UNIVERSITY OF SAO PAULO
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**Analysis of the expression of neurotrophins during regeneration of
peripheral nerves in rats with vein graft**

BAURU
2013

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Analysis of the expression of neurotrophins during regeneration of
peripheral nerves in rats with vein graft

Tese apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Estomatologia e Biologia Oral.

Orientador: Prof. Dr. Antonio de Castro Rodrigues

BAURU
2013

Ah52a Ahmed, Farooque
Analysis of the expression of neurotrophins during regeneration of peripheral nerves in rats with vein graft/ Farooque Jamaluddin Ahmed. – Bauru, 2013.
83 p. : il. ; 31cm.

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Prof. Dr. Antonio de Castro Rodrigues

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Data: 18 de outubro de 2011

DEDICATION

I dedicate this work of mine to the Almighty GOD, my Creator. I would have never reached so far without His mercy and blessings; to my late grandfather, who always wanted me to become a successful doctor; and last but never the least my beloved parents, who did everything they could have done to make me reach where I am today.

ACKNOWLEDGEMENTS

My parents back home in India, whom I miss every single day. They have supported me since my birth and have played a great role in attaining my career goals. There are no words to express their unconditional love.

My beloved wife for lot of courage and patience while being away from me. Her encouragement and motivation has helped me a lot in achieving my goals.

I would like to thank Prof. Dr. Antonio de Castro Rodrigues; my guide, my guardian, my best friend here in Brazil. I look up to him as a great human being who has shown me how to come out as a winner even in times of hardship and difficulty. I would always value his constant supervision throughout my life.

Very special thanks to **The academy of sciences for the developing world (TWAS) and Conselho Nacional de Desenvolvimento Científico e Tecnológico - "National Counsel of Technological and Scientific Development" (CNPq)** for providing me the postgraduate scholarship which allowed me to fulfill the dream of a PhD from a well renowned University.

To professors: Jesus, Rogerio, Marilia, Lauris, Conti, Carol, Carlos, Gustavo, Gerson, Izabel, Camila, Rodrigo and Heitor for supporting me throughout my PhD program

A Special thank to Andre (Shino), Cadu, Rafael, Andre (Meleca) Vitor, Thiago, Flavia and Felipe for always being eager to help me whenever I needed them.

To my fellow colleagues in the department of Anatomy; Roamario, Daniela, Vera, Gustavo, Lori, Geraldo, Daniel, Leticia, Cleuber, Jessica, Iris, Mizael, Idvaldo Karine for sharing those lovely and funny moments while being at work in the last 2 years making it as an enjoyable experience.

“Take up one idea. Make that one idea your life - think of it, dream of it, live on that idea. Let the brain, muscles, nerves, every part of your body, be full of that idea, and just leave every other idea alone. This is the way to success.”

Swami Vivekanand

RESUMO

Análise da expressão de neurotrofinas durante a regeneração de nervo periférico de rato por enxerto venoso

Enxertos de veias têm sido empregados para preencher lacunas em nervos periféricos transeccionados para melhor recuperação funcional. No entanto, vários inconvenientes, como a constrição do enxerto secundário foram observados. Uma nova alternativa para esta técnica foi desenvolvida. Simplesmente invertendo a veia de dentro para fora, chamado do “ Inside- out vein graft”. As neurotrofinas são uma família de fatores neurotróficos conhecidos por desempenhar um papel significativo na regeneração de nervos periféricos. A família da neurotrofina é constituído por fator de crescimento nervoso (NGF), fator neurotrófico derivado do cérebro (BDNF), Neurotrofina-3 (NT-3) e Neurotrofina-4 (NT-4). No campo da neurobiologia, vários autores têm utilizado a técnica de PCR a fim de obter mais informações sobre os nervos regenerados. Neste estudo, foi utilizada a técnica de biologia molecular para explorar o papel e o nível das neurotrofinas durante a regeneração de nervos periféricos com enxerto de veia. O nervo isquiático de ratos foi seccionado e reparado com enxerto de veia invertida (IOVG) e técnicas de enxerto de veia padrão (SVG). No grupo controle, os ratos foram operados e o nervo isquiático foi mantido intacto. Os animais foram sacrificados após 6 e 12 semanas e os enxertos foram colhidos para observar o nível das neurotrofinas. Músculos EDL e Sóleo foram excisados e pesados para determinar a diferença de peso entre os grupos. Um pequeno segmento dos cotos distais de ambos os grupos SVG e IOVG também foram excisados e foram processados histologicamente para examinar a quantidade de axónios regenerados. Além disso, um outro pequeno segmento do coto distal foi processado para RT-PCR para analisar o nível das neurotrofinas nesta área. A técnica do “walk track analysis” foi realizada para determinar o índice funcional do nervo isquiático nos grupos. Em 6 semanas, não ocorreu crescimento neuronal significativo no coto distal dos dois tipos de enxertos, porém um crescimento foi observado em 12 semanas. Não houve diferença significativa na massa muscular entre IOVG e SVG em ambos os períodos de tempo. No entanto, um aumento significativo na massa muscular foi observado a partir de 6 a 12 semanas nos grupos IOVG e SVG. Um aumento significativo na produção de NT-3 foi observado no grupo de SVG em ambos, enxerto e o coto distal quando comparados a partir de

6 a 12 semanas, no entanto, não houve aumento observado no nível de neurotrofinas dos outros tipos (NGF e NT-4) . Surpreendentemente, não houve aumento significativo da NT-3 no grupo IOVG. Conclui-se que, entre as neurotrofinas avaliadas neste estudo, não há nenhuma diferença significativa no seu nível de RNAm entre os dois grupos, exceto NT-3. Finalmente, uma vez que o nível de RNAm de NT-3 aumenta significativamente entre 6 e 12 semanas no grupo SVG e não no IOVG, observado por estas duas técnicas de nível molecular, estudos adicionais necessitam serem feitos para decifrar o mecanismo exato.

