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DOCTORAL THESIS

CLINICAL AND EXPERIMENTAL MULTIMODAL APPROACH TO THE INFLUENCE OF ADIPOSE TISSUE ADDITION ON THE REGENERATION OF DAMAGED PERIPHERAL NERVE

ABSTRACT

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Key Words: *peripheral nerve, nerve regeneration, stem cell, adipose tissue.*

1. Introduction, Motivation

The nervous system is considered to be the most complex system of the body; due to its complex functions, it alerts the body regarding internal and external changes and generates responses to effector organs. Complex traumas and major post-excision defects in the upper limb may have an important functional impact. Reconstructive surgery has the task of restoring the function as close as possible to the initial one. Nervous lesions are an important part of this chapter and often the most difficult to solve, being responsible for the existence of important sequelae. The clinical expression and type of lesion are given by the damaged structure; the different component structures of a peripheral nerve respond differently to the lesion and have different possibilities for regeneration.

The arguments for approaching such a research theme are due to the high incidence of clinical cases encountered at the Timișoara Plastic Surgery Clinic, where I have been working since January 2009. Nerve injuries are both post-traumatic and post-surgical, with the need for a reinnervation or resuscitation. The implications of such interventions are major, both functional (upper limb, lower limb), social through socio-economic and professional reintegration, but also psychological (facial reanimation). Repeated clinical observation led to a question: what causes mediocre results in peripheral nerve surgery, why are the results of primary neurorrhaphy sometimes unpredictable?

2. General part

Specialty literature lists a number of such causes, ranging from technical, material to physiological causes, both local and general. A possible answer would be that at this point peripheral nerve surgery, or nerve microsurgery, may have reached its limits, with the focus being automatically directed to new resources such as tissue regeneration. The post-lesion nervous regeneration, especially guided by Schwann cells, appears to be impinged by the appearance of scar tissue before the axonal growth. But scar tissue is normal in response to any injuries, and is implicitly encountered throughout the body. Again, current clinical practice can provide answers and correlations. It has become common in recent years to use lipoaspirate (the

remaining tissue together with the solution used in liposuction) to correct some volume deficiencies; it has also been used in areas with volume deficiency after scarring processes. The clinical observation was that the appearance of scarring improved considerably. Taking things further, why can lipoaspirate influence a scar? The presence of adipose-derived stem cells in this lipoaspirate has been demonstrated and their effects are multiple, from a local anti-inflammatory effect, to producing factors that can drive regeneration of different lines; these are of course incompletely understood and known processes. The study of the adipose-derived stem cell began with the reference article published by Zuk et al. in 2002 in Molecular Biology of the Cell. The ability of the adipocyte-derived stem cell to be pluripotent, not multipotent, as well as that derived from bone marrow, has been demonstrated. This observation has greatly extended the scope of applicability, given the ease with which this tissue can be harvested and processed, its availability and low morbidity. Also, there is a major difference in the quantity obtained after harvesting, if approximately one mesenchymal stem cell is obtained from the bone marrow from 25,000 to 100,000, 2% of the lipoaspirate cellularity is made up of stem cells derived from adipose tissue. The next step was isolation, which was initially done under laboratory conditions, but these processes are often lengthy and costly. Raposio's study followed - he compared the classical method, based on collagenase, and a simpler, mechanical, centrifugal-based alternative. A series of initial effects were observed in surgical use, thus adipose tissue graft obtained via liposuction was used in augmentation of certain atrophic areas, implicitly also in scars and, as a result, in addition to the mechanical filling of the area, an improvement in the appearance of scars was also observed. Reference studies in this regard were undertaken by Coleman, who used the tumescent technique for harvesting and centrifugation by 3,000 rpm for 3 minutes.

The correlation between these two clinical procedures was made and the logical subsequent question was what happens with primary neurorraphy, and implicitly with neuroregeneration, if we add processed fat? The present study has been developed with this in mind; experimental medicine will be used as follows in order to develop a model that can answer this question. Scientific literature contains numerous such studies of nerve regeneration and adipose tissue processing or various growth factors with influence on regeneration.

