it is critical to consider both components of the disease, the immune system and the nervous system. Targeting the cause of the initial immune response could lead to a decrease in lesion formation; however this method would have no benefit

for pre-existing lesions, which are the source of patients' symptoms. Instead, enhancing remyelination would heal these lesions therefore reducing symptoms and preventing further axonal damage which occurs from a lack of remyelination and contributes to the progressive nature of this disease (Irvine & Blakemore, 2008). While there are currently no widely accepted forms of remyelinating therapy, research focuses on two main types; cell transplantation and endogenous restoration (Jadasz et al., 2012). Cell transplantation is complicated for several reasons, but primarily due to the fact that viable cells may not function properly because they are being placed into an environment that is, for some reason, not conducive for proper function and survival. Therefore, researchers Huang et al. (2011), suggest that remyelination therapies should aim to rejuvenate endogenous cells.

Just as chronic MS displays a decrease in the efficiency of remyelination; increased age has shown to have this same effect (Kuhlmann et al., 2008). Therefore, researchers speculate that studying age-associated deficits of remyelination in an acute form of MS can help shed light on why remyelination fails in cases of chronic MS (Kuhlmann et al., 2008; Ruckh et al., 2011). In their study, Kuhlmann et al. (2008) found that a failure of OPC differentiation is at the root of the remyelination issue. While these cells continue to migrate to the site of the lesion, they fail to differentiate into the necessary oligodendrocytes. This finding places a high priority on studying these precursor cells, and determining the underlying cause of failed differentiation.

A study conducted by Ruckh et al. (2011) investigated the changes undergone by OPCs with age, and sought to discover whether these cells lost their innate capability to differentiate, or if they could be rejuvenated in some way and restored to their proper function. Through exposure to a youthful systemic environment, this study found that OPCs can indeed be restored to a youthful

state and therefore increase the success of remyelination. In particular, they identified the macrophages from the young systemic environment as the key factors behind this change.

This finding is consistent with another study performed by Kotter, Setzu, Sim, van Rooijen and Franklin (2001) who found that when they depleted the macrophage population in a demyelinated animal model, remyelination was significantly impaired. The importance of these macrophages is based on experimental findings that show myelin debris, which occurs as a result of demyelination, to inhibit remyelination (Kotter, Li, Zhao, & Franklin, 2006). Typically, it is the role of macrophages to phagocytize, or consume, this debris, therefore allowing for efficient remyelination. While the study performed by Ruckh et al. (2011) showed the ability of young macrophages in an aged model to clear this debris and enhance remyelination, their technique of joining two systemic environments through parabiosis, the surgical joining of two animals, is not a practical form of therapy. Therefore, methods of treating aged macrophages in order to restore them to youthful function should be analyzed.

Similar to how OPCs cannot differentiate without the right surrounding environment, macrophages require the proper environmental support to function and, more specifically to phagocytize myelin debris (Kearns et al., 2012). In a study conducted on cells of the circulatory system, Kearns et al. (2012) found that vascular endothelial growth factor (VEGF) increased the phagocytic activity of macrophages. While this study was not performed on the nervous system, several other studies tie VEGF and endothelial cells to the environment and cells of the CNS. Endothelial progenitor cells (EPCs) were shown by Jickling et al. (2009) to have an indirect relationship to age-related white matter decline, such that a decreased number of EPCs was associated with an increased amount of age-related decrements in the white matter. Additionally, Arai and Lo (2009) studied the effect of endothelial cells on the survival and proliferation of OPCs,

and found that it enhanced the cells in these aspects. While this study is targeting the differentiation of OPCs, this previous literature indicates that endothelial cells may be a critical player in the environment of these cells. Also, while not directly tied to enhanced macrophage function, a study performed by Chu et al. (2005) showed an enhancement of vascularization in the cerebrum due to treatment with VEGF. Taking all of this literature into account, it is reasonable to assume that VEGF may be able to increase the phagocytic activity of aged macrophages in the CNS, therefore increasing OPC differentiation and remyelination.

In order to appropriately study the effects of VEGF on remyelination, it is critical to choose an appropriate model of demyelination. Since MS is a disease unique to humans, and is complicated by the involvement of two different systems, there is not a perfect animal model. Experimental autoimmune encephalitis (EAE) is the closest equivalent, but is largely ineffective in studying remyelination because it is complicated by the inflammatory component and, due to poor spatial and temporal predictability, it is difficult to assess the extent of remyelination (Ransohoff, 2012). Thus, in his review of the various animal models for MS, Ransohoff (2012) supports the use of a toxin induced model for this type of research because the experimenter can create one lesion in a specific location at a documented time, such that the rate of remyelination can be examined. This injection is typically given into the dorsal funiculus, which is a white matter tract of the CNS and has only a sensory component, such that lesioning this tract would not result in the loss of functional motor skills that may occur from lesioning other white matter tracts.

Thus, using a toxin induced focal demyelination model in the rat spinal cord; this study aims to examine the effect of VEGF on aged macrophages and determine if injections of this growth factor can lead to increased phagocytic activity. Secondly, if injecting VEGF causes effects these cells in such a way, will remyelination then be enhanced? While there is a strong experimental link

between endothelial cells and white matter function, this relationship has not been tested in regards to increasing phagocytosis of myelin debris, or in regards to enhancing remyelination. Answering this question is critical to finding a viable remyelinating therapy, which would relieve patients' symptoms as well as prevent further axonal damage. Even more so, this study takes into account age-related effects, which are important to consider in MS research since those living with this disease often live until old age, having their lifespan shortened by less than 5 years (Jadasz et al., 2012). Based on review of previous literature and the current understanding of the cellular interactions involved, it is hypothesized that VEGF will encourage remyelination in the CNS by rejuvenating aged macrophages.

Method

Animal Subjects

Animal subjects will consist of 12-month-old female Sprague-Dawley rats, which will be obtained from Charles River Labs. This strain and sex were chosen due to their use by previous researchers in similar studies (Kotter et al., 2001; Kotter et al., 2006). A total of 60 animals are needed, with 15 in each experimental group. An animal model is necessary to accurately reflect the variety of systemic factors which effect remyelination; this study uses just enough animals to obtain statistical power in the results. A veterinarian will be on call to maintain the health and care of all animal subjects. Surgical procedures will be performed in compliance with the Institutional Animal Care and Use Committee (IACUC) of the Neuroscience Research Institute. Animals will be handled regularly in order to reduce the stress associated with experimental procedures. All anesthesia will be given by inhalation of isoflurane, which, in addition to be used by other researchers in similar procedures, reduces animal handling, reduces recovery time and has a large margin of safety (Ruckh et al., 2011).

Facilities and Major Equipment

All experimental procedures and analyses will be performed at the Neuroscience Research Institute: Center for disease management. This facility contains an animal facility, which is maintained on a 12 hour light/dark cycle between 24-25 degrees Celsius. Additionally, this facility contains the equipment necessary for this study. A surgical area will be required, containing: an isoflurane vaporizer, a flowmeter, an induction chamber, a facemask, oxygen and a gas regulator. The demyelination of lysolecithin will require a micromanipulator and glass micropipette, allowing for precise and accurate delivery of the toxin. A cryostat and several fluorescence microscopes are also necessary for obtaining and analyzing data and can be found in the Neuroscience Research Insititute.

Research Design and Data Analysis

Animal subjects will be randomly assigned to four different experimental groups. Groups 1 and 2 will both undergo demyelination by way of a lysolecithin injection; Group 1 will be injected with VEGF while Group 2 will serve as a control, and will be injected with saline solution. Groups 3 and 4 will both undergo a sham surgery, in which they receive a surgery similar to that of the lysolecithin injection; however there is not actual injection of the toxin. As with Groups 1 and 2, Group 3 will be injected with VEGF and Group 4 with a saline solution. The use of sham surgeries will ensure that the effects of the surgery itself do not skew the results of the study.

The research being conducted will require approximately 5 weeks to complete. Surgical procedures will be carried out on Day 1 and injections given on Day 3, but animals will not be sacrificed until Day 21, as this is known to be the length of time required for complete remyelination in this model (Ruckh et al., 2011). The rest of the experimental procedures will require: two days for

the harvest and preparation of the tissue, about a week for immunohistochemistry and an additional week for data quantification and analysis.

All data will be analyzed using 2x2 between-subjects ANOVA, with surgical procedure received and injection received as the two independent variables and the quantification of the cell population of interest as the dependent measure. No post-hoc analyses will be needed since there are only two levels of each factor.

Lysolecithin injection.

Prior the beginning of the procedure, animals will be anesthetized by way of isoflurane inhalation. Once fully anesthetized, a small incision will be made along the back, exposing the space between the second and third thoracic vertebrae. Then, a dorsal laminectomy will be used to remove the lamina above the spinal cord. An incision should be made to cut through the dura mater followed by an injection of 1 µl of lysophosphatidyl choline, for groups 1 and 2, and 1 µl of saline, groups 3 and 4, given through a small hole in the pia mater. These injections will be given using a micromanipulator with a glass micropipette attached at the tip, and will be injected into the dorsal funiculus. In order to provide for future identification of the injection site, the adjacent epaxial muscle will be marked with a suture. Then, the incision will be sutured closed and the animal will be removed from anesthesia. These procedures are in accordance with other demyelinating procedures performed by Ruchk et al. (2011) and Woodruff et al. (2004).

Delivery of vascular endothelial growth factor.

Two days after the demyelinating and sham procedures are carried out, the animals will receive a single injection; groups 1 and 3 will be injected with VEGF, while groups 2 and 4 will be injected with saline solution (Bieber et al., 2002). The solution, VEGF or saline, dependent on

Group, will be infused into the tail vein at a rate of $1\mu\text{g}/(\text{kg min})$ using a micro-infusion pump. Human recombinant VEGF₁₆₅ will be used for the VEGF injection at a dose of $50\mu\text{g}/\text{kg}$, the maximum dosage which can be used without causing hypotension, an abnormal decrease in blood pressure. This method of injection and appropriate dosage of VEGF used are modeled after the procedures used in a study conducted by Chu et al. (2005), where researchers were examining the effects of VEGF on cerebral ischemia, indicating that their procedure is effective for delivery of the growth factor to the central nervous system.

Tissue preparation.

Three weeks after receiving surgery, all animals will be anesthetized through inhalation of isoflurane. The time frame is designated as such because this is the length of time required for remyelination in the lysolecithin model (Ruckh et al., 2011) All animals will then undergo perfusion-fixation, a method used to fixate the tissue and make it viable for analysis and will be accomplished using 4% paraformaldehyde, following the procedure described by Ruckh et al. (2011). After this procedure, the spinal cord of each animal will be dissected out, and cryosectioning will be used to obtain a $12\mu\text{m}$ thick section containing the lesion. This methodology is also described by Ruckh et al. (2011) and is accomplished by packing the spinal cord in dry ice and then using a cryostat to section it.

Immunohistochemistry.

Methods and techniques used here are modeled after those used by Ruckh, et al. (2011). Prior being incubated with the appropriate antibodies, each section will be blocked with goat serum and undergo permeabilisation with 0.3% Triton-X 100. These techniques prevent non-specific

binding and allow fluorescent tags to bind to the primary antibodies. Following these preparations, the sections will be incubated for 24 hours at room temperature with the primary antibody. Then, the secondary antibody, which tags the first with fluorescence, will be added for approximately 1 hour at room temperature.

Primary antibodies to be used include: mouse anti-CC1, rabbit anti-Olig2+, rat anti-CD34+ and rat anti-MAC1. Secondary antibodies include: donkey anti-mouse Alexa Fluor 594, donkey anti-rabbit Alexa Fluor 488, donkey anti-rat Alexa Fluor 594 and donkey anti-rat Alexa Fluor 647. All antibodies were chosen based on previous research which stained for the same cell types using these antibodies (Ruckh et al., 2011).

Viewing and Quantification of Cells.

Cells will be quantified using density as a measure, rather than exact cell count, in order to account for varying lesion sizes. By using a Zeiss Obeserver. A1 Axio fluorescence microscope, densities will be taken from three different sections of each lesion and averaged together to find the most accurate representation.

Results

Injection of VEGF Increases Endothelial Cell Density in an Aged Model of Demyelination.

If the proposed hypothesis is accurate, the following results will be obtained. A two-way ANOVA comparing the effects of growth factor treatment and surgical procedure on the density of endothelial cells, see Figure 1, showed a significant main effect of both these factors. Additionally, there is a significant interaction between these two variables such that the effect of VEGF on endothelial cells is dependent on the surgical procedure the subject received. The p value for each of these F statements is less than .05.

These results indicate that treatment with VEGF increases endothelial cell density in Groups 1 and 3, however this increase is greater in Group 1 than in Group 3, because Group 1 had this cell population depleted by inducing demyelination.

Injection of VEGF Increases Macrophage Cell Density in an Aged Model of Demyelination

A two-way ANOVA comparing the effects of growth factor treatment and surgical procedure on the density of macrophage cells, see Figure 2, showed a significant main effect of both these factors. Additionally, there is a significant interaction between these two variables such that the effect of VEGF on macrophage cells is dependent on the surgical procedure the subject received. The p value for each of these F statements is less than .05.

These results indicate that treatment with VEGF increases macrophage cell density in Groups 1 and 3, however this increase is greater in Group 1 than in Group 3, because Group 1 had this cell population depleted by inducing demyelination.

Increases in Endothelial and Macrophage Cell Density Lead to an Increase in Remyelination in Aged Animals.

A two-way ANOVA comparing the effects of growth factor treatment and surgical procedure on the extent of new myelin formation, see Figure 3, showed a significant main effect of growth factor treatment, but not the surgical procedure performed. Additionally, there is a significant interaction between these two variables such that the effect of VEGF on the extent of new myelin formation is dependent on the surgical procedure the subject received. The p value for each of these F statements is less than .05.

These results indicate that treatment with VEGF causes an increase in remyelination in Group 1 as compared to Group 2. There is not effect of VEGF between Groups 3 and 4, because these animals have not experienced any demyelination.

Discussion

The experimental results expected to be obtained from this study will show that aged macrophages can be rejuvenated through the injection of VEGF. A study conducted by Kearns et al. (2012) demonstrated the importance of cell environment on the phagocytic function of macrophages. In their study, these researchers found that treatment with VEGF enhanced macrophage clearance of apoptotic cells in the lungs. While the current study is examining the clearance of myelin debris, not apoptotic cells, and occurs within the CNS, not the lungs, these results indicate that VEGF will function by a similar mechanism as seen in the previous literature.

Such literature has found the primary mechanism of VEGF to be based on its role as a positive regulator, which interacts with receptor 1 for VEGF (VEGF R1) (Kearns et al., 2012). This interaction is necessary for activation of Rac1, an important protein, which is responsible for regulating many cellular processes including the cell-cycle and epithelial differentiation (Ridley, 2006). Treatment with VEGF thus increases the activation of Rac1, which in the case of this study, may have a variety of effects. As in the study by Kearns et al., (2012), this interaction is expected to increase phagocytic activity of macrophages, thus explaining the proposed experimental results of increased remyelination. However, if the results of this study are found significant, future research should analyze the other various mechanisms in which Rac1 can affect remyelination, such as potentially encouraging differentiation of OPCs as occurs with endothelial cells (Ridley, 2006).

This increase in phagocytic activity of macrophages, as a result of a VEGF injection, is then expected to be seen through an increase in clearance of myelin debris. Since previous research has

shown myelin debris to be a significant inhibitor of OPC differentiation, and defined the primary function of macrophages as phagocytosis of this debris, this is logical progression of effects (Kotter et al., 2006). Therefore, if phagocytic function is enhanced as expected, the rate and efficiency of remyelination will also be improved, making injections of VEGF a potential therapy for promoting remyelination.

However, before this step could occur, additional research should be conducted. The effects of injecting VEGF must be analyzed more fully in order to gain an appropriate understanding of how each system is affected. Furthermore, high doses of VEGF have resulted in adverse side-effects in animals, such as hypotension, or a significantly lowered blood pressure (Chu et al., 2005). Thus, it would be critical to determine if any additional side effects exist and if so, how they could be avoided.

Additional studies manipulating factors of various animal models should also be examined. The model used in this study injects a toxin into the dorsal funiculus, a sensory tract of white matter in the spinal cord. However, this model does not represent the effects that such demyelinated lesions have on the motor function of the animal subjects. Manipulating the location of toxin injection in future studies can help further our knowledge as to how these lesions effect function and how well this function can be regained with remyelinating therapy. Also, as previously discussed, Ransohoff (2012) supported toxin-induced demyelination for studying remyelinating therapies; however this model does lack the inflammatory component of MS and therefore cannot show the true effectiveness that a treatment may have in a patient with MS. Therefore, VEGF should be injected into an EAE model in order to obtain a more accurate view on its implications for treating MS.

While previous literature supports this study's hypothesis that VEGF will increase the phagocytic activity of macrophages, therefore enhancing remyelination, there is the potential that the results will not represent the effects described above. Under these circumstances, a lack of effect of VEGF on macrophage function may be the result of a fundamental difference between the mechanism described by Kearns et al. (2012), and the mechanism in which VEGF effects macrophages of the nervous system. Failure to rejuvenate aged macrophages would result in a failure to enhance OPC differentiation and remyelination. If the experimental procedures in this study have these results, several different possibilities may exist, with previous literature highlighting two in particular: VEGF functions by a different mechanism in the lungs than in the nervous system or, the function of aged macrophages cannot be rejuvenated. Such possibilities create new experimental questions for researchers and incite additional research into the mechanism of VEGF and other possible factors for rejuvenating aged macrophages.