Palavras-chave: enxerto de veia. Neurotrofinas. Nervo isquiático

ABSTRACT

Analysis of the expression of neurotrophins during regeneration of peripheral nerves in rats with vein graft

Vein grafts have been employed to bridge the gap in transected peripheral nerves to produce better functional recovery. However several disadvantages such as secondary graft constriction were observed and a new alternative to this technique was developed by simply reversing the vein inside out. Both inside out and standard vein grafts were successfully used in recovering the sensory segmental defect in humans. Neurotrophins are a family of neurotrophic factors known to play an important role in the regeneration of peripheral nerves. The neurotrophin family consists of Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4). In the neurobiology field, several authors have been using PCR technique in order to gain more information regarding regenerated nerves. In this study, we employed this molecular biology technique to explore the role and level of the neurotrophins during the peripheral nerve regeneration with vein graft. The sciatic nerve of rats were sectioned and repaired with Inside out vein graft (IOVG) and standard vein graft techniques (SVG). In the control group the rats were sham operated wherein the sciatic nerve was kept intact. The animals were euthanized at 6 and 12 weeks and the grafts were harvested to observe the level the neurotrophins. EDL and Sol muscles were excised and measured to determine any weight difference between the groups. A small segment of the distal stumps from both the SVG and IOVG groups were also excised and were subjected to histological process to examine the amount of regenerated axon. In addition, another small segment of the distal stump was processed for RT-PCR to further examine the level of the neurotrophins in this area. At 6 weeks, no significant neuronal growth was observed in the distal stump of both graft types but a distinct growth was seen at 12 weeks. Walk track analysis showed poor motor function recovery in the experimental groups during both time intervals. Morphometric analysis demonstrated no significant differences in the amount of myelination between both the groups. There was no significant difference in the muscle mass between IOVG and SVG in both time periods. However, a significant increase in both the muscle mass was observed from 6 to 12 weeks in the IOVG and SVG groups. A

significant increase in the production of NT-3 was observed in SVG group in both the distal stump and graft segment when compared from 6 to 12 weeks; however there was no observed increase in the level of other neurotrophins (NGF and NT-4). Surprisingly, no significant increase of NT-3 was noticed in the IOVG group. We conclude that amongst the neurotrophins evaluated in this study, there is no significant difference in their mRNA level between both groups except NT-3. Also, since mRNA level of NT-3 increases significantly between 6 and 12 weeks in SVG and not in IOVG, it suggests that the mechanism by which these two techniques operate at a molecular level may differ and further studies need to be done to decipher the exact mechanism.

Key words: Vein graft. Neurotrophins. Sciatic nerve

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LIST OF ABBREVIATIONS AND SYMBOLS

BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
CNS	Central Nervous System
DNA	Deoxyribo Nucleic Acid
EDL	Extensor Digitorum Longus
EGF	Epidermal Growth Factor
EIT	Experimental Intermediate Toe Spread
EPL	Experimental Print Length
ETS	Experimental Toe Spread
FGF	Fibroblast Growth Factor
HGF	Hepatocyte Growth Factor
IOAG	Inside Out Artery Graft
IOVG	Inside Out Vein Graft
IGF	Insulin Growth Factor
mRNA	Messenger Ribo Nucleic Acid
NGF	Nerve Growth Factor
NIT	Normal Intermediate Toe Spread
NPL	Normal Priint Length
NTS	Normal Toe Spread
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PNS	Peripheral Nervous System
PTFE	Poly Tetra Flouro Ethylene
RT	Reverse transcription
SC	Schwann Cells
SFI	Sciatic Functional Index
SOL	Soleus
SVG	Standard Vein Graft
TAA	Total mean Axon Area
TAD	Total mean Axon Diameter

TFA	Total mean Fiber Area
TFD	Total mean Fiber Diameter
TGF- β	Transforming Growth Factor- Beta
TMA	Total mean Myelin Area
TMT	Total mean Myelin Thickness
TOF	The swing of the Opposite limb

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1 Introduction

1 INTRODUCTION

The repair of peripheral nerve defects is traditionally accomplished using an autologous nerve graft. This provides continuity of the stumps, with minimal or no tension, and supports axonal regeneration whilst protecting against surrounding scar tissue formation. Generally, sensitive nerves are employed as grafts. Whilst this technique usually provides good functional results, it does require an extra-surgical procedure that may lead to secondary damage created by the withdrawal of a healthy nerve (surgical incisions in sound areas, sensory residual deficits, etc.). Further, graft material is limited (in terms of length) especially in cases requiring the repair of extensive lesions, such as brachial plexus lesions. Therefore various surgical alternatives have been experimented and employed. These procedures are based on the use of both biological and synthetic graft tubes (tubulization).

Blood vessel grafts were proven successful as a conduit for nerve repair at the beginning of the twentieth century. LUNDBORG (1982) first underlined the advantages of tubulization showing the spontaneous search of regenerating axons for their target (chemiotropism) through artificial endothelial chambers. Specific chemiotropism, of motor and sensory fibers respectively, was shown by BRUNELLI (1993) using vein grafts and by RATH (1991) by means of skeletal muscle. Several other authors have utilised blood vessels and skeletal muscle as grafts with good results (GLASBY et al., 1986; JIMMING et al., 1986).

Vein grafts have been used successfully by various researchers to bridge the gaps in peripheral nerves (FERRARI ET AL., 1999; RODRIGUES and SILVA, 2001). However, WANG et al. (1993) came up with an alternative technique where the vein grafts were pulled inside out and expose the collagen rich adventitial surface to the regenerating axon. Various studies on inside out vein graft (IOVG) have since been carried out and it has been proved that IOVG provides a favorable microenvironment for the regeneration of peripheral nerves than the standard vein or the inside out artery graft. Several histological, electromyographical studies have been carried out to show the beneficial effect of the vein graft over other grafts however not many studies have been carried out showing the involvement of the neurotrophins in such grafts (MARCONI et al., 2003).

The discovery of the Nerve growth factor (NGF) led to the advancement in the field of neuroscience dealing with neurotrophic factors. The neurotrophic factors

includes groups of protein families such as the Transforming growth factor (TGF- β), Insulin growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) interleukin-6, Bone morphogenetic protein (BMP), Platelet-derived growth factor (PDGF). Among these, the factors which are of utmost importance in the survival, development of the neuronal population in the Central Nervous System (CNS) and Peripheral Nervous System (PNS) is the family of neurotrophins (LESSMANN et al., 2003).

NGF was the first neurotrophin to be discovered during the search of survival factors responsible for the maintenance of balance between the size of a target organ and the corresponding innervating neurons (Levi-Montalcini, 1987). After NGF, TURNER et al. (1982) managed to purify Brain Derived Neurotrophic Factor (BDNF) from pig brain, in an effort to search for a factor responsible for the survival of several neuronal populations not responsive to NGF. Later several other members of this family were discovered and are collectively known as Neurotrophins. In addition to NGF and BDNF, this family now also contains Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5).

In the neurobiology field several authors have been using PCR technique in order to get more information about regenerated nerves. In our study, we employed this molecular biology technique to explore the role and level of the neurotrophins during the peripheral nerve regeneration with vein graft. This would give us an overview of which neurotrophins gets elevated or gets diminished during this process.

2 Literature review

2 Literature review

2.1 Nerve injury repair techniques

Nerve injuries without any segmental loss or with a very short gap are usually treated by an end-to-end coaptation technique. The proximal and distal stumps in short gaps can be stretched without creating significant tension and connected by means of end-to-end coaptation technique. Major neural injuries with significant gap pose a major clinical challenge and necessitate the use of a graft to bridge the two stumps of the injured nerve. The gold standard to bridge these defects are the autologous nerve graft which provides Schwann Cells (SC), growth factors and basal lamina components. Donor nerves are usually taken from less functional nerves such as the sural nerves, superficial cutaneous nerves, etc (JOHNSON and SOUCACOS, 2008). However, these grafts are generally associated with complications such as scarring, neuroma formation and loss of sensation at the donor site. Thus, the search for an ideal graft which would pose minimal complication is still underway. With the view of achieving better clinical outcome, several artificial and biological grafts have been tried to bridge the inter-stump gap.

In the last few decades, many synthetic materials have been evaluated as an alternative to autologous nerve graft. Synthetic nerve conduits can be divided into biodegradable and non-biodegradable materials. Non-biodegradable Materials such as silicone (expanded polytetrafluoroethylene (PTFE) and polypyrrole (LUNDBORG, 1981), have been tested for use as nerve conduits and thought to provide favourable environment for growing axons. However, problems such as compression syndromes are often observed due to the non-degradable nature of the materials and inability to adapt to the nerve growth and maturation (MOHANNA et al., 2005).

Biodegradable grafts made of collagen, polyurethane, poly(lactic acid-epsilon-caprolactone) (COLIN and DONOFF, 1984; DEN, 1995) etc have been used extensively to promote regeneration of nerves. Collagen tubes have been successfully used as a nerve conduit to repair short nerve gaps. Collagen tubes provide a suitable alternative to nerve autografts due to its bio-compatibility and also it can be manipulated to provide an appropriate diameter matching the injured nerve thereby guiding the axonal growth (COLIN and DONOFF 1984). Even though these

materials facilitate the regeneration of axons, they are not very cost effective and demonstrate less regeneration efficacy and functional recovery than the autografts.

The advantage of immunological compatibility makes biological graft a more suitable alternative to repair nerve gap defects. Biological grafts contain cells which naturally provide the growth factors and cytokines to promote regeneration of nerve. A range of tissues have been used successfully to regenerate axons. These include blood vessels, muscles and combination of both (CHIU, 1982; GEUNA, 2000; MEEK et al., 2004).

2.2 Vein graft

Since the introduction of graft to treat the peripheral nerve injuries, many different types of natural and artificial grafts have been employed to achieve regeneration of the nerve.

WEISS and TAYLOR (1944) introduced the use of vein and arteries to repair large nerve defects in the experimental animals. Since then, many studies have been undertaken in order to augment the proposal of using vein as a substitute for autogenous nerve grafts.

Advantages such as no donor morbidity, easy harvesting and transplanting, ease of availability, etc makes vein graft close to an ideal mode of bridging gaps in transected peripheral nerves (BENITO-RUIZ, 1994). However with further studies, few complications like fragility, tendency to collapse, and physical obstruction of the growing axons by valves were reported (HEIJKE, 1993; KELLEHER, 2001).

In an attempt to eliminate these complications, WANG et al. (1993), came up with the new alternative idea of introducing IOVG as a means to bridge the gap between the peripheral nerves. In this study, the team uses a modification of the standard vein graft (SVG) wherein the vein is inverted inside out and is connected to the proximal and distal nerve stumps. The idea was to expose the collagen rich adventitial surface to the regenerating axons once the inner surface of the vein was pulled out. The result produced was significant as axon regeneration was faster with IOVG when compared with the SVG and polyethylene nerve guide (WANG, 1993).

Subsequently Wang et al compared the regeneration of nerve using two conduits: IOVG and autogenous nerve graft and concluded that the result obtained with IOVG is superior to the nerve graft. (WANG, 1995)

The encouraging results obtained by the use of IOVG in regeneration of peripheral nerves motivated researchers in the other parts of the world to carry out subsequent work on this alternative conduit. Since, the structure of arteries is similar to that of the veins, BACELOS et al. (2003) compared the IOVG with IOAG and found that even though both the conduits showed similar morphometric results, in general, IOVG presented a closer to normal organization than IOAG.

Encouraging results obtained by the use of autologous vein graft led to its use in human clinics too. RISITANO et al. (2002) carried out grafting of the sensory nerves with autologous vein graft and observed positive results in 20 out of 22 patients involved in his study. Similar study was carried out as recently as 2011 by ALLIGAND-PERRIN et al. and positive results were seen in the patients treated for sensory recovery of the digital nerves.

JEON et al. (2011) for the first time used IOVG to treat segmental sensory nerve defects in humans and exhibited beneficial results.

All the clinical studies with vein grafts have been performed to treat only sensory nerve defects. One of the major reasons which could be attributed to this is the lack of motor functional recovery observed with the use vein graft.

2.3 Peripheral nerve degeneration and regeneration

Peripheral nervous system has the potential to regenerate to some extent unlike the CNS. The axotomy of a nerve is followed by the retraction of the proximal and distal stump, leakage of the axoplasm and collapsing of the damaged membrane. Once there is injury to the nerve, various cellular events get triggered leading to the activation of macrophages and SCs (PERRY et al., 1987; BURNETT AND ZAGER, 2004). The macrophages get recruited at the injured site and causes phagocytosis of the myelin and subsequent Schwann cell proliferation (BEUCHE AND FRIEDE, 1984).

In the distal stump of the injured nerve, degeneration of the axon takes places by the means of Wallerian degeneration, wherein, the axon degenerates and their myelin sheath gets degraded. These degradation products along with the

macrophage secretion stimulate the proliferation of the Schwann cells within the basal lamina tubes to form the Schwann cells columns or “bands of Bügner (SALZER AND BUNGE, 1980). Schwann cells are critical for the process of axonal regeneration and one of their important roles is to secrete neurotrophic factors. The SC and basal lamina scaffold acts as a guide and provides a microenvironment for the regenerating axons in the proximal nerve stump to grow across the lesion (IDE, 1996).

In the proximal nerve stump, the axons degenerate retrograde until the first node of Ranvier. Several neuronal sprouts arise from the injured nerve within few hours of injury, whose number is more than original amount of nerve fascicles (WONG and MATTOX, 1991). This episode increases the chances of each neuronal cell reaching its target. Some of these sprouts will ‘die back’ through axonal pruning because of the lack of sufficient growth factors (BRUSHART, 1993). The terminal end of the growing axon (also known as the ‘growth cone’) actively searches for a suitable environment to support the axonal growth. Schwann cells present in the distal stump produces neurotrophic factors which attract and direct the axon growing from the proximal stump (BIXBY et al., 1988).

2.4 Neurotrophins

Neurotrophins are growth factors which help in the neuronal development, function and survival. They are found most prominently in central and peripheral nervous system and include four important members: NGF, BDNF, NT-3 and NT-4/5 (PEZET, 2006).

2.4.1 NGF

NGF was the first neurotrophin to be discovered during the search of survival factors responsible for the maintenance of balance between the size of a target organ and the corresponding innervating neurons (reviewed in Levi-Montalcini 1987). In the review from KORSCHING in 1993, it was demonstrated that the NGF was secreted by sympathetic and sensory target organs. From these sources, it is captured in the nerve terminals and is transported to the neuronal cell body through the way of axons thereby providing neuronal survival and differentiation.

NGF acts by binding to two receptors on the cell surface; the high affinity receptor TrkA and low affinity receptor p75. There has been evidence that NGF circulates throughout the whole body and is important in maintaining the homeostasis (LEVI-MONTALCINI, 2004).

Along with peripheral nerve regeneration, it also seems to promote myelin repair (ALTHAUS, 2004). It has shown promising results in reducing or preventing some neurodegenerative diseases in animal models which have motivated its use in human clinical trials (SUN et al., 2009).

Source: <http://betarhythm.blogspot.com.br/2008/09/neurotrophins.html>. Accessed: 03 Nov 2012

Figure 1 - A schematic diagram showing the binding of different neurotrophins to their respective low affinity and high affinity receptors on the surface of a cell.

2.4.2 BDNF

The next neurotrophin to be discovered after NGF happened to be BDNF. As the name suggests this neurotrophin was derived from pig's brain and has different antigenic and functional properties than NGF (SENDTNER et al., 1982). BDNF binds to two receptors on the cell surface that are capable of responding to it: TrkB and P75.

In addition to the CNS and PNS, BDNF has been expressed in range of tissues and cells such as kidney, prostate, and retina (BRONZETTI, 2008; MANDEL, 2009).

BDNF has been shown to play a role on long term memory and is linked to many disorders such as depression, schizophrenia, dementia, etc (ARANCIO and CHAO, 2007; BRUNONI et al., 2008; XIU et al., 2009).

2.4.3 NT-3

NT-3 is the third neurotrophic factor to be discovered after NGF and BDNF (YANCOPOULOS, 1990). It acts by acting on the low affinity receptor P75 and high affinity receptor TRKC.

NT-3 is a target derived neurotrophic factor which plays an important role in the survival and differentiation of PNS neurons. During both perinatal and postnatal period, NT-3 plays an important role in the survival and differentiation of sensory neurons (LEWIN and BARDE, 1996). It is present in a significant amount in adult skeletal muscle (MAISONPIERRE, 1990) and plays an important role in the survival of muscle sensory neurons (HORY-LEE, 1992). Neuromuscular spindles present in the muscles are known to secrete NT-3, which in turn helps in their differentiation (OAKLEY, 1997; CHEN, 2003). Furthermore, it acts as a short-distance axon guidance molecule for muscle sensory afferents as they approach their proper targets (GENC, 2004)

2.4.4 NT-4

NT-4 is the most recently discovered member of the neurotrophin family. It binds to two receptors TrkB and low affinity neurotrophin receptor p75LNGFR for efficient signalling and retrograde transport in neurons (CURTIS, 1995; RYDÉN et al., 1995;)

Upon nerve transection, the NT-4 mRNA level virtually disappears from the skeletal muscle (FUNAKOSHI, 1993). However, electrical stimulation of either nerve or muscle significantly increases the NT-4 mRNA and protein level. Thus, it can be inferred that the level of NT-4 in skeletal muscle is controlled by muscle

activity. Furthermore, it has been reported that intramuscular administration of NT-4 induced sprouting of intact adult motor nerves (FUNAKOSHI, 1995).

In addition to its role in nerve regeneration, NT-4 has also been attributed to have an impact on bipolar disorders and primary open-angle glaucoma (PASUTTO et al., 2009; WALZ et al., 2009)

Since neurotrophic factors play an important role in the regeneration of nerves, studies need to be conducted to understand its role in regeneration with autogenous vein grafts. In our literature search, it seems that role of neurotrophins with respect to vein grafts have not been studied extensively. This motivated us to perform studies which would ultimately provide us with data pertaining to the role of various neurotrophins in animal models where vein graft has been employed to close the gap between the two ends of a transected nerve.

3 Proposition

3 PROPOSITION

The ultimate goal of this study is to compare and measure the level of neurotrophins in standard and inside out vein graft during peripheral nerve regeneration.

4 Material and methods

4 MATERIAL AND METHODS**4.1 Animals and surgical procedures**

All procedures were carried out in accordance with Brazilian society on Animal experimentation (COBEA) and was approved by the Animal Research Ethics committee of Bauru dental school, USP (CEEPA-Proc. 032/2011). The experimental model consist a total of 36 male Wistar rats weighing around 300—350 g of around 90 days of age.

Groups	Rats with IOVG	Rats with SVG	Sham operated
Weeks			
6 weeks	6	6	6
12 weeks	6	6	6

Table 1- Distribution of the animals in each group

The rats were divided into two experimental (IOVG and SVG) groups and a Control (Sham operated) group. Each group was then further divided into two periods of 6 and 12 weeks (n=6). The surgical procedure consisted of trichotomy of the ventral surface of the neck and right thigh region (Figure 2) followed by the removal of about 14 mm of the right external jugular vein of the rats from the experimental groups through a paramedian neck incision. The grafts were washed in physiological solution and were then inverted inside out by pulling it down the cannula with microsurgery tweezers. Thereafter, the right sciatic nerve was exposed through median thigh and muscle-splitting incisions (Figure 3-A) and a 10 mm segmental nerve was excised. The transected nerve was then repaired using graft made of the segment of external jugular vein in the site of lesion (Figure 3-B). The proximal and the distal stumps were inserted 2mm into the graft. The grafts were then sutured using four stitches of 10-0 monofilament nylon for each stump. In the sham operated group, the right sciatic nerve was exposed and the muscles and the facial layers were subsequently sutured using 4-0 monofilament nylon sutured without undergoing any transection.

Figure 2 - Photographic image of trichotomy of the (A) ventral surface of the neck and (B) right thigh region of the rat.

Figure 3 - Photographic image of (A) the exposure of the sciatic nerve following median thigh muscle splitting incision and (B) Vein graft sutured with the two ends of the sciatic nerve

Post-surgery, the animals were kept in groups of four animals per cage in a temperature and humidity controlled environment with 12 hour light-dark cycles. They had food and water ad libitum. The animals were euthanized after 6 and 12 weeks with overdose of anesthesia and the grafts were removed (Figure 4) and kept in RNAlater Solution. From the distal stump of the sciatic nerve, a section of approximately 3 mm of the nerve was harvested and kept in RNAlater solution for RT-PCR analysis and another 3 mm section of it was stored in karnovsky's fixative solution for histological analysis. From the control group, around 10 mm of the nerve was excised for RT-PCR and another 3 mm of it were kept for histological sections.

Figure 4 - Photographic image of a harvested graft after the experimental periods.

4.2 Functional assessment of sciatic nerve

Walking track analysis was carried out after 6 and 12 weeks of surgery before euthanasia. The rats were made to walk on transparent track made of plastic (Cat walk track) and they were filmed with the help of a camera while the rats moved from one part of the track to the other end. The film was transformed into individual images and the best image from each animal was chosen to measure the distance between the toes. The distances were measured with the help of software Image Pro Plus 6.2 (Figure 5). To calculate the Sciatic Functional Index (SFI), the formula proposed by BAIN et al. (1989) was used.

$$SFI = -38.3 \left(\frac{EPL - NPL}{NPL} \right) + 109.5 \left(\frac{ETS - NTS}{NTS} \right) + 13.3 \left(\frac{EIT - NIT}{NIT} \right) - 8.8$$

An SFI of 0 in normal and an SFI of -100 is considered as total impairment, which would result from a complete transection of the nerve.

Figure 5 - Photographic image of rat feet subjected to an image analyzing software to measure the distance between the toes. L1- NTS, L2-NIT, L3-NPL, L4-ETS, L5-EIT, L6-EPL, L7-TOF

4.3 Histological and morphometric analysis

The harvested nerves and control specimens kept in karnovsky's solution were washed with running water overnight to be free from any excessive fixative solution. Tissues were then treated with 70% and 95% alcohol respectively for 2 hours before subjecting it to the treatment for resin embedding. All samples were carefully oriented to permit ultrathin sections of 5-7 μm , perpendicular to their axis. Subsequently, the specimens were stained with toluidine blue to permit visualization of the nerve fibres under microscope.

