Advances in molecular diagnosis and therapy of muscle disorders

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Molecular diagnosis is a powerful tool to dissect clinical entities and might impact on therapy (in particular in dystrophinopathies). Diagnostic advances are prominent in Limb girdle muscular dystrophies (LGMD). The molecular diagnosis of LGMD is challenging because mutational analysis is cumbersome because of widespread mutations in a number of genes. LGMDs in fact include at least 14 different genetic entities with autosomal recessive inheritance (LGMD type 2) and 7 autosomal dominant forms (LGMD type 1). The latter account for less than 10% of LGMD, whereas recessive forms are the most common and include the most severe phenotypes; while LGMD2I is the most common form of all LGMDs in Northern Europe, LGMD2A is the most prevalent in southern Europe. In a cohort of over 500 cases we used a quantitative protein analysis in muscle and a subsequent gene mutation screening to diagnose LGMDs. We demonstrated that the likelihood of obtaining a molecular diagnosis is much higher when a protein defect has been found. About 60% of LGMDs were diagnosed, but when one defective protein was found we diagnosed 87% of cases. New treatments are being developed in the field of muscle disorders: a series of molecular treatments are going to be used in dystrophinopathies such as antisense oligonucleotides and PTC to skip null mutations. However so far only one successful new therapy has been in metabolic myopathies enzyme replacement therapy for glycogenosis type II, which is useful in infantile and in some adult cases. Molecular diagnosis and diet with bezafibrate therapy seem useful in myoglobinuric episodes from CPTII deficiency.

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Fibromyalgia and spasmophilia: two aspects of the same syndrome?

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The aim of our work is to compare the signs and symptoms and biochemical characteristics of fibromyalgia (FM) and spasmophilia (SP), because of the lack of information on this topic. Fibromyalgia syndrome (FM) is a chronic, generalized pain condition with characteristic tender points on physical examination, often accompanied by a number of associated symptoms such as fatigue, sleep disturbance, mood disorders and is often accompanied by a latent tetany also called spasmophilia. Spasmophilia is a series of symptoms characterized by painful muscle cramp that derives from enhanced neuromuscular excitability due to hypocalcemia, hypomagnesemia or alkalosis. Typical symptoms of tetany include carpopedal spasm, laryngospasm and generalized seizure. Many diseases including endocrine disorders like hypoparathyroidism and alkalosis by hyperventilation can cause spasmophilia. We compared 300 patients: 233 FM and 67 SP. Our SP patients resulted normocalcemic. Sex ratio (M/F) was different between FM and SP patients: 1/7 in SP patients, 1/9 in FM patients. SP patients showed the following concentrations significantly different comparing to FM patients: IL10 levels were higher, Mg concentrations lower, cortisol higher, growth hormone higher, tender points count lower. Psychiatric comorbidity was not different among these subgroups (panic disorder: 48% in SP vs 47.2% in FM), nor FIQ and HAQ. Moreover they had the same incidence of thyroiditis and the same positivity to surface electromyography (SEMG) test (62.5% SP vs 60% FM). In conclusion patients with SP were more tired, showed more muscle weakness than FM patients, and unexpectedly they did not have a more frequent incidence of panic disorder.

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Effect of GH/IGF-1 on neurogenesis and neuroregeneration processes

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Growth hormone (GH)/Insulin-like growth factor-I (IGF-I) axis is involved in the regulation of brain growth, development, and myelination. Plasticity in the central nervous system (CNS) is characterized by the functional interaction between neurons, astrocytes, and oligodendrocytes. The neuroprotective effects of GH and IGF-I have been demonstrated in different experimental models of CNS injury. IGF-I stimulates the progenitor cell proliferation and new neurons, oligodendrocytes, and blood vessels in the dentate gyrus of the hippocampus. IGF1 plays vital roles also in the myelin synthesis and finds application in the treatment of demyelinating diseases such as Multiple Sclerosis, a chronic disorder characterized by inflammation, demyelination, and axonal degeneration. Oligodendrocytes at the edges of demyelinated plaques display enhanced immunoreactivity for IGF-I, IGF-I receptors, IGFBP5s and -6. Because increased expression of IGFBP5s and -6 has been associated with impaired synthesis of myelin proteins in oligodendrocyte lineage cells, pharmacological approaches to reduce their expression might be useful for promoting remyelination of axons in MS lesions. IGF1 found a clinical application in the Alzheimer's dementia showing a protective effect of anti-inflammatory and cholesterol-lowering drugs gave way to clinical trials with these compounds. GH/IGF-1 presents a unique opportunity for therapeutic intervention in...
disorders of the motor neuron, such as the loss of spinal cord motor neurons in amyotrophic lateral sclerosis or the degeneration of spinal cord motor neuron axons in peripheral neuropathies. IGF-1 receptors are present in the spinal cord, like members of the neurotrophin receptor family. They are involved in brain function, such as learning and exercise, and in stress and ageing. GH is used in patients suffering various neurogenic diseases and dominates an important area of research.

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FES of denervated muscle: Up-date of a difficult-to-stimulate case of Conus-Cauda Syndrome