Regardless of the results obtained by this study, the research conducted will contribute to our knowledge about MS; a debilitating progressive disease which effects millions of individuals (Jadasz et al., 2012). Finding effective remyelination therapies is crucial to promoting functional recovery in patients with this disease as well as protecting axons from further degeneration (Irvine & Blakemore, 2008). The theory and methodology behind this study add to this level of importance as they highlight age-related aspects of the disease. With MS, age is very important to consider because patients life expectancies are only shortened by about 5 years, meaning many people afflicted with MS live into old age. Treatments being developed should account for age-related affects in order to increase their effectiveness and range of application. If the hypothesis proposed by this study is supported by the experimental results, this will mean great progress for developing remyelinating treatments. However, a failure to support the proposed hypothesis will also contribute to the field of research as it will provide new questions as described above.

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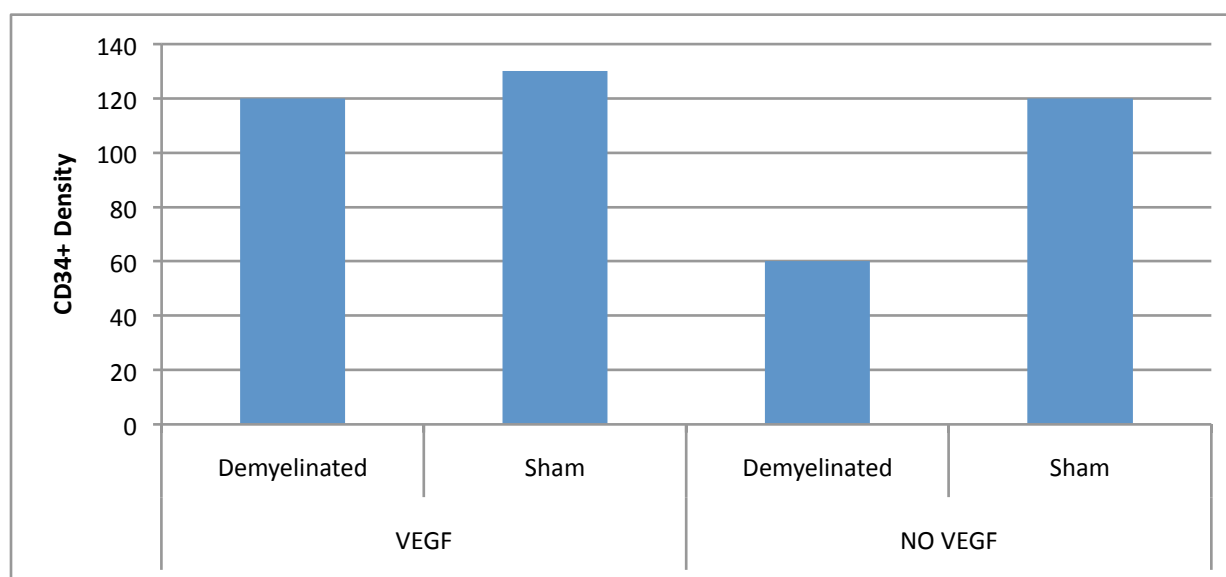


Figure 1. Interaction between growth factor injection and surgical procedure on endothelial cell density.