The nerve sections were observed under light microscope (Olympus BX50) at a magnification of 40X and images were captured with the help of a camera (Olympus DP-71) fitted with the microscope. Morphometric analysis was carried with the help of an image analyzing software (Image Pro Plus 6.2) connected to a computer. The regenerated fibers were analyzed to determine the mean fiber area, mean axon area, mean fiber diameter, mean axon diameter, mean myelin area and mean myelin thickness in each group of animals.

4.4 Muscle mass measurement

Once the grafts from the experimental group and the nerve from the control samples were harvested; the muscles of the rats supplied by the sciatic nerve i.e, Soleus (Sol) and Extensor Digitorum Longus (EDL) were also excised and their weight was measured with the help of a laboratory electronic weighing scale (Bel mark 3500) to determine the level of atrophy.

Figure 6 - Photographic image of excised EDL and SOL muscles after the experimental periods.

4.5 RT-PCR

Total RNA was isolated from the harvested grafts and distal nerve stump along with the control nerve samples kept in RNALater solution using an RNAeasy™ kit (Qiagen, USA). The concentration of mRNA was determined by absorbance at 260 and 280 nm in nanodrop machine (Nanodrop 1000 spectrometer, Thermo scientific, USA). All samples exhibited absorbance ratios (260/280) > 1.7. RT was carried out in thermal cycler (Applied Biosystems Verti-96-well thermal cycler, USA). The RT products were then subjected to real time PCR for NGF, NT-3 and NT-4. Amplification was carried out in duplicate for samples using the ViiA 7 real time PCR system (Applied biosystems).

4.6 Statistical analysis

Raw data was analyzed using SPSS, STATISTICA V5.1., Tusla, USA) and MS Excel software. Analysis between the groups in different experiments was carried out using one way, two ways or three way ANOVA (Analysis of Variance) depending on the number of parameters.

All pairwise multiple comparisons were done with the help of Tukey test/ Fisher LDS method/ Shapiro WILK tests as suggested by the softwares.

In all the analyses, P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$) were considered as statistically significant.

5 Results

5 RESULTS

Histological observation demonstrated that there were no regenerated nerve fibers at the distal stump of both the groups, 6 weeks post surgery. Very few regenerated axons were seen in only one of the animals from the IOVG group. Whereas, distinct regenerated nerve fibers were easily noticed at the distal stump in both IOVG and SVG groups, 12 weeks post surgery.

Figure 7 - Microscopic image of nerve fibers of sciatic nerve taken at 6 weeks post surgery. (Sham operated)

Figure 8 -Microscopic image of nerve fibers of sciatic nerve taken at 6 weeks post surgery (SVG). No nerve fibers are noticed during this period.

Figure 9 - Microscopic image of nerve fibers of sciatic nerve taken at 6 weeks post surgery (IOVG). No nerve fibers are noticed during this period.

Figure 10 - Microscopic image of nerve fibers of sciatic nerve taken at 12 weeks post surgery (SVG). Nerve fibers are clearly observed during this stage.

Figure 11 - Microscopic image of nerve fibres of sciatic nerve taken at 12 weeks post surgery (IOVG). Nerve fibers are clearly observed during this stage.

5.1 Morphometric analysis

Since, no regenerated nerve fibers were observed at the distal stump of the nerve of the animals from 6 weeks post surgery group; morphometric analysis was performed of only the samples from 12 weeks post-surgery group.

The data obtained after analysis of the nerve fibers are as follows:

5.1.1 Total Mean Fiber Area (TFA)

Significant decrease in the TFA of the nerve fibers in both the IOVG and SVG groups was seen when compared with the control group.

No significant difference in the TFA was noticed in between the SVG and IOVG groups.

Graph 1 - Histogram showing the difference in the total fiber area between the groups. Significant difference was seen between the control and the experimental groups. No difference was noticed between IOVG and SVG. P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$).

5.1.2 Total Mean Axon Area (TAA)

Significant decrease in the TAA of the nerve fibers in both the IOVG and SVG groups was seen when compared with the control group.

No significant difference in the TAA was noticed in between the SVG and IOVG groups.

Graph 2 - Histogram showing the difference in the total mean axon area between the groups. Significant difference was seen between the control and the experimental groups. No difference was noticed between IOVG and SVG. P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$).

5.1.3 Total Mean Fiber Diameter (TFD)

Significant decrease in the TFD of the nerve fibers in both the IOVG and SVG groups was seen when compared with the control group.

No significant difference in the TFD was noticed in between the SVG and IOVG groups.

Graph 3 - Histogram showing the difference in the total mean fiber diameter between the groups. Significant difference was seen between the control and the experimental groups. No difference was noticed between IOVG and SVG. P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$).

5.1.4 Total Mean Axon Diameter (TAD)

Significant decrease in the TAD of the nerve fibers in both the IOVG and SVG groups was seen when compared with the control group.

No significant difference in the TAD was noticed in between the SVG and IOVG groups.

Graph 4 - Histogram showing the difference in the total mean axon diameter between the groups. Significant difference was seen between the control and the

experimental groups. No difference was noticed between IOVG and SVG. P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$).

5.1.5 Total Mean Myelin Area (TMA)

Significant decrease in the TMA of the nerve fibers in both the IOVG and SVG groups was seen when compared with the control group.

No significant difference in the TMA was noticed in between the SVG and IOVG groups.

Graph 5 - Histogram showing the difference in the total mean myelin area between the groups. Significant difference was seen between the control and the experimental groups. No difference was noticed between IOVG and SVG. P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$).

5.1.6 Total Mean Myelin Thickness (TMT)

Significant decrease in the TMT of the nerve fibers in both the IOVG and SVG groups were seen when compared with the control group.