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A 41-year-old male patient (08Rise2-Friuli-01LS) had a spinal cord injury (fracture-dislocation D12-L1) after an accident at work (April 11, 2008). After 15 days from trauma he was discharged from Neurosurgery and he was admitted in our Spinal Unit. At the clinical examination he had a flaccid paraplegia, A at the ASIA Impairment Scale (Motor-sensory level D12). Somato-sensory evoked potentials and motor evoked potentials were silent. He began the rehabilitation program directed to autonomy in wheelchair locomotion and ADL. The physical rehabilitation was directed to the strengthening of upper limbs, trunk control and to preventing lower limb muscle atrophy with patterned Electrical Stimulation (quadiceps and tibialis anterior). Several different stimulation parameters (i.e., pulse width, train duration, between train intervals, method of application) were applied, but we obtained at best a weak muscle “fibrillation, not the usual sustained tetanic contraction achievable in ‘innervated SCI patients” using standard commercial electrical stimulators. Only visible tremor of the thigh muscles occurred. Meantime some reinnervation of leg muscles occurred, demonstrated by random spontaneous activity of foot muscles (more evident on the right side), but voluntary and electrical stimulation-induced muscle contractions did not appeared. From early March 2009 (ten months from trauma), the patient is following the Vienna Strategy for home-based Therapeutic Electrical Stimulation (TES) of denervated muscle. An early result of the muscle TES was the impressive reduction of the leg edema. At the beginning of the program we tested (1) O2 maximal consumption at the crank ergometer stress test with respiratory gases analyzer (VO2000, Medgraphics-USA); 2) quadriceps isometric torque during PES with isokinetic dynamometer (Biodex System 4); 3) using plicometry we calculated the thigh muscle area. After six month of training we repeated the same measures. Actually we verified an increase of the thigh muscle area (from 154.01 to 163.37 cm2), an increase of quadriceps thigh torque (from 3.5 to 6.8 Nm) and an increase of the aerobic performance at the stress test (from 15.4 to 17.5 ml/Kg/min). Supporting results of Functional Echo Myography demonstrate that all extensor leg muscles benefited of h-b FES training. Indeed, muscle thickness of quadriceps and tibialis anterior increased (Table) and twitch muscle contraction improved, but not normalized, confirming that extensor muscles are denervated. Furthermore, intramuscular blood perfusion substantially increased during and after 15 min of electrical stimulation. While at rest the perforating arteries are hardly identified, after stimulation blood perfusion of several of them appear in the ultra sonogram and displaying a high pulsatory perfusion. We believe that the FES training increases not only stimulated muscle mass and function indexes, but also physiological parameters and patient performance.

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Paraneoplastic myopathy and inflammatory myopathy: when autoimmunity meets tumor immunity

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Patients with idiopathic inflammatory myopathies (IIM) present an increased risk of developing malignancy (breast, ovarian, lung and colorectal tumors at most). The temporal relationship between malignancy and myositis can vary:
malignancy may occur before or concurrently (paraneoplastic myositis - PNM) or following the diagnosis of myositis (cancer-associated myositis - CAM). In PNM the immune mediated destruction of muscle may be a sort of paraneoplastic manifestation of the immune system’s response to the cancer. PNM may be associated with autoantigens histidyl-tRNA synthetase (Jo-1), SRP (signal recognition particle) and the 218 kDa helicase (Mi-2) which are expressed at very low levels in normal muscles, whereas they are found at high levels in autoimmune myositis muscle, particularly in regenerating muscle cells suggesting that tumor cells and regenerating cells are antigenically similar. Anti-SRP antibodies identify neoplastic patients with severe myopathy, poor response to treatment and histological findings of muscle cell necrosis, often lacking inflammatory infiltrates as in necrotizing myopathy one of the PNM subtypes in addition to dermatomyositis and polymyositis. Cancer-associated myositis (CAM) occurs years after myositis onset, suggesting that cytotoxic agents in the treatment of myositis may be implicated. Case report. We describe the case of a 56 years old lady who presented a cancer-associated myositis (colorectal cancer) two years after myositis onset and chronic immunosuppressive therapy with mofetil mafenolate and steroids. Patient presented a rapid clinical worsening with severe proximal limbs weakness, dysphagia and poor response to the therapy. She was positive for anti signal recognition particle (SRP), a myositis-specific autoantibody, usually associated with paraneoplastic inflammatory myopathies. *****

**Differential diagnosis between Facio-Scapulohumeral Dystrophy and Inflammatory/paraneoplastic Myositis**

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Facio-Scapulohumeral Dystrophy is the second most frequent muscular dystrophy in adults. Inherited with autosomal dominant transmission, it involves the facial muscles, the shoulder girdle and the pelvic girdle in the most severe cases. Histopathological changes in FSHD biopsies are not specific and include fiber atrophy, increasing of fiber size variability and chronic inflammatory response. Over 40% of biopsies show a mononucleated infiltrate very similar to the one observed in polymyositis. SRP (signal recognition particle) and the 218 kDa helicase (Mi-2) which are expressed at very low levels in normal muscles, whereas they are found at high levels in autoimmune myositis muscle, particularly in regenerating muscle cells suggesting that tumor cells and regenerating cells are antigenically similar. Anti-SRP antibodies identify neoplastic patients with severe myopathy, poor response to treatment and histological findings of muscle cell necrosis, often lacking inflammatory infiltrates as in necrotizing myopathy one of the PNM subtypes in addition to dermatomyositis and polymyositis. Cancer-associated myositis (CAM) occurs years after myositis onset, suggesting that cytotoxic agents in the treatment of myositis may be implicated. Case report. We describe the case of a 56 years old lady who presented a cancer-associated myositis (colorectal cancer) two years after myositis onset and chronic immunosuppressive therapy with mofetil mafenolate and steroids. Patient presented a rapid clinical worsening with severe proximal limbs weakness, dysphagia and poor response to the therapy. She was positive for anti signal recognition particle (SRP), a myositis-specific autoantibody, usually associated with paraneoplastic inflammatory myopathies. *****

