

RESTORING THE CARDIAC CONTRACTILE FUNCTION USING IMPLANTS WITH NEURAL BUNDLES IN THE SINOATRIAL NODE OF THE HEART IN DILATED CARDIOMYOPATHY AT DOGS

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Abstract: The survey deals with a diagnostic method of dilated cardiomyopathy in dogs, related to a new method of treatment. Dilated cardiomyopathy is the main disease-causing death in large dog breeds, having genetic determinism and palliative methods of treatment. The disease consists in the dilation of the cardiac muscle and the progressive decreasing of the heart contraction strength. The hypothesis of the treatment, which offers hope to be revolutionary, is based on the histological similarity between nodal cells and neurons from the brain. The treatment is in a theoretical phase and consists in using culture neurons in order to supply the contractil function and the cardiac automatism.

Introduction:

Dilated cardiomyopathy is a progressive¹ disease of the myocardium, the dilatation initially concerning the left ventricle², but it extends to all cardiac cavities, being accompanied by alterations of ventricular kinetics and arrhythmias³. Dilated cardiomyopathy is the most common of chronic cardiomyopathies¹. It is demonstrated the predisposition of large dog breeds towards dilated cardiomyopathy (Doberman, Pincher, Great Dane, Cane Corso, Presa Canario, Bulldog American, Leonberg, Bullmastiff, Mastiff Tibetan, Schnautzer²⁶, Irish wolfhound¹⁹) and the genetic transmission of this disease⁴.

Currently applied treatment regimens are symptomatic of secondary heart failure and arrhythmias, as an adjuvant or in a prophylactic way⁶. There is no form of treatment for *in situ* changes⁵.

The purpose of this article is to present a new concept for the treatment of dilated cardiomyopathy, dealing with the contractile dysfunction of the heart⁶. Developing and testing this hypothesis raise tremendous possibilities for the future, including the adaptation to other cardiac diseases or human

medicine. In order to be able to compensate for the contractile function, it is necessary to thoroughly study the structure that naturally deals with this - the nodal system. Cardiac tissue regeneration attempts using human induced pluripotent stem cell (hiPSC)¹⁸ or cellular cardiomyoplasty¹⁵ using stem cells residing in the heart are known.

The study presents current attempts to restore the cardiac contractile function⁶ and brings to light a new hypothesis: the use of culture neuronal cells⁷ to supplement the nodal system⁵ and stem cells to replace the decompensated myocardiocytes¹ in the cardiac dilatation process.

The treatment hypothesis is based on the histological resemblance⁸ of nodal cells to neurons in the encephalus⁹. The method of treatment is in an early phase, the theoretical one, and cumulates aspects and hypotheses of existing studies to establish the most effective technique in restoring the cardiac contractile function in dogs affected by dilated cardiomyopathy.

Materials and Methods

The treatment consists in the use of culture neurons⁵⁴ to compensate the contractile

function⁶, the cardiac automatism¹⁰ and autologous stem cells by creating patient-specific hiPSC¹⁸ systems. The clinical translation¹⁵ requires the isolation of stem cells from surgical or biopsy samples¹¹, ex vivo expansion, and relocation¹⁹ in the affected heart.

1) Stem cells - the highest rate of success was achieved in the monostatum approach using Roswell Basal Medium (RPMI), ascorbic acid, recombinant human albumin and Wnt pathway inhibitors¹⁸. After obtaining functional myocardiocytes *in vitro*¹⁹, from stem cells, relocation to the left ventricular myocardium can be achieved by ecoguided puncture²² through the transverse wall of the ribs¹⁴.

2) Cultures of neuronal cells - maintenance on modified polypeptide media (PNW) in microfluidic system¹⁵. They will be transplanted by injection³³ into the sinoatrial node.

3) The recipient organism of the two cell types, respectively the dog with dilated cardiomyopathy²⁴, requires preoperative preparation¹² and complex induced immunosuppression¹⁵, to avoid the immune reaction of graft rejection³⁴.

It is necessary to use the flow cytometry for sorting cells according to the markers expressed on the surface⁴⁵, the selection of neurons⁵⁴ which have the capacity to bind to nodal cells and myocardiocytes multiplied *in vitro*³⁵, with the highest cardiac regeneration potential³⁶.

The biopsy puncture has made numerous contributions¹² to the knowledge of the structural aspects of myocardial fibers in chronic cardiomyopathies¹⁴. It was possible to establish the localization, nature and dynamics of structural and ultrastructural changes¹⁴ in chronic cardiomyopathies and the correlation with the clinical signs¹⁶. The study proposes using the same type of puncture in the sense of introducing reconstructive elements to the myocardium.