The structure of the thesis is a conventional one, with an introductory part, a general part and a special part, followed by the corresponding bibliography. The general part consists of four distinct chapters, which aim at informing on the topic of the normal and injured peripheral nerve, but also the

adipose-derived stem cell, namely the angiogenesis role in the nerve regeneration. These chapters are: macroscopic and microscopic aspects of the peripheral nerve with implications in peripheral nerve physiology, peripheral nerve response to the lesion, approached microscopically and surgically, adjunctive surgical therapy techniques in peripheral nerve regeneration: the adipose-derived stem cell and aspects of angiogenesis in the regeneration of the peripheral nerve by adipose tissue addition.

3. Special part

The special part consists of reasons for choosing this theme, materials and methods, results and final conclusions.

We have developed an experimental model based on the Pius Brânzeu Experimental Surgery Center facility, UMFT; the chosen study animal was the Wistar breed rat. The nerve chosen for the experimental study was the sciatic nerve due to its diameter corresponding to a microsurgical suture and anatomical topography, a relatively easy dissection exposure. In order to minimize the possibility of different neuroregeneration by other factors than the applied method, it was decided to use both sciatic nerves from the same animal, applying the two different methods. In the right sciatic nerve there was a microsurgical termino-terminal neurorrhaphy, and in the left sciatic nerve, after the neurorrhaphy was completed in the same way as the right side, the sutured nerve is surrounded by a fragment of adipose tissue harvested from the inguinal region and processed by centrifugation for 3 minutes at 3,000 rotations per minute. A pre-established protocol was divided into three consecutive steps: stage 0 (day 0), when the microsurgical part was performed, stage 1 (day 30), when the effects of applied and sampled methods from the first 5 animals were assessed and stage 2 (day 70), when the effects were evaluated again and samples from the last 5 animals were taken; all animals were subsequently euthanized. The tracking and quantification of the results was accomplished by two means: the evaluation of the motor response of the gastrocnemius muscle - distal and innervated by the sciatic nerve -, while observing the evolutionary diameter of this muscle via musculoskeletal ultrasound, as well as via histological evaluation of the treated nerve fragment, along with a fragment of muscle distal to injuries. From the histological point of view, several types of staining were made: common (hematoxylin-eosin, Masson's trichrome), histochemical (Gordon-Sweet and

Luxol Fast blue arginine impregnation), but also immunohistochemical (GFAP, NFAP, Prox1, AC133, OCT3 / 4, PGP 9.5, CD34 and MCT).

The results chapter summarizes the various conclusions drawn after the ultrasound and histological evaluation. The first subchapter, referring to ultrasound, observes the diameter of the muscles corresponding to the injured nerve, quantifying the atrophy and its regeneration. There is a better evolution in the left gastrocnemic muscle, adjacent to the sural nerve treated by suture and addition of processed adipose tissue compared to the right one; the recovered percentage difference is more significant in the first batch (4 weeks). Subsequently, a comparison was made between the ultrasound and the histological evaluation, with statistically significant correlations.

The next subchapter is the morphological analysis of sutured nerve fragments and adjacent muscle by hematoxylin-eosin staining, Masson's trichrome and argentic impregnation. The following conclusions have been drawn: adipose tissue adhesion around neurorrhaphy has an antifibrotic and anti-inflammatory effect, the addition of minimally processed adipose tissue stimulates the growth of the Schwann cell proliferation cone and the rapid realization of the interconnection of the two nerve ends, causes a hypervascularization of the micromedium around the neurorrhaphy, which may be involved in stimulating normal nerve regeneration. The histochemical methods used demonstrate in part the stimulation of not only the growth of the Schwann cell proliferation cone, but also the stimulation of the intraneuronal synthesis of neurofibrils that have previously invaded the Schwann cell guiding cone in the treated group, compared to the group untreated with adipose tissue. The morphological and histochemical studies of specimens harvested at 10 weeks demonstrate the ability of self-adherence and fixation of adipose tissue processed around neurorrhaphy, this aspect having a possible impact on the surgical technique, namely the removal of other currently used techniques in adipose tissue adition around neurorrhaphy (and viability).