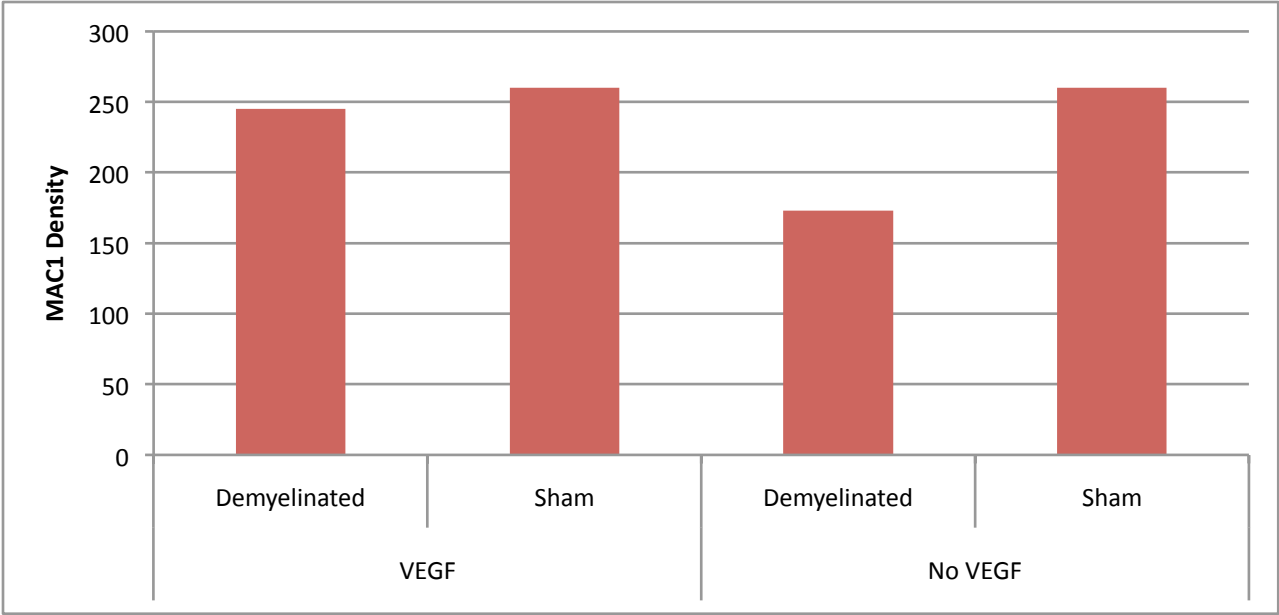


Figure 2. Interaction between growth factor injection and surgical procedure on endothelial cell density

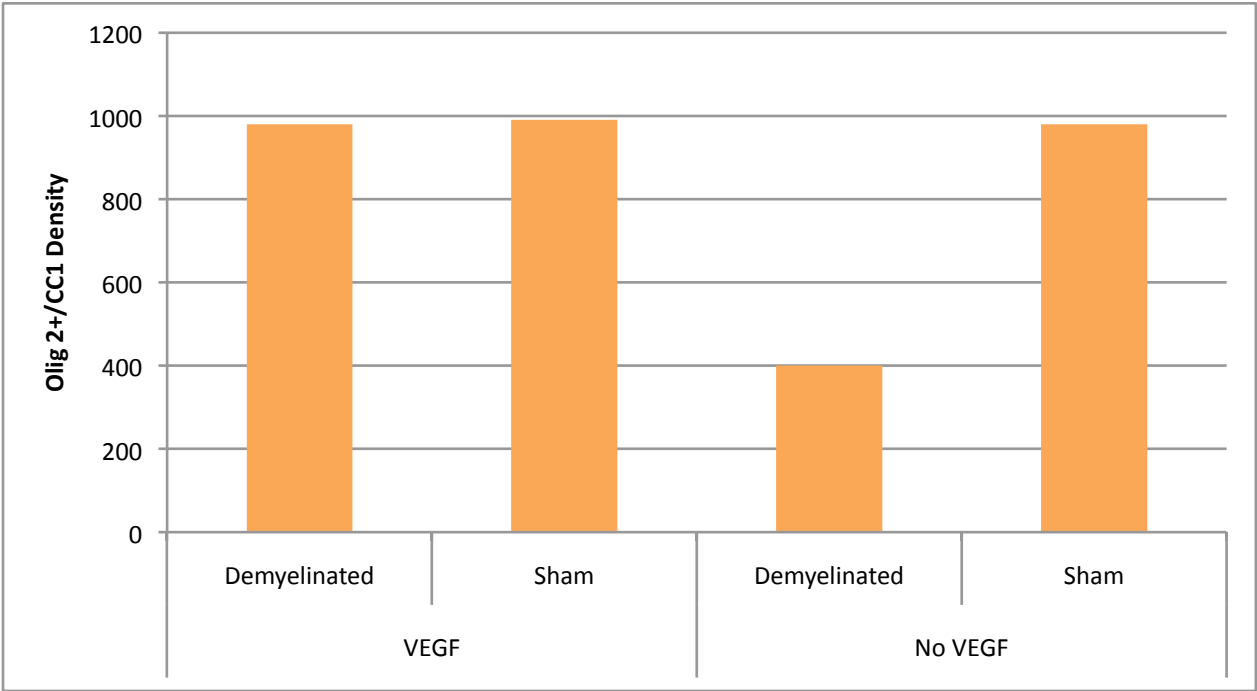


Figure 3. Interaction between growth factor injection and surgical procedure on extent of new myelin formation.