**To mimic reinnervation of long term denervated muscle. Results of animal research**

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Long-lasting complete denervation of an anatomically defined muscle is the result of lower motor neuron or peripheral nerve lesions that have major consequences on the innervated muscle. One of the important conclusion of the fundamental studies published in the book “The Denervated Muscle”, edited by Ernest Gutmann in 1962 [16], is that after a certain period of time (6 months in the rat), denervated muscle undergoes some irreversible changes that inhibit its full restoration even after reinnervation. Works on a different experimental model, free autotransplantation of leg muscles, lead to the same conclusion: rat muscle denervated up to 2 months is restored as well as grafts of control muscles, but between 2 and 7 months of denervation, the restorative capacity decline continuously before leveling out at a very low level at 7 months of denervation [8]. These pioneering observations were translated to human muscle, without critical analysis of the biological difference between small rodents (mice is the extreme case) and large or very large mammals, humans included. On the other hands, loss of function, gross anatomy and clinical neurophysiology seems to suggest that behavior of denervated human muscle and his potential for reinnervation are similar to that observed in the rat, establishing a dogma that continues to influence clinical managements of muscle denervation/reinnervation. Contrary to general expectation (our own, included) our recent results show that what happens in months in rodents occurs in years in large mammals, humans included: the denervated muscle undergo early progressive atrophy, spontaneous activation (muscle fibrillation) and then un-excitability by surface electrical stimulation with standard commercial stimulators. After years of sever atrophy, the human muscle fibers finally disappear, substituted by adipose and fibrous connective tissues. We will describe the time course of human denervated muscle atrophy and their long-lasting ability to respond to home base electrical stimulation using custom-designed electrodes and stimulators developed in Vienna,
Austria [7,17-19] and new muscle monitoring methodology that we have develop to follow-up the difficult-to-measure changes in muscle function and structure [13,14,21,24]. These results of human trials are strongly supported by animal research in rat [2,8-13,22], rabbit [3-6], sheep [1,11,20] and pig [Winfried Mayr, personal communication]. All these results points to disuse and to deregulation of atrophy-hypertrophy-myogenesis factors to explain the early and mid term atrophy. The final tissue degeneration could be the result of additional late events: i) extensive duplications of the basal membrane of muscle capillary network in response to chronic ipoperfusion, stasis and ipoxia (in early denervation muscle perfusion at rest is increased in comparison to innervated resting muscle [16]), and ii) progressive perimisium and endomisium fibrosis [2,12,14,17], which are worsened by joint immobilization and related decrease of passive muscle stretching. They suggest also that the modulator effects of spontaneous, voluntary or electrical stimulation-induced muscle contractions on muscle mass, dynamic properties and fiber types are the result of how long last the induced changes (in particular in muscle perfusion), much more that the total daily activation or the extent of changes during contractions [1-6,8,13,20,22]. The new muscle monitoring methods we developed to follow-up human muscle function and structure [13,14,21,24] will translate these results to clinical studies. In long-lasting denervation, preventing muscle tissue degeneration by home based functional electrical stimulation may open new perspectives: a multi-task strategy may have a clinical impact on reinnervation potential of human denervated muscles, at least in the cases of nerve lesion far from the target muscle.

References


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Vibration therapy for denervated patients

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Vibratory stimulations were investigated in the past for their positive action in eliciting muscle activity. It is well known in fact, that localized vibrations applied to tendons produce a reflex muscle response induced by stretching action of the applied vibration, the tonic vibration reflex. Vibration therapy could be applied to maintain bone density [1,3], to hold size and internal structure of denervated muscles and eventually to improve muscle activity. Recently, literature has moved to the study of vibrations (applied locally or to the whole body) as a rehabilitation treatment in different central and peripheral neurological pathologies. Besides electrical stimulation the application of vibration has become an alternative and/or a complementary treatment method in neurological rehabilitation. The guiding idea is to apply vibration to generate involuntary muscular activation in order to achieve different outcomes. Nevertheless, vibration treatment could be also used to modify the release of neurotrophic factors. A rise of this release increases nerve growth potential and thereby accelerates rehabilitation in neuro-trauma [2]. Some recent studies found that release of these factors can be influenced by exercise. Since vibration applications are known to stimulate muscle spindles it is possible to speculate that this treatment could promote the release of those factors [2]. Further studies engaging patients are however necessary. As no negative effect on the nerve sprouting is then known, this therapy could be applied to neurological patients also during the first reinnervation phase.

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Calsequestrin-I: a new candidate gene for malignant hyperthermia (MH) and exertional/environmental heat stroke (EHS)

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Malignant hyperthermia (MH) and exertional/environmental heat stroke (EHS) in humans present as similar life threatening crises triggered by volatile anesthetics and strenuous exercise and/or high temperature, respectively. Many families (70-80%) diagnosed with MH susceptibility (MHS), and a few with EHS, are linked to mutations in the ryanodine receptor type-1 gene (RYR1), the Ca2+ release channel of the sarcoplasmic reticulum (SR) of skeletal muscle and key protein in excitation-contraction (EC) coupling. However, mutations in the RYR1 gene are not found in all MH families, suggesting that alternative genes remain to be identified. In our laboratory we have recently characterized a novel knockout model lacking skeletal muscle calsequestrin (CASQ1), a SR Ca2+-binding protein that modulates RYR1 function, and investigated whether these mice present a MH/EHS-like phenotype. Ablation of CASQ1 results in remodeling of the EC coupling apparatus and in functional changes, which in male mice causes a striking increase in the rate of spontaneous mortality and susceptibility to trigger MH-like lethal episodes in response to halothane- and heat-stress. The demonstration that ablation of CASQ1 results in MH- and EHS-like lethal episodes validates CASQ1 as a viable candidate gene for linkage analysis in MH and EHS families where mutations in RYR1 are excluded.

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Muscle modifications in fibromyalgic (FM) patients revealed by surface electromyography (SEMG)

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The aims of this study were to evaluate muscle modifications by SEMG analysis in FM women with respect to a sample of healthy controls and to investigate the relationships between SEMG parameters and the clinical aspects of the disease. SEMG was recorded in 100 FM women (48.10±11.96 yr) and in 50 healthy women (48.60±11.18 yr), from the tibialis anterior and the distal part of vastus medialis muscle during isometric contraction. Initial values and rate of change of median spectral frequency (MDF) and conduction velocity (CV) of the SEMG signal were calculated. The clinical parameters evaluated were: “Fibromyalgia Impact Questionnaire”, pain, tender points, and tiredness. MDF absolute values and the Fatigue Index (FI) were significantly lower (p<0.001) in both muscles studied in FM patients (MDF: 93.2 mV; FI: 1.10, 0.89) with respect to healthy controls (MDF: 138.2 mV; FI: 2.41, 1.66) and a smaller reduction in the percentage values of MDF was observed in FM patients vs controls (22% vs 38%). A significant correlation was found between the SEMG parameter decrement of normalized median frequency (MNF) (%) and seriousness of FM. We have found some interesting muscle modifications in FM patients with respect to healthy controls, regarding MDF, CV and FI values which resulted significantly lower in FM. Patients might have different fiber recruitment or a possible atrophy of type II fibers suggesting that they are not able to reach muscle relaxation.

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Regulation of Transient Receptor Potential Channel 1 (TRPC1) by sphingosine-1-phosphate in C2C12 myoblasts: relevance for a role of mechanotransduction in skeletal muscle differentiation

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C1-type transient receptor potential, TRPC1, channels provide cation and Ca2+ entry pathways, which have important regulatory roles in many cell processes. We provided the first experimental evidence that TRPC1, beside acting as a store-operated channel, represented an essential component of stretch-activated channels (SACs) in C2C12 skeletal myoblasts, as assayed by whole-cell patch-clamp and atomic force microscopy, AFM, pulling. The channel’s activity was potentiated by sphingosine-1-phosphate (S1P), a bioactive lipid involved in muscle biology. Such enhancement was paralleled by a TRPC1-lipid rafts association that was increased by stress fibre formation elicited by S1P and abolished by the treatment with the actin-disrupting dihydrocytochalasin-B, suggesting a role for cytoskeleton in TRPC1 membrane recruitment. Moreover, the increased expression of TRPC1 also improved the connexin 43 (Cx43) gap-junction expression and functionality as assayed by dual whole cell voltage patch-clamp in paired myoblasts. Consequently, TRPC1 channel activity is required for S1P-induced Cx43 expression in differentiating C2C12 myoblasts. Finally, the reduction of TRPC1 expression as well as of Ca2+ influx by Gd3+ strongly impair myoblasts differentiation denoting an important role of the Ca2+ influx trough TRPC1/SACs. From the biological point of view, the SACc function of TRPC1 was essential for C2C12 myogenesis and resulted to be up-regulated by S1P. Collectively, all these data suggest that modulation of TRPC1 expression and localization into lipid microdomains may represent critical mechanisms by which S1P exerts its pro-myogenic effect and offer new tools for experimental strategies of a stem cell transplantation therapy.

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Muscle tissue composition and macrostructural changes by 3D-Color Tomography for denervated muscles

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In order to study the changes in tissue composition on a larger muscle section, with the intent of supporting and validating histological analyses from minute tissue biopsies, non-invasive methods are developed to analyze macroscopic and microscopic structural changes of human skeletal muscle. The advantages of using such segmentation techniques and 3D modeling are mainly two: 1) the analysis of whole muscle; and 2) the potential to isolate single muscle bellies such as Rectus Femoris. Additionally, quantitative color 3D analysis is performed; it enables researchers to evaluate the specific tissue content of muscle structures [1,2]. This is extremely important because the ratio of muscle to adipose to connective tissues is an invaluable indicator of muscle health. Here, we apply color 3D CT imaging to the evaluation of FES treatment on normal and denervated muscles.

References


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**Paraneoplastic myopathies and muscle cachexia**

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Patients with cancer can have muscle involvement of several types. Most frequently observed is cancer cachexia, which associated with weight loss and type 2 fiber atrophy. Several paraneoplastic causes as dermatomyositis, much rarer polymyositis and necrotizing myopathy occur. Rarely hormonal changes as ectopic hormone production cause myopathy. Although muscle tissue is a large part of the body volume, skeletal muscles are rarely the site of metastasis. Also hematological diseases rarely affect muscle tissue, amyloidosis of muscle tissue can be observed in association with paraproteinemias. Also treatment can cause myopathies, most frequently by steroid treatment, but also taxanes can cause a proximal myopathy. The phenomenon of muscle wasting can be observed in starvation and associated with several severe diseases as sepsis, debilitating internal disease and AIDS and possibly common pathways exist. Several pathways as cytokines, and most recently myostatin have been suspected to play major factors. This is of particular interest, as the progression of cancer cachexia may be a bad prognostic factor in cancer survival. Several therapies as hypercaloric nutrition, glucocorticoids, prostaglandins drugs and antiserotonergic drugs have been used.

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**Nutritional influences on the hormonal responses to exercise and training**

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Skeletal muscle performance during a physical exercise session is influence by a variety of factors. Similarly, there are a variety of factors that influence the muscle adaptation process (e.g., "myoplasticity") to exercise training. Two critical, interacting and overlapping factors that have an influence on both these acute and chronic exposures to exercise are: 1) nutritional status and, 2) hormonal responses. This presentation will address aspects of how nutrition status such as dietary macronutrient intake-content, ingestion manipulations, and substrate availability can have direct and indirect effects on the hormonal responses of the endocrine system to physical exercise. Specific points to be addressed in the presentation will be the key roles protein and carbohydrate ingestion can play with respect to exercise for: 1) influencing those anabolic-androgenic and catabolic hormones involved within the protein synthesis and breakdown processes; 2) impacting on those hormonal elements involved in controlling substrate utilization; and 3) improving physical performance by skeletal muscle which can lead to enhanced sports performance in athletes.

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**A non homogeneous growth in muscle in electrical stimulation treatment**

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Three patients with lower motor neuron lesion in the lumbar region have been in electrical stimulation treatment for six years. They are treated according to a protocol developed in the European project RISE. Their thigh muscles where degenerating for 10 months (P1), 4 years (P2) and 7 years (P3) respectively before the treatment. This is due to the total inactivity following the flaccid paralysis and can end in a total loss of contractile elements in the muscle. Other consequences of a flaccid paralysis are thinner skin, lower capillary density and lower bone mineral density. It was shown in the RISE project that the muscle volume, the muscle tissue density and to a great extent the muscle force can be restored with electrical stimulation treatment. In this work the three patients have been differently therapy compliant. Two of them P1 and P3 have not been therapy compliant but P2 has been. Each thigh has been stimulated with electrodes 12 x 15 cm in size. In the sitting position, i.e. the hip and the knee joint are in 90 degree position, they cover most of the upper side of the thigh. They are placed with about 1 to 3 cm apart. The distal electrode is about one to five cm above the patella. The thigh muscles have been monitored throughout the treatment with spiral CT scans three times a year. These scans allow the measurement of volume, density and shape of the muscles. The results from measurements of P2 of the rectus femoris muscle, which is a bipennate muscle, having short muscle fibres going form a central tendon to a surrounding layer, show a very non homogeneous growth. The muscle fibres in the middle of the muscle have grown the most and increased their density. These are the muscle fibres below the slot between the two stimulating electrodes, the place where to expect the highest current density. The muscle fibres at the proximal and the distal end of the muscle has not grown and even lost density during the electrical stimulation treatment. This indicates that they have not been stimulated by the electrical current and have therefore not bee contracting. They have only been stretched passively by the other muscle fibres in the same muscle. This suggests that in order to grow the muscle fibre has to contract itself.

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Effect of passive movement on regeneration degeneration processes in the long-term denervated rat leg muscles

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Irreversibility of the late denervation atrophy still remains an open question. Recently, we found that a passive movement during the 2-month denervated rat leg muscles and attenuate some of the factors which were believed to be the reasons of atrophy irreversibility, as for example, an increase in the number of capillary blood vessels or muscle fiber nuclei. Morphology of the contractile apparatus was also improved and the amount of myosin heavy chains significantly increased after the training. In the soleus muscle, the concomitant decrease in the number of severely damaged muscle fibers and amount of collagen were observed, while in the extensor digitorum longus (EDL) muscle training caused an increase of damage within the contractile structure and a number of the degenerating fibers. In the present study 1-month locomotor training on a treadmill was applied, as previously, but muscles were denervated up to 3 months. We examined the following 3-month denervated soleus and EDL muscles: 1) in the second month trained, decapitated one month after training, 2) trained during the third month of atrophy, 3) untrained and 4) intact controls. In the 3-month-denervated muscles atrophy was more advanced and the number of degenerating and regenerating muscle fibres, adipocytes and collagen accumulation were increased as compared to the 2-month-denervated muscles. Positive effect of training during the third month after denervation was much lower than that during the second month. One month after training (performed during the second month), progress of atrophy was slightly slower than in the untrained muscle, and some positive effects of training were still visible; number of damaged fibres was kept at the same level as just after training. However, unfortunately, in the soleus an increase (no significant) of fat and collagen was observed. It has to be pointed out that also a positive effect was observed: in both muscles the number of myotubes / young fibres increased and was significantly higher than that of immediately after training as well as in the 3-month denervated untrained muscles. In conclusion, passive movement seems to attenuate some of the pathological processes within the denervated muscle and increases its regenerative possibility. However, danger of some damage and degeneration should be also taken into account.

Time course and dose-dependence of transcriptional changes in electrically stimulated skeletal muscle

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Electrical stimulation of muscle induces both quantitative and qualitative changes in protein synthesis. Quantitative changes result from global increases in transcriptional and translational activity. These increase the rate of protein synthesis [2] but also the rate of protein degradation, so that muscle mass may increase, remain stable, or even decrease. This response is mediated largely or entirely through pathways related to the generation of force [4]. Qualitative changes affect specific proteins and protein isoforms, resulting, for example, in an increase in mitochondrial enzymes and a decrease in enzymes of anaerobic glycolysis [3], and the sequential replacement of myosin heavy chains Types 2B and 2X by Types 2A and 1 [1]. They depend on the aggregate amount of impulse activity delivered to the muscle. There is a good understanding of these changes at the protein level, but much remains to be learned about the underlying signaling events. We have used Real-Time Quantitative PCR with custom-made primers to investigate, in a targeted way, specific pathways believed to be involved in protein synthesis, protein degradation, isoform expression, and mitochondrial biogenesis. Some results will be presented to show the time course of these changes and their dependence on the pattern of stimulation.

References

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MRI of the peripheral nerve network

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Clinical high field (3T) MR allows a detailed visualization of the peripheral nerve structure in vivo. The advanced MR imaging technique of diffusion tensor imaging (DTI) probes the diffusion of water molecules, thereby reflecting the structural geometry of different tissues. 3D models of
peripheral nerves can be created by postprocessing of the high resolution DTI data ("tractography"). In animal models DTI was shown to mirror axonal growth, well corresponding to functional regeneration after peripheral nerve damage. Furthermore it has been proposed, that it may indirectly quantify axonal functional integrity. In the clinical setting, DTI allows a 3D evaluation of the exact location and severity of pathological nerve involvement in a variety of disorders. In traumatic nerve lesions and nerve compression syndromes DTI offers a quantification of nerve damage and additionally depicts the structural continuity/discontinuity of the nerve at the site of the lesion. Preliminary results show a high anatomical correspondence of tractography data with the surgical anatomy in cases of peripheral nerve tumors. Using the high sensitivity in detecting acute and chronic muscle denervation, this method and other advanced MR imaging tools will further improve our understanding of human nerve de- and regeneration in vivo. Now ongoing clinical correlation has to validate animal data in order to prove, if advanced 3T MR can aid in the prediction of functional consequences of peripheral nerve damage.

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Rehabilitation strategies on denervated muscles in human
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After complete Spinal Cord Injury (SCI), causing complete disconnection between the muscle fibers and the nervous system, the denervated muscles become excitable with commercial electrical stimulators [1,2,4] within several months and undergo severe atrophy and disorganization of pathological nerve involvement in a variety of disorders. Using the high sensitivity in detecting acute and chronic muscle denervation, this method and other advanced MR imaging tools will further improve our understanding of human nerve de- and regeneration in vivo. Now ongoing clinical correlation has to validate animal data in order to prove, if advanced 3T MR can aid in the prediction of functional consequences of peripheral nerve damage.

indicating that the shorter the time span between SCI and the beginning of h-b FES, the larger were the number and the size of recovered fibers. The study demonstrates that h-b FES of permanent LMN denervated muscle is an effective home therapy that results in rescue of muscle mass, function and perfusion. Additional important benefits for the patients are the improved cosmetic appearance of lower extremities and the enhanced cushioning effect for seating. The biological and clinical values of these findings are strengthened by recent results obtained from animal studies [2,3]. Concluding can be said that prevention of tissue degeneration in long-lasting denervation will open new perspectives for reinervation of human long-term LMN denervated muscles.

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**Myofiber regeneration in denervated human muscle up to 3.5 years after post-traumatic free flap reconstruction**

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We here report two clinical cases of muscle free flap for repair of a post-traumatic soft tissue loss. A 51 years old man was treated in 2007 with a gracilis muscle free flap for repair of a post-traumatic soft tissue loss at the sole and lateral side of the left foot, with calcaneum fracture and exposure. The gracilis muscle main vascular pedicle was anastomised with the anterior tibial vessels, after 2 years to the Clinic for a reshaping of the flap, which was otherwise totally satisfying. During the operation, a muscle biopsy was collected and snap-frozen with liquid nitrogen. A co-existence of fibro-fatty areas with isles of muscular tissue is evident in the H&E stained sections. By morphometry analyses the muscle fibers have an average minimum diameter of 26.9±11.4 µm, a value that is not generally expected in a two-year denervated muscle. Furthermore, the ATPase histochemical staining for pH 4.35 (slow type fibers) and pH 9.4 (fast type fibers) demonstrate the presence of both types of myofibers is confirmed also by positivity of the mitochondrial SDH histochemical reaction. MH Cemb-positive fibers are demonstrated by the specific monoclonal antibody. The observation of muscle regeneration in this likely-traumatic denervated muscle is in full agreement with the results described in paretic muscles after complete spinal cord injury involving the lower motor neurons. The presented cases are further evidence of the long term viability of denervated myofibers, to which myofiber regeneration seems to provide an important contribution. Indeed they are in full agreement with the results of the EU Project RISE: Use of electrical stimulation to restore standing in paraplegics with long-term denervated degenerated muscles (Contract No: QLG5-CT-2001-02191) demonstrating in a cohort of long-term Conus Cauda cases that: 1. denervated muscle fibers maintain excitability-contractility much longer (in tens of months) than expected; 2. denervated muscle fibers survive much longer (in years) than expected; 3. denervated muscle tissue shows sustained regenerative myofiber events, maintaining the pool of myogenic stem cells much longer (in tens of years) than generally expected.

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**“RISE” to “Reinnervation after peripheral nerve lesions”**

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This contribution is intended to foster a coordinating discussion within the Autumn Padova Muscle Days 2009 for a project application to the actual FP7 Health call. This text is extracted from draft material in preparation of the proposal and was contributed by members of the application group. Peripheral nerve lesions are frequent and generate significant deficits. The peripheral nervous system has the capacity to regenerate, but despite optimal surgical care and adequate postoperative therapy very often sensorimotor recovery is neither satisfying nor well predictable. Limiting factors are, slow axonal regeneration across the injury site, progressive decline in the regenerative capacity of axotomized neurons, progressive failure of reactive Schwann cells to provide trophic support, loss of endplate morphology, degeneration of muscle fibres and central nervous system neglect. When the nerve to a muscle is damaged the muscle undergoes severe atrophy, with a reduction of up to 80% in cross-sectional area. In the absence of reinnervation the fibres degenerate after 12-18 months and are replaced progressively and permanently by fibrofatty tissue. In the case of traumatic injury to a peripheral nerve, surgical repair is feasible. However, in large mammals nerve regrowth may take many months, during which time the condition of the muscle declines seriously, so the therapeutic result is far from optimal. The concept of the proposed project is based on recent research work by members of the consortium in the field of (1) surgical nerve reconstruction (e.g. end-to-side anastomosis leading to better nerve regeneration) and (2) Functional Electrical Stimulation (FES) of denervated muscle (European project RISE, maintenance and training of denervated muscles demonstrated in principle). Consequently, the outcome of these recent findings shall be a translational effort for a step forward in treatment of peripheral nerve lesions focusing on (1) improved treatment of the nerve lesion.
Autophagy is required to maintain muscle mass

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The ubiquitin-proteasome and autophagy-lysosome pathways are the two major routes for protein and organelle clearance in eukaryotic cells. In skeletal muscle both systems are under Akt-FoxO regulation and their excessive activation induces severe muscle loss 2-4. Conversely altered autophagy has been observed in various myopathies with accumulation of inclusions and vacuoles5. However the role of autophagy in skeletal muscle has not been determined by specific loss-of-function approaches. Here we specifically deleted Atg7 gene in skeletal muscle and we analyzed the contribution of autophagy to homeostasis of organelle and proteins and its role in muscle wasting. Genetic ablation of Atg7 resulted in profound muscle atrophy because of increased expression of atrophy-related genes. Physiological studies revealed an important decrease in absolute and specific force which is age-dependent. Morphological analysis showed accumulation of abnormal mitochondria, sarcoplasmic reticulum distension, disorganization of sarcomere and formation of aberrant concentric membranous structure. Autophagy inhibition exacerbated muscle loss during denervation and fasting and induced sarcocellular instability which resulted in myofiber death. Thus maintenance of autophagy flux is important to preserve muscle mass and to maintain myofiber integrity. Our results suggest that inhibition or alteration of autophagy can contribute to muscle degeneration in some muscular dystrophies characterized by accumulation of abnormal mitochondria and inclusions bodies.

 Muscle involvement and IGF-1 signaling in genetic disorders: new therapeutic approaches

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The prolongation of skeletal muscle strength in aging and neuromuscular disease has been the objective of numerous studies employing a variety of approaches. In the last decade, dramatic progress has been made in elucidating the molecular defects underlying a number of muscle diseases. With the characterization of mutations responsible for muscle dysfunction in several inherited pathologies, and the identification of novel signaling pathways, subtle alterations in which can lead to significant defects in muscle metabolism, the field is poised to devise successful strategies for treatment of this debilitating and often fatal group of human ailments. Among growth factors, the insulin-like growth factors 1 (IGF-1) has been implicated in many anabolic pathways in skeletal muscle, where it plays a central role during muscle regeneration and it has been considered a promising therapeutic agent in staving off advancing muscle weakness during ageing and in several muscle diseases. Here we discuss the roles of IGF-1 isoforms in myogenesis and the potential therapeutic role of local IGF-1 isoforms on muscle aging and diseases.

Mitochondria calcium signaling in cell life and death


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Mitochondria rapidly accumulate Ca2+ through a low-affinity uptake system (the mitochondrial Ca2+ uniporter, MCU) because they are exposed to high [Ca2+]i microdomains generated by the opening of ER Ca2+ channels. These rapid [Ca2+] changes stimulate Ca2+-sensitive dehydrogenases of the mitochondrial matrix, and hence rapidly upregulate ATP production in stimulated cells. Ca2+ also sensitizes to cell death mediators, e.g. ceramide. Accordingly, we demonstrated that Bcl-2 reduces the state of filling of ER Ca2+ stores, and this alteration is effective in reducing the sensitivity to apoptotic challenges. I will here review our latest data focusing on: 1) The effect on mitochondrial Ca2+ homeostasis of other signaling pathways involved in autophagy and apoptosis (Akt, FHIT). 2) The signaling route that links oxidative stress to the activation of p66shc, an isoform of a growth factor adapter acting as apoptotic inducer. PKCβ, activated by the oxidative challenge, induces p66shc phosphorylation, with ensuing alteration of mitochondrial structure and function. We also showed that this route is involved also in adipose differentiation of muscle-derived precursors, highlighting a novel process of utmost interest in pathophysiological conditions. 3) The molecular elements of the mitochondria-ER Ca2+ connection.
functions of VDAC isoforms in autophagy and apoptosis. I will discuss the role of VDAC in rapidly channeling Ca\(^{2+}\) through the outer mitochondrial membrane and the specific functions of VDAC isoforms in autophagy and apoptosis.

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How aerobic training can modulate exercise-related oxidative stress in mitochondrial myopathies

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Mitochondrial myopathies represent a wide group of disorders due to a biochemical defect of mitochondrial respiratory chain that results in reduction of energy cell production and excess reactive oxygen species generation, with consequent oxidative stress and cell damage. Aerobic training has been showed to increase the muscle performance in patients with mitochondrial myopathies. Aim of our study has been to evaluate, in patients affected by mitochondrial disease, concomitantly to lactate exercise curve, the occurrence of oxidative stress, as indicated by circulating levels of lipoperoxides, in rest condition and as effect of exercise, and also, to verify if an aerobic training program was able to modify, in these patients, ox-redox balance efficiency. At rest and before training blood level of lipoperoxides was higher compared to controls (P<0.05). During exercise blood level of lipoperoxides did not increase, but maintained significantly higher compared to controls. After an aerobic training of 10 weeks the blood level of lipoperoxides decreased by 13.7% at rest (P<0.01) and 10.4%, 8.6% and 8.5% respectively at the corresponding times during the exercise test (P=0.06). These data indicate that, in mitochondrial patients, oxidative stress occurs and that aerobic training partially reverts this condition.

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Fatal multisystem failure in Danon disease

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Danon disease, an X-linked dominant disorder, results from mutations in the lysosome-associated membrane protein-2 (LAMP2) gene and presents with hypertrophic cardiomyopathy, skeletal myopathy, and mental retardation. We identified three novel families (including one affected mother) with unreported LAMP2 gene null mutations. Clinically, males were more severely affected and had a variable combination of cardiomyopathy, skeletal myopathy, hepato-pancreatic disease, retinopathy and mental retardation. Three out of four patients suffered from transient ischemic attacks or embolic strokes, leading to death or severe disability. LAMP-2 protein deficiency was detectable in various tissues, including muscle, fibroblasts and leukocytes, explaining the multisystem clinical involvement. Skeletal muscle immunopathology showed extensive autophagic build-up and vacuolar membranes expressed sarcosomal-specific proteins. The degree of muscle fiber vacuolization correlated with clinical muscle involvement. In our female patient, muscle histopathology and LAMP-2 protein analysis was inconclusive, indicating that diagnosis in females requires mutation identification. The random X-chromosome inactivation found in muscle and leukocytes excluded the possibility that selective involvement of some tissues in females is due to skewed X-chromosome inactivation. Therefore, biochemical analysis of leukocytes might be used for screening in male patients, but genetic screening is required in females.

Influence of diabetic polyneuropathy in muscle activation during gait

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Diabetic sensorimotor polyneuropathy (DSP) is a long-term diabetic complication, involved in the pathogenesis of the diabetic foot, which is a major cause of morbidity and mortality. The study aims to investigate the effects of diabetic polyneuropathy on muscle activation during gait throughout surface electromyography (SEMG) analysis. So far SEMG of 28 subjects during gait have been acquired: 10 control subjects, 37 diabetics (20 DSP and 17 without DSP=NODSP). The subjects underwent clinical and instrumental analysis to assess DSP, postural and morphological global exam, gait analysis and SEMG. Six cameras BTS motion capture system synchronized with a SEMG system (Pocket Emg, BTS) and 2 Bertec force plates were used. SEMG data of the rectus femoris, tibialis anterior, gastrocnemius mediais, peroneus longus, extensor digitorum and gastrocnemius medialis were collected. The signal was band pass filtered between 10 and 450 Hz with a Butterworth filter and full wave rectified. The rms was computed with a moving window of 50 ms and the signal was normalized to the mean in the gait cycle, integral and linear envelope were also determined. The SEMG data were analyzed relatively to other gait parameters (stride period, stride length, gait). In order to compare the different populations 1-way-ANOVA was adopted. Gastrocnemius, rectus and peroneus longus revealed delay or not activation in timing of their EMG activity with respect to the gait cycle phase both in NODSP and DSP subjects. In extensor digitorum, peroneus longus and tibialis anterior prolonged activation timing was revealed. These preliminary results show an altered SEMG pattern both in DSP and NODSP which should be considered when planning prevention protocols.