The following subchapter proposes to find a phenotype corresponding to adipose-derived stem cells involved in nerve regeneration by studying specific markers: Prox1, AC133 and OCT3/4. A specific phenotype is detected: AC133 positive 3+, 3/4 +/- and Prox1 +/-, characterize the untreated group at 4 and at 10 weeks. Instead, for the treated group, at week 10, an Ac133 -, Prox1 3+ and Oct 3/4 + phenotype is identified, unlike week 4 for the treated group, where we noticed Ac133+, Prox1 with 3+ and Oct 3/4 with 3+. It has also been observed that nerve regeneration by addition of adipose tissue also influences the reactivity of satellite muscle cells, which tend to express a phenotype similar to those identified in the nerve repair cone but

also perineural fat, but at this time one cannot release the hypothesis that they differentiate into nerve fibers.

The following study theme consisted of the variability of GFAP expression, NFAP in nerve traumatic lesions. After the analysis and interpretation of the results it was concluded that the thread regeneration is chaotic at 10 weeks, consisting of a possible remodeling, the process being incomplete.

Further, PGP 9.5 expression was observed in the regenerated nerve, and PGP 9.5 was found to be useful for identifying mesenchymal cells in adipose tissue with differentiation to the neuronal line and that a correlation between expression of PGP 9.5 in adipose tissue, correspondent muscle, regenerative nerve, and ultrasound data could be a useful marker for assessing motor function recovery in patients with peripheral nerve injuries.

A special and interesting study consisted of assessing the presence and possible roles of mast cells in nerve regeneration. This was done by Luxol fast blue staining and mast cell tryptase evaluation (MCT). The findings were the following: mast cells are directly involved in nerve regeneration, their number being stimulated by adipose tissue addition; accumulation of mast cells compared to normal nervous tissue has an early onset at 4 weeks after neurorrhaphy and progressively increases at 10 weeks; mast cells influence not only nerve regeneration but also the stimulation of muscle function, as evidenced by the statistically significant correlation found between the diameter of the gastrocnemius muscle and the number of mast cells in the group treated with addition of adipose tissue; the increase in the number of mast cells in the group treated by addition of adipose tissue at 10 weeks - both intranervous , as well as in perinervous adipose tissue, suggests that the latter is a source of mast cells with direct involvement in stimulating nerve regeneration.

Last but not least, the nerve angiogenesis process was evaluated in regeneration by CD34 expression; it has been shown that vascular microdensity plays a major role in the regeneration of the peripheral nerve and that peripheral nerve regeneration angiogenesis is independent of mast cell activation and activation of mast cell tryptase.

4. Conclusions

• Nerve regeneration is a multistep process that includes a series of particular cell and molecular events specific to this type of regeneration.

• Adipose tissue addition around neurorrhaphy has multiple positive effects on this regeneration, starting from its anti-inflammatory effect, stem cell source with differentiation potential for activated Schwann cells, to indirect action on the corresponding muscle.

• Stem cells involved in nerve regeneration have a versatile phenotype that varies depending on the time elapsed since neurorrhaphy and adipose tissue addition.

• In the first 4 weeks, the regeneration by addition of adipose tissue is active, characterized by the persistence of the Schwann cell immature phenotype; the regeneration process is incomplete at 10 weeks, but unlike the first stage, the nerve maturation is predominant, a fact proven also by the diameter increase of the muscle in the adipose tissue treated group at 10 weeks.

• Angiogenesis is mandatory in nerve regeneration, being stimulated by adipose tissue addition; in addition to endothelial cells, mast cells play an important role in nerve regeneration, their number increasing in the adipose tissue group (which suggests that it can be a source of mast cells), but which paradoxically does not function by mast cell tryptase.

• The decrease of mast cell tryptase in the adipose tissue group and in the second part of regeneration (weeks 5-10) supports the anti-inflammatory effect. Most likely, the increase in the total number of mast cells is determined by their attractiveness through a range of factors secreted at the injury site, and their most likely role is to synthesize NGF, a fact that will later need to be proven, as it is not part of the scope of this study.

The chosen field of study proved to be surprising and abounding in results. It initiated the opening of new horizons with regard to the research and improvement of peripheral nerve regeneration, with possible direct implications in current clinical practice.