Muscle denervation atrophy is a result of lower motor neuron injury, thus an early restitution of muscle stimulation is essential in prevention of atrophic changes. The aim of the study was to evaluate the new application of naturally occurring epineural sheath conduit in repair of the peripheral nerve gap to prevent development of muscle denervation atrophy.

Material and methods. We used the model of 20 mm sciatic nerve gap, resulting in denervation atrophy of the gastrocnemius muscle in the diabetic rats (DM type 2, n=42, Zucker Diabetic Fatty strain). We applied the epineural sheath conduit created from the autologous sciatic nerve for gap repair. Muscle atrophy was assessed with the Gastrocnemius Muscle Index (GMI) and microscopic muscle morphometry (mean fiber area) at 6 and 12 postoperative week. Muscle regeneration in the experimental group was compared to the gold-standard technique of autologous nerve grafting for the repair of created nerve gap.

Results. The GMI evaluation revealed comparable muscle mass restoration in groups with nerve repair using both epineural sheath and standard autologous nerve grafting (reaching 28 and 35% of contralateral muscle mass at 12 postoperative week, respectively, p=0.1), and significantly better restoration when compared to the negative control group (no repair, 20%, p<0.01). Micromorphometry confirmed significantly larger area of the regenerated muscle fibers in groups with both nerve grafting and epineural sheath conduit repair (reaching for both ca. 42% of the non-operated side), when compared to severe atrophic outcome when no nerve repair was performed (14% of the control fiber area, p<0.0001). The effectiveness of epineural conduit technique in muscle mass restoration was observed between 6 and 12 weeks after nerve repair – when gastrocnemius muscle mass increased by 12%.

Conclusions. Peripheral nerve gap repair with naturally occurring epineural sheath conduit is effective in prevention of muscle denervation atrophy. This method is applicable in diabetic model conditions, showing results of regeneration which are comparable to the autologous nerve graft repair.

Key words: muscle denervation atrophy, peripheral nerve repair, diabetes, animal model

Atrophy of skeletal muscles following loss of stimulation by the nervous system (denervation) is a pathological response of muscle cells that markedly reduces their functional capacity. It is manifested by loss of muscle mass and increase of connective tissue:muscle tissue ratio in the area supplied by motor fibers of the damaged nerve (1). Mechanism of denervation atrophy is based on two reactions – loss of neural stimuli that normally stimulate muscles to work and loss of inflow of myotrophins – growth and nutritional factors for

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muscles, produced in the neuronal body and transported to muscles via rapid axonal transport (2, 3). Loss of communication between the nervous system and muscle cells results in changes in the latter cells, manifesting as inhibited protein synthesis and activated proteolysis of structural proteins, transmitted by ubiquitin and lysosomal pathways and supported by the caspase system. Concurrently, myocyte apoptosis is initiated via the mitochondrial pathway, manifesting as loss of subsarcolemmal mitochondria and destabilization of endoplasmic reticulum as well as several-fold increase of oxygen free radicals, DNA degradation and breakdown of cellular nuclei. There is a reactive activation of muscle satellite cells that proliferate and coalesce with myocytes, initiating the regeneration process that is of limited effectiveness due to persistent state of deprivation of trophic neurogenic factors. Published research studies do not indicate role of necrosis or presence of inflammatory factors in the initiation and propagation of neurogenic muscle atrophy (4-8, 14, 16).

Denervation can result from multiple mechanisms that mainly include degenerative diseases of the lower motor neuron, peripheral neuropathy (toxic and metabolic, including diabetic) as well as injuries of the lower motor neuron – peripheral nerve. Approximately 2.5% of population throughout their lifetime sustain a severe, posttraumatic injury of a peripheral nerve that most commonly affects the upper limb and radial nerve (9, 10). Literature indicates that the most common causes of nerve injury in adult subjects differ depending on local specificities, however generally they include: traffic accidents (40-46%), accidents sustained by pedestrians (9%) and gunshot wounds (7-32%). Approximately 44% of these cases require surgical repair (9, 11). Primary repair of the injured nerve, using tension-free end-to-end anastomosis or nerve graft, most commonly sural or cutaneous nerve of the forearm, when an approximation of both nerve ends is impossible, is the gold standard therapy. The latter method has its limitations, including: differences in the nerve diameter, requirement for surgical manipulation at two anatomical locations, denervation of the graft donor site, requirement for immunosuppression in case of allogenic grafts. Furthermore, in patients with immune or wound healing disorders as well as with coexisting inflammatory or diabetic neuropathy, nerve transplantation carries higher risk of complications. Therefore alternative methods of surgical repair of nerve gaps are being sought (12).

The first choice method of prevention of denervation muscular atrophy is as fast as possible and effective restoration of neural stimulation by repairing the nerve gap. Studies demonstrate that time to functional regeneration of the injured nerve is the key factor affecting inhibition or limitation of the muscle atrophy (13, 16). Loss of muscle mass in the denervated muscle rapidly progresses, at a rate of approximately 1% daily, achieving stabilization within several weeks at the level of 30% initial muscle mass. In the light microscopy these changes are manifesting as reduced cross section area of the denervated muscle fibers. Loss of muscle mass and protein mass, loss of muscle strength, increased insulin resistance, change of the type of the fibers from slow to fast fibers and easy fatigue of the muscles are the clinical manifestations of denervation (5-8, 16). Early restoration of neural stimulation and muscle rehabilitation provide a chance to limit or even reverse this process. Reinnervation of the muscle by approximately 30% of motor neural fibers normally supplying this muscles was shown to be sufficient to preserve its function and inhibit atrophy (15).

The aim of the study was to use a natural epineural nerve sheath to surgically repair a nerve gap in an animal model and assess efficacy of regeneration in the prevention of development of denervation muscle atrophy in the diabetic condition.

MATERIAL AND METHODS

Forty two male ZDF (Zucker Diabetic Fatty, Charles River Laboratories) rats were used in the study – their average body weight was 370 g (range 321-449, no differences between the experimental groups) on the day of the surgical procedure. This strain is an animal model of type 2 diabetes mellitus (congenital leptin-resistance, hyperglycemia, insulin-resistance). During the study the animals were maintained at an animal facility, at room temperature, with 12-hour light/dark cycle, 2 rats per cage, with ad libitum access to water and food – diet Purina 5008 according to recommendations of the manufacturer, and litter
was replaced daily. After 3 days of quarantine after the arrival, all animals had serial measurements of blood glucose concentration, with blood collected from the middle tail vein in the morning and in the afternoon (glucometer Alphatrak Blood Glucose Meter, Abbott Laboratories, Inc., Alameda, CA, USA – calibrated for rats). The eligibility criteria for the study included overt diabetes mellitus – two post-prandial blood glucose values ≥200 mg/dl and at least one blood glucose value after 16 hours of fast ≥ 110 mg/dl (17). Surgical procedures were performed under the general anesthesia, using inhaled isoflurane (Classic T3 Vaporizer system, SurgiVet, Norwell, MA, USA), at a concentration of 2-2.5% vol., mixed with oxygen, and buprenorphine 0.2 mg/kg body weight, SC. 5% isoflurane was used for the anesthesia induction. In all animals, under aseptic conditions, a surgical incision through the gluteal muscles was used to expose the right sciatic nerve between the sciatic notch and popliteal fossa. Subsequently the nerve branches were ligated and cut and blood vessels were removed from the nerve surface, while bifurcation to final branches was left intact. A 20-mm segment of the exposed nerve was resected in each rat and the proximal nerve incision was made approximately 3 mm below the sciatic notch.

The rats were divided into 3 experimental groups: group 1 – the gap was left unrepaired (n = 10), group 2 – the gap was repaired with orthotopic implantation of a cut nerve segment (n = 16), group 3 – the gap was repaired through implantation of an epineural sheath conduit created from the resected fragment of the sciatic nerve via removal of all nerve fibers (n = 16) (fig. 1). A nerve or its sheath was implanted using a microsurgery technique of epineural “end-to-end” suture, using a surgical microscope and sterile microsurgery instruments, and non-absorbable nylon suture 10-0 (Black Monofilament, Surgical Specialties Corporation, Reading, PA, USA). Following the implantation, an empty epineural sheath was filled with normal saline (0,1 cc) using a microirrigator, from the distal side before the last suture was tied. The wound was closed in layers, using an absorbable braided suture 5-0. All procedures were performed by the same operator (author M.Ł.). The rats were observed after the surgical procedure with particular emphasis put on signs and symptoms of inflammation and mechanics of denervated muscles.

The animals were not subjected to any motor rehabilitation or any other muscle stimulation.

Half of the animals from each group was euthanized after 6 weeks, and the other half after 12 weeks, using intraperitoneal pentobarbital 100 mg/kg body weight. Subsequently bilateral gastrocnemius muscles were collected from each rat and weighed on an accurate laboratory scale (College Balance, Mettler, Toledo, OH, USA), and the whole muscles were fixed in 10% formalin solution. GMI (Gastrocnemius Muscle Index), mass of the gastrocnemius muscle on the operated side divided by mass of the contralateral muscle (R/L), was calculated. Following the fixation, histological slides were prepared using a transverse section throughout the half of the belly of each of the studied muscles. The slides were stained in a conventional manner, with hematoxillin and eosin, and subsequently two images were taken of randomly selected high magnification fields from the light microscope (Leica DM4000B, W. Nuhsbaum, Inc., McHenry, IL, USA) for each muscle. The images were digitized and analyzed by an independent observer, who evaluated 30 cross section areas of the muscle fibers in each sample (Image-Pro Plus, Ver 6.3.0.512, Media Cybernetics, Bethesda, MD, USA) (fig. 2). The results were averaged and compared. To avoid interindividual variability, averaged cross section areas of muscle fibers from the operated (R) side were compared to that on the left, unoperated side (L) and expressed as a R/L ratio in each rat. Statistical analysis was conducted with SPSS Statistics software (v20, IBM Corp.). Differ-
ences between the study groups were compared using variance analysis (ANOVA) with post-hoc analysis: Tukey’s and Bonferroni’s tests; p < 0.05 was considered statistically significant. Variance homogeneity was tested using Levene’s test. The experiments were conducted at the Microsurgery Laboratory of the Cleveland Clinic Department of Plastic Surgery (Cleveland Clinic Main Campus, Cleveland, OH, USA). The study protocol was approved by the Cleveland Clinic Institutional Animal Care and Use Committee (IACUC no. A3047-01).

RESULTS

During the postoperative observation, the calf muscles of the study rats did not demonstrate any signs or symptoms of inflammation or edema, and gait mechanics was disturbed in a similar manner in all study subjects. Following the euthanasia, the gastrocnemius muscles on the operated side demonstrated clear signs of atrophy as compared to unoperated muscles on the control side (fig. 3). The figure demonstrated average values of GMI index in individual study groups 6 and 12 weeks after the surgery (fig. 4, 5). After 6 weeks the largest mass loss was observed in the group without repair of the nerve gap (researching to 22% of the control mass), while the corresponding indices in group 2 and 3 were similar (approximately 24%). However, these differences between the study groups were not statistically significant. Progressive atrophy could be seen in muscles collected 12 weeks after the surgical procedure in group 1, where a relative mass of the denervated muscle dropped to 20%.
However, in two remaining study groups, restoration of muscle mass could be seen, that was 12% over the 6 weeks between the observations for the group with implantation of epineural sheath (group 3). GMI rose to approximately 35% in the group of implantation of the whole nerve. Statistical analysis demonstrated significant differences in the GMI between the study groups (p = 0.004), and post-hoc tests demonstrated that there were no statistically significant differences between groups 2 and 3 (p = 0.08 to 0.10).

Histomorphometric testing of the muscle samples gave similar results and confirmed muscle atrophy at the microscopic level (fig. 4, 5). Six weeks after the denervation, reduction of muscle fiber size was evident in all study groups. What needs to be emphasized, the smallest loss of muscle fibers was found in the group with repair using an epineural sheath conduit, where an average myocyte cross sectional area was by 14% larger than in the reference group, in which the gap was repaired with a nerve graft. However, these differences were not statistically significant. Microscopic examination of muscles 12 weeks after the surgery demonstrated potent regeneration of muscle fibers as compared to results obtained 6 weeks after the surgery, both in the group of epineural sheath conduit and a whole nerve implantation. In both groups the size of a muscle fiber was approximately 42% of the area of the non-denervated muscle. Further progression of atrophy of the muscle fibers was also demonstrated in the group without nerve repair – the fiber size index was 14% of the corresponding value for the unoperated side. Statistical tests demonstrated significance of these differences at the p < 0.0001 level. Post-hoc tests demonstrated such significance for results for both groups with surgical repair (2 and 3) versus the negative control group (group 1). Comparison of average values for groups 2 and 3 had p = 0.99. Changes of muscle mass and size of the denervated muscle fibers in all study groups, 6 and 12 months after the surgery, are summarized on fig. 6 and 7.

**DISCUSSION**

The model used in this study is based on iatrogenic injury of a peripheral nerve and concurrent repair of the nerve gap during the same surgical procedure. It is essential for the regeneration and reinnervation to occur as early as possible after the muscle denervation. A study by Canadian authors demonstrated that the number of motor neurons adjacent to a muscle is reduced exponentially with chronic denervation, after the nerve cutting, after one year amounting to 10% of the baseline value before the nerve cutting. On the other hand, the reinnervated muscle is a factor that supports further regeneration of its motor neuron (18). The current standard management is to repair large nerve gaps with transplantation of sural nerve or cutaneous nerve...
of forearm (these are strictly sensory nerves). However, studies have demonstrated that the regenerative potential and efficacy in the prevention of the muscle atrophy with sensory nerves are markedly poorer than with nerves of motor nature (19).

Recently published studies involving “end-to-side” anastomosis in the nerve reconstruction demonstrated worse functional results of regeneration than with conventional “end-to-end” anastomosis (19, 20). In view of these data, we proposed repair of a nerve gap using “end-to-end” implantation of an epineural sheath conduit as a prophylactic method that prevents muscle atrophy. An epineural sheath that is devoid of blood vessels and nerve fibers, is non-immunogenic (21) and in the future could be used in the clinical setting in the allogenic human nerve model. An issue whether clinical results with epineural sheath conduit prove comparable to or better than results with currently used autologous transplantation of sensory nerves requires further studies.

In the 1990’s intensive studies were undertaken to develop grafts formed from synthetic materials or derivatives of natural products that could be an alternative to methods used in the nerve gap reconstruction. Reports were published, documenting use for this purpose of veins with similar diameter to that of the injured nerves, collagen, silicon or chitin tubes, absorbable biopolymer scaffolds and other substances of synthetic origin. The problems that the authors emphasized, included difficult access to natural materials and their immunogenic nature and requirement for immunosuppressive treatment in allogenic models, low durability of biodegradable materials and inflammatory reactions to synthetic grafts that inhibited the process of nerve regeneration (22-25). Therefore, in our study we used a material of natural origin, nonabsorbable and durable that, after preparation proposed by us, was used to repair a nerve gap which length made its primary approximation impossible. In our study we used a model of autologous nerve sheath, although we believe that epineural sheath conduits, devoid of blood vessels and nerve fibers, could be used in the future as potentially non-immunogenic materials, in allogenic grafts from the human tissue banks, without the requirement for immunosuppression.

In our study we used rats with developed type 2 diabetes mellitus, because regeneration of nerves in this metabolic disease is impaired. This results from existing organ and systemic complications of diabetes mellitus, such as peripheral neuropathy and ischemic microvascular angiopathy. Impaired blood flow in the regenerating nerves, tissue hypoxia as well as detrimental effects of hyperglycemia and insulin deficiency make a nerve repair in the setting of diabetes mellitus a surgical and diabetological challenge (26). One can expect that peripheral nerves affected by neuropathy are unsuitable as nerve grafts and as a consequence, may result in worse functional outcome of regeneration. We observed such process in our study, where 6 weeks after the surgical procedure, outcomes in the group of
transplantation of an autologous nerve were worse than in the group of epineural sheath conduit. This may result from the fact that a nerve harvested for the transplantation had been injured before the surgical procedure by diabetes-related mechanisms, so that after its implantation, further partial degeneration ensued (first 6 weeks after the surgical procedure) with abnormal involvement of macrophages (27), and only subsequent reorganization and remyelination of nerve fibers was found (weeks 6-12). In case of an empty epineural sheath conduit, organized ingrowth of axons was found from the very beginning of regeneration and axons reached the distal end of the nerve within approximately 3 weeks after the transplantation (rate of growth: 1 mm/day) (28), and subsequently their myelination occurred. Thus regeneration in week 6 was more advanced in this group than in the group of autologous transplantation of a neuropathic nerve. Until week 12 after the surgical procedure, the regeneration sped up, because it had larger number of supporting cells (Schwann cells) and higher concentration of growth factors. Therefore results of measurement of denervation atrophy in week 12 are similar in both study groups. Our study indicates that use of epineural sheath conduit for the repair of a nerve gap and in the prevention of atrophy of denervated muscles may be more effective than a nerve transplantation in diabetic patients, in particular with long-term complicated metabolic disorders related to prominent peripheral neuropathy. Our study is the first such study in the literature that assessed possibility of repair of a peripheral nerve gap and prevention of muscle atrophy caused by denervation in a model of type 2 diabetes mellitus.

Alternative methods of protection of denervated muscles and their stimulation include: motor rehabilitation, electrostimulation of muscles and nerve, supply of the muscle by another motor neuron (so called Babysitter procedure) and transplantsations of nervous ganglia in the region of atrophic muscle (14). The latter two methods have not been clinically adopted. Among the above listed methods, obviously rehabilitation through kinesitherapy is commonly accepted and used. However, its use does not preclude attempts undertaken to restore stimulation from the nervous system; on the contrary, both methods should be used simultaneously since they result in release of myo-derived growth factors for the regenerating nerve (3). Studies of electrical stimulation of denervated muscles with high intensity electrical current had preliminary promising effects in the prevention of atrophy and regeneration of muscle mass (approximately 25% increase of muscle mass after 1 year of stimulation; project RISE) (29, 30). However, this is a strictly symptomatic method, targeting only an effector of the neuro-motor pathway. Furthermore, safety of such stimulation in the clinical setting has not been fully elucidated. Our study suggests that it is possible to repair an injured diabetic nerve using a new method with an epineural sheath conduit, allowing for restoration of neuro-muscular stimulation, thus eliminating the cause that underlay the development of muscle atrophy.

CONCLUSIONS

This experimental study supports the possibility and effectiveness of use of an epineural sheath conduit in the surgical repair of a peripheral nerve gap in the diabetic settings. Study of atrophy in the diabetic model demonstrated that 12 weeks after the nerve reconstruction, denervation indices (GMI, area of muscle fibers) for the technique with epineural sheath conduit was comparable to indices of muscle atrophy found in the standard model of autologous nerve graft.

REFERENCES