No significant difference in the TMT was noticed in between the SVG and IOVG groups.

Graph 6 - Histogram showing the difference in the total mean myelin thickness between the groups. Significant difference was seen between the control and the experimental groups. No difference was noticed between IOVG and SVG. P-value significance levels *($P < 0.05$), **($P < 0.01$), ***($p < 0.001$).

5.2 Functional assessment of the Sciatic nerve

To analyze the functional recovery of the sciatic nerve, walk track analysis was performed to determine the SFI of each rat.

The data obtained exhibited that there was significant difference ($p < 0.05$) in the functional recovery of both the SVG and IOVG groups when compared with the control group.

Meanwhile, no statistically significant difference was seen between the time periods (6 ad 12 weeks) of both the experimental group.

Graph 7 - Line chart showing the mean SFI of the groups at both the time period. Significant difference was noticed between the control and the experimental groups.

No significant difference was seen between the time periods in all the groups.

A score of 0 is normal and -100 represents total impairment of the nerve.

5.3 Muscle mass measurements

To determine the amount of atrophy of the Sol and EDL muscles in both the experimental groups, the final weight of the muscles were measured with an electronic scale.

The data obtained were analyzed with ANOVA followed by tukey test and demonstrated that there was significant decrease in the muscle mass in both IOVG and SVG groups. There was no significant difference in the muscle mass between IOVG and SVG.

A significant increase in both the muscle mass was observed from 6 to 12 weeks in the IOVG and SVG groups.

Graph 8 - Line chart showing the difference in the muscle mass measurements between the groups (Soleus). Significant decrease in the muscle mass was seen in the IOVG and SVG when compared to control. However, the muscle weight increased significantly from 6 to 12 weeks.

Graph 9 - Line chart showing the difference in the muscle mass measurements between the groups (EDL). Significant decrease in the muscle mass was seen in the IOVG and SVG when compared to control. However, the muscle weight increased significantly from 6 to 12 weeks.

5.4 Real time - PCR

Neurotrophins play an important role in the formation and maintenance of nerve fibres. During the denervation and re-innervation period, there is alteration in the levels of these neurotrophins. To investigate such variations during the regeneration process of the nerve fibers with vein grafts, we decided to perform RT-PCR to quantify the levels of NGF, NT-3 and NT-4.

5.4.1 Level of neurotrophins in the vein graft

From the data obtained following RT-PCR, we observed that the level of NT-3 was significantly upregulated between 6 and 12 weeks. No significant changes were noticed in the level of other neurotrophins in both the graft types between the time periods.

Graph 10 - mRNA expression level of NGF: Bar graph demonstrating no significant changes in the level of NGF in both the graft types between 6 and 12 weeks.

Graph 11 - mRNA expression level of NT-4: Bar graph demonstrating no significant changes in the level of NGF in both the graft types between 6 and 12 weeks.

Graph 12 - mRNA expression level of NT-3: Bar graph demonstrating upregulation in the level of NG-3 in SVG between 6 and 12 weeks.

To determine if this upregulation with time in the level of NT-3 was also present in distal stump of the nerve, RT-PCR was performed on the distal stumps of the grafts and the control nerve sample.

As expected, an increase in the distal stump of the SVG was noticed in the level of NT-3 in SVG. On the contrary, no such increase was noticed in IOVG.

Graph 13 - mRNA expression level of NT-3: Bar graph demonstrating upregulation in the level of NG-3 in SVG between 6 and 12 weeks. P value significance level *, # ($P < 0.05$) No such variation was seen between the time periods in the control and SVG group. A significant difference was also noticed between control and IOVG at 12 weeks.

6 Discussion

6 DISCUSSION

Injury to peripheral nerves can result in considerable distress. Many of the injuries are mild and can be treated appropriately within a considerable timeframe. However, some injuries are severe and can amount to considerable loss of function. Complete recovery of such injuries is often difficult and involves expenditure of a considerable amount of revenue. To treat injuries involving complete nerve transection with tissue loss of more than approximately 3 cm in length, a graft is required to provide an environment for the regenerating nerves to grow. (HUDSON et al. 1979; MILLESI, 1982). Many artificial grafts have been used in animal experimental studies and some with positive results. However, they are not cost effective in many countries. Thus, the use of autologous biological graft is an appropriate alternative for treatment of such injuries.

In the last few decades, many studies on animal models have shown that vein graft can be successfully used as a nerve conduit (CHIU, 1982; HEIJKE et al., 1993). These positive results encouraged its subsequent use in human trials. However, some complications such as the tendency of the vein wall to collapse and physical obstruction of the regenerating axons by the valves were reported and a modification to this technique was suggested (WANG et al., 1993; KELLEHER, 2001). The vein was turned inside out, exposing the tunica adventitia to the regenerating axons. Tunica adventitia is a rich source of collagen and the middle muscle layer is rich in laminin, both of which have been known to promote nerve regeneration. Also, by turning it inside out, the probability of vein wall collapse is also reduced. The use of IOVG has demonstrated promising results, with some studies even grading it better than the standard autologous nerve graft (WANG, 1995).

Both the SVG and IOVG have been used clinically to treat transections in sensory nerves (ALLIGAND-PERRIN, 1997; RISITANO et al., 2002; JEON et al. (2011). The results obtained are very similar, with both techniques providing good sensory recovery. To determine any difference between the modes of function in these two types of techniques at a molecular level, we performed this study to analyze the variance in the level of different neurotrophins.

From previous studies it was evident that in animal models with a 1 cm gap in peripheral nerves, regenerating nerves managed to transverse from the proximal stump to the distal stump through the nerve conduit in approximately 12

weeks (WANG, 1993; FERRARI, 1999; BARCELOS, 2003). Therefore, utilizing a time period of 6 and 12 weeks would aim to assist in determining the level of neurotrophin midway through the regeneration process.

Walk track analysis of rats demonstrated that there wasn't any significant improvement in the motor function of rats in both the experimental groups. This could be the reason that most of the clinical trials with vein grafts have only been tried in the sensory nerve defects.

Histological analysis of the distal stump of the nerves at 6 weeks demonstrated no regenerating nerves in both experimental groups. However, distinct growth of the nerves was observed during the 12 week post surgery period. Thus the result obtained was in accordance with the previously conducted similar studies. Furthermore, in order to observe if there was any significant difference in the degree of myelination in the regenerated nerves, morphometric analysis of all the samples was performed.

Significant difference in the level of total mean fiber area, total mean fiber diameter, total mean axon area and total mean fiber diameter was noticed between the control and experimental (IOVG and SVG) groups. However, no significant difference was observed in these parameters within the IOVG and SVG groups.

In order to examine any variation in the muscle mass supplied by the sciatic nerve, measurement of the SOL and EDL muscle was performed. Similar to the results obtained in morphometric analysis, no significant difference in the weight of SOL and EDL were noticed between IOVG and SVG groups. However, there was significant increase in the muscle mass in both the experimental groups between 6 and 12 weeks. Thus it can be perceived that regeneration process maybe gets accelerated between these 2 time periods.

The regenerative process of nerves involves the interaction of cellular events. This leads to the activation of macrophages and Schwann cells which in turn releases various cytokines and neurotrophic factors. Of these neurotrophic factors, neurotrophins play a crucial role in the whole regeneration process. In mammals, four important types of neurotrophins have been identified; NGF, BDNF, NT-3 and NT-4/5. Various studies on these neurotrophins have been conducted with the aim to elucidate its role in the regenerative process of sensory and motor neurons (MICHALSKI et. al., 2008; CHU et. al., 2008).

To decipher the molecular mechanism involved in the regeneration process in the nerves using IOVG and SVG techniques, this study observed the mRNA concentration of the neurotrophins in different time periods.

To analyze the level of neurotrophins, RT-PCR was performed from the grafts harvested from both the groups. Between the time periods of 6 and 12 weeks, only NT-3 showed a significant increase in the SVG group. No such increase in the level on NT-3 was illustrated in the IOVG group and no significant alteration was observed in the level of other Neurotrophins (NT-4 and NGF). Furthermore, to investigate if this increase in NT-3 was just not limited to the graft, an RT-PCR of the distal stump of all the groups were performed.

A significant increase in the level of NT-3 in the SVG group at the distal stump was observed between 6 and 12 weeks, similar to that obtained in the grafts. Surprisingly, no such increase was seen in the IOVG group.

According to literature, NT-3 has been known for its role in the survival and differentiation of sensory neurons. In addition to this, it also acts as a short-distance axon guidance molecule for muscle sensory afferents as they approach their proper targets (GENC ,2004). Thus, NT-3 seems to play an important role in the sensory recovery of the nerves with SVG.

From the results obtained from the RT-PCR of both the vein grafts and distal nerve stump, we hypothesized that the mechanisms by which both the techniques function at a molecular level may be different.

Even though we were not able to notice significant difference in the histological, morphometrical and muscle mass measurements between the two experimental groups, the difference in the mRNA concentration of NT-3 indicates that the functional recovery process might have a different approach in both the techniques.

Another hypothesis is that the regenerating axons from the proximal stump when they come in close proximation with the tunica intima of the SVG, they secrete growth factors in different proportion than that of the IOVG wherein, the regenerating axons are in close proximation with the tunica adventitia of the vein.

Also, since it is known that the muscles secrete neurotrophins during denervation and reinnervation of nerves, it is also possible that the muscles in both the IOVG and SVG techniques secrete different growth factors during their respective recuperation (HENDERSON et al., 1993; MAGNUSSON et al., 2005). This idea has

been supported by various findings which demonstrated that there is indeed increase in several neurotrophic factors such as hepatocyte growth factor (HGF), fibroblast FGF, BDNF, etc following either nerve or muscle damage (WALLENIOUS et al., 2000; WEHRWEIN et al., 2002; YAMAGUCHI et.al., 2004).

Many studies need to be conducted to decipher an exact mechanism by which these two grafts help in the recuperation of the transected nerve.

7 Conclusion

7 CONCLUSION

From the results obtained from the experiments conducted in this study we can conclude that:

1. IOVG and SVG acts a suitable nerve guide in achieving regeneration of the transected nerve.
2. The regeneration process seems to get elevated between 6 and 12 weeks in both the techniques
3. No significant differences were seen in the results of both the techniques except in the mRNA level of NT- 3
4. Also, since mRNA level of NT-3 gets increased significantly between 6 and 12 weeks in SVG and not in IOVG, suggesting us that the mechanism by which these two techniques operate at a molecular level could be different.

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Annex

**Universidade de São Paulo
Faculdade de Odontologia de Bauru**

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 032/2011

Bauru, 20 de outubro de 2011.

Senhor Professor,

O projeto de pesquisa encaminhado a esta Comissão de Ética no Ensino e Pesquisa em Animais, denominado **Análise da expressão de neurotrofinas durante a regeneração de nervos periféricos de ratos por enxerto venoso invertido**, de autoria de Farooque Jamaluddin Ahmed e colaboração de André Luis Shinohara, foi enviado ao relator para avaliação e considerado **APROVADO** em reunião desta Comissão realizada no dia **18 de outubro de 2011**.

Solicitamos que ao final da pesquisa seja enviado, para avaliação desta Comissão, um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Prof. Dr. Gustavo Pompermaier Garlet
Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Antonio de Castro Rodrigues
Docente do Departamento de Ciências Biológicas

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