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Functional Echo Myography (Ultra Sonography) of denervated/reinnervating muscle

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In this study we followed with ultrasound patients with permanent denervation to evaluate changes in morphology, thickness, contraction and vascularisation of muscles undergoing the electric stimulation program of the Rise 2 Italy project. During a period of 1 year for the first subject and 6 month for the second subject we studied the denervated muscle with ultrasound comparing it (when possible) to the contralateral normal one. We evaluated: i) Changes in morphology and sonographic structure of muscle; ii) Muscle thickness in response to the electrical stimulation therapy; iii) Short-term modifications in muscle perfusion characteristics and patterns of arterial vascularisation after electrical stimulation; iv) Contraction-relaxation kinetic induced by electrical stimulation. Morphology and ultrasonographic structure of the denervated muscles changed during the period of stimulation from a pattern typical of complete denervation-induced muscle atrophy to a pattern which might be considered “normal” when detected in an old patient. Thickness improved significantly more in the middle third than in the proximal and distal third of the denervated muscle, reaching in the middle third of the first subject approximately the same thickness as the contralateral innervated muscle (see also Bizzarrini et al. [1]). In the chronically denervated muscle the ultrasonographic analysis has the potential to record twitch contractions that are not recognized by palpation and visual inspection. Contraction-relaxation kinetic, measured by recording the muscular movements during electrical stimulation, showed an abnormal behavior of the chronically denervated muscle during the relaxation phase, which resulted to be significantly longer than in normal muscle (880 msec in the denervated muscle vs 240 msec in the contralateral innervated TA). In all the long-term denervated muscles analyzed with Echo Doppler for this study, at rest the arterial flow showed a low-resistance pattern, and a more pulsed one during and after electrical stimulation. This second result is more similar to the trifasic high-resistance pattern of normal muscle, and is a reliable pathognomonic sign of chronic muscle denervation. Furthermore, the electrical stimulation-induced hyperemia lasts longer than the stimulation period. The very high energy of the delivered electrical stimuli of the Vienna home-based Functional Electrical Stimulation strategy (h-b FES) needed to stimulate the denervated muscles demonstrated that the explored muscles were still almost completely denervated. In conclusion, this pilot study confirms the usefulness of Functional Echo Myography in the follow-up and the positive effects of h-b FES of denervated-reinnervating muscles.


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Mass spectrometry and tissue biopsies in biomedical science/applications

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Nowadays mass spectrometry is one of the most interesting analytical techniques in biomedical world, being able to give important information of the proteomics and metabolomics of living organisms. This has been due to the developments of new instrumental approaches, mainly matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), whose validity has been recognized by the scientific community with the assignment in 2002 of Nobel Prize for Chemistry to Koichi Tanaka and John B. Fenn for “their development of soft ionization methods for mass spectrometric analyses of biological macromolecules”. These approaches have been addressed mainly in two directions: i) research of marker of disease, i.e. of molecules over- or under-expressed in presence of pathological states; ii) ion imaging experiments, devoted to tissue mapping of endogenous or exogenous molecules of interest. After a first optimistic period, in which all seemed to be easy, these analytical approaches have been critically evaluated and now the most of the scientists working in the field are conscious of the pros and cons of the methods. In this presentation some applications of mass spectrometry in points i) and ii) will be given, emphasizing either the instrumental approaches or their possible limits.

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Subclinical non-inflammatory myopathy in rectus abdominis muscle biopsy harvested during diagnostic laparoscopy for colorectal cancer

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We evaluated skeletal muscle biopsies from asymptomatic patients affected with early stage colorectal cancer, in order to identify pathologic features which may be indicative of tumor associated muscle disorders. Morphometry, histochemistry, and immunohistochemistry with anti-NCAM and anti-MHCemb antibodies were performed on biopsies of the rectus abdominis muscle obtained from 10 patients during...
laparoscopic tumor resection and 10 healthy subjects as controls [1]. In patients’ biopsies, we observed a surprising high percentage of myofibers with internal nuclei compared to controls (9.15±0.9 vs 0.19±0.37, mean±SD p<0.001), together with 18.6% of muscle atrophy compared to controls (p<0.001). In the 30% of patients, myofibers expressing MHCemb have been identified (0.4±0.5 fibers/mm², mean±SD), while in the 50% of patients NCAM positive fibers have also been detected (0.7±1.1 fibers/mm², mean±SD) suggesting that investigated muscle biopsies exhibit evidence of muscle injury/regeneration and/or denervation, but not inflammation [2]. In control biopsies none MHCemb and only one NCAM positive muscle fibers have been detected. These findings indicate that patients’ biopsies display early signs of myopathy. Follow-up studies of this patients’ cohort will elucidate the clinical relevance of our observation, and further analyses investigating the molecular mechanism underlying this early cancer-associated myopathy, will hopefully provide some pathogenetic clues leading to the identification of potential therapeutic targets to prevent tumor cachexia.

References


New 3D Hyaluronan vased scaffold for in vitro reconstruction of the rat sciatic nerve

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For peripheral nerve regeneration, three-dimensional distribution and growth of cells within the porous scaffold are of clinical significance. The purpose of this study was to test in vitro a novel hyaluronic acid (HA) based tubular conduit (HYAFF-11™ biomaterials –1 x 10 mm) as nerve guide. At confluence, human fibroblasts, RN22 Schwann cell line, human umbilical vein endothelial cells (HUVEC), and primary nerve cells, obtained from neonatal rat sciatic nerve were harvested and seeded at density 3x10⁵ cells/cm² on HYAFF-11™ devices. Histological (haematoxylin-eosin) and immunohistochemical (antibodies to S-100, CD 31 and von Willebrand factor) analyses were performed after 7 and 14 days from cell seeding onto biomaterials to study the morphology of the constructs and the behavior of cells. MTT-based (thiazolyl blue) cytotoxicity test was performed to observe the biocompatibility of the cells cultured within the biomaterial devices. We subjected the conduits to an in vitro fibroblast cytotoxicity test and concluded that the conduits were not cytotoxic. We demonstrate that cultured RN22 Schwann cells and rat Schwann cells grow in vitro on new artificial nerve conduits, concluding that HYAFF-11™ conduit is a suitable biomaterial that can support in vitro nerve cell growth and, after 14 days of cultivation, remained circular with a round lumen, maintaining the size and shape of its original architecture. Finally, attachment and proliferation of endothelial cell attested the feasibility to develop a co-culture system to favor in vivo integration of a microvascularized nerve substitute.