Justification of the theoretical concept

Generally, the pathology of the heart in veterinary medicine has a high frequency and involves major therapeutic problems. Cardiospheres¹⁵ or stem cells obtained

directly from the heart of the patient provide a potential source for myocardial regeneration therapy, lowering the degree of immunosuppression required for the intervention³⁶.

The cardiac dilatation process leads to overcoming the adaptive functions of the myocardium⁶. Its response consists in the elongation of supra modum myocardiocytes⁷ to functional disability or destruction¹³. It is necessary to replace them and ensure a rate of gradual replacement⁸ of the destroyed ones, by the hiPSC systems⁴⁴.

These can be adapted on the basis of cardiospheres⁴⁰ to increase the compatibility with the myocardium at implantation¹⁷ and the rate of transformation of these stem cells into adult myocardiocytes.

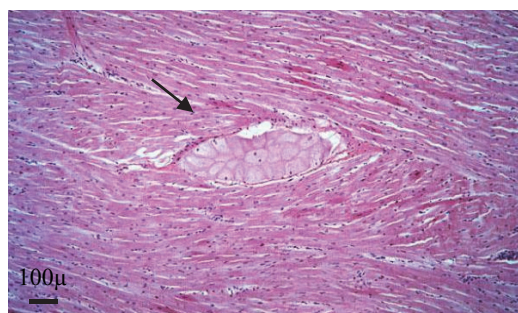
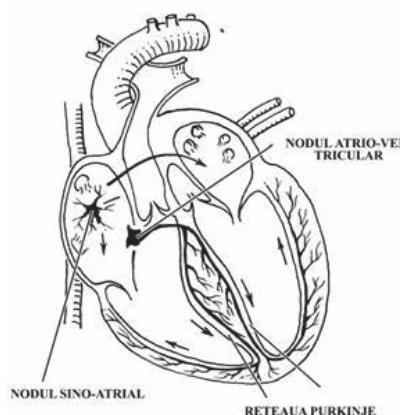


Figure 1 | The histostructure of the nodal tissue vs. Organizing the nodal tissue in the heart (scheme). The HE colour was used for the preparation, the cells are larger than the myocardiocytes, and they are slightly coloured. Objective View 60X.

For the restoration of the nodal tissue, a cell with functional capabilities and a nodal cell-like structure is required. The neuron is, in turn, a cell with electric potential²¹ and its adaptability to the surface markers²⁷ of other cells is known⁵⁴. These aspects support the study hypothesis and make the neuron the perfect candidate for restoring the cardiac contraction function.

The nodal tissue (or the excito-conductive system of the heart) is considered to be part of the myocardium¹, although the nodal cells are very different from the myocardial muscle fiber²; the nodal tissue is characterized by the preservation of the embryonic self-contractile properties²⁷.

The excito-conductive system of the heart is a complex of highly specialized cells³ with the role of generating rhythmic excitations and of leading them into the myocardium, providing rhythmical and continuous contraction of the heart⁴.

It consists of a dispersed portion - the Purkinje network¹ represented in the form of a fine subendocardial network and a dense voluminous portion². The agglomerations form the sinoatrial node⁴, located in the wall of the right atrium and the atrioventricular node, located at the base of the interatrial septum⁵. The atrioventricular node continues, without a precise demarcation, with the atrioventricular bundle.

The nodal tissue consists of 3 cell types: P or the pacemaker – it produces the electric impulse, T – it transmits and propagates the pulse produced by the P cell and the Purkinje cell – it has the role of stopping the access of premature or ectopic electrical impulses.

P cells show branches of the cell membrane and form anastomoses with adjacent cells⁷, making electron exchanges at this level (Ca^{2+} , Na^{+} , K^{+}). The potential for action spreads from one myocardial cell to the other through intercalated discs⁴.

A particular feature of the cardiac muscle excitability is the long duration of action potency of 100-250 msec²¹, 100 times more than the potential of the skeletal muscle, as it is determined by the change in the membrane permeability for potassium, sodium and calcium ions⁸. The duration of a contraction is almost equal to the duration of the action

potency¹², thus equal to the duration of the refractory period³².

Due to the long refractory period, the cord cannot sustain a continuous contraction and prevents the fusion and summation of the cardiac contractions (phenomena encountered in the skeletal muscle)²⁷. This provides the time to relax the myocardium²³ and to refill the cavities with blood, between systole⁶.

The existence of the refractory period, also known as the "law of periodic inexcitability of the heart," explains the impossibility of tetanus occurring in the myocardium. The cardiac muscle does not respond to stimuli during the absolute refractory period.

If stimulus is applied during the period of excitability⁴, before the physiological nodal stimulus, they can cause premature depolarization of the heart muscle¹⁷, with interruption of the heart relaxation and the appearance of a systole outside the normal rhythm - an extrasystole⁶.

This prevents, through its own refractory period³, the cardiac response to the next sinus stimulus, which is why the extrasystole is followed by a prolonged rest, the myocardium contracting to the next sinus stimulus⁴. The potential for action generated by the cells of the sinoatrial node propagates in the atrial mass at a speed rate of 0.3-0.5m/sec³².

Atrioventricular node cells²² represent the only functional link between atria and ventricles²⁷. They are separated by a layer of connective tissue that does not produce or drive potential action⁵, so an insulator²⁵.

The speed rate of propagation of action potentials through the atrioventricular node is only 0.02-0.05 m / sec³². The reduced speed ensures the temporal gap between the atrial and ventricular contractions⁵, respectively the end of the atrial systole before the ventricular systole begins⁶. The cellular cardiomyoplasty or the grafting of cardiomyocytes¹⁵ is an attempt to regenerate the affected myocardium³³, bringing a new hypothesis.

The mammalian cord is in a continuous state of cellular fluctuation³⁴ and has intrinsic regenerative potential, but it is limited²³. In adulthood, the myocardium also stores small populations of multipotent cardiac-progenitor cells³⁵, identified by surface expression of the

receptor for stem cell factor (c-kit), P-glycoprotein, and the antigen stem cells 1 (Sca-1)³⁵. Cardiac stem cells were identified using flow cytometry³⁶. Cells expressing Sca-1 and not expressing CD31 had the greatest potential for differentiation³⁷.

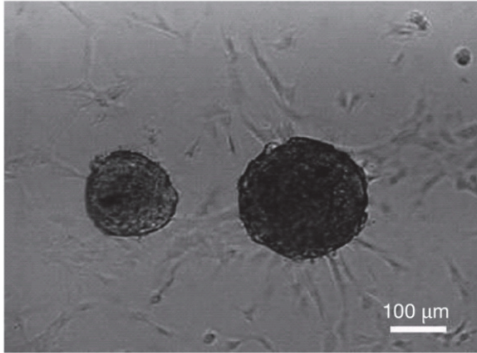


Figure 2 | Morphology of human cardiospheres. Isolated by myocardial biopsy and grown *in vitro*. The cardiospheres in the image have 5 days after harvesting and form an adherent monolayer.

A distinct population of cardiac stem cells was isolated in the adult rat heart. These primitive cells do not express markers for blood lines and express the c-kit³⁹. They are renewed, multipotent by cloning⁴⁰, and have the ability to generate myocytes, smooth muscle cells and endothelial cells *in vitro* (in the presence of 10% fetal calf serum and dexamethasone) and *in vivo*¹⁵. They were injected into the border area 5 hours after the induction of myocardial infarction in adult rats¹⁵, and they induced the formation of myocytes, capillaries and arterioles, in the infarct area³⁵.

The improvement of the cardiac dysfunction was found 5 weeks after the infarction¹⁵. A Sca-1¹⁵-like protein was detected by confocal microscopy and Western blot tests in human and dog myocytes³⁶. Like Sca-1, it belongs to the Ly6 family of surface antigens and it is still unraveled¹⁵. A population of cardiac stem cells having the Isl-1 transcription factor¹⁵ has been described; they have shown an extremely efficient conversion to adult myocytes in the absence of cell fusion⁴⁰. However, up to this

point, they have been detected only in neonatal samples in humans and rats¹⁵. Residual cardiac stem cells are involved in the slow replacement of myocytes⁷, replacing cells lost occasionally⁸ but inaccessible to the rapid regeneration of a large number of cells in response to lesions such as myocardial infarction¹⁵.

In addition, bone marrow stem cells have been found to undergo a process of mobilization towards the cord after the myocardial infarction³³. However, in myocardial damage, the lymphoproliferative response produces a fibrous tissue.

The isolated cells form groups called cardiospheres¹⁵, in the suspensions of cultures. They mimic the biological situation *in vivo* when incorporated in a three-dimensional environment⁴⁰, they develop upper-order intercellular structures facilitating proliferation, differentiation and angiogenesis¹⁵. The cardiospheres extended as adherent monolayer¹⁵ were transplanted to mice with severe induced immunodeficiency in the pre-infarcted area⁴¹.

The replacement of the necrotic tissue with neofunction myocytes and the functional improvement of the myocardium occurred within 18-20 days¹⁵.

There are attempts, quoted in literature (Chen I. Yan, 2016), regarding the use of pluripotent stem cells in cardiovascular disease called hiPSC (human induced pluripotent stem cell)¹⁸.

The possibilities were unlimited, the cells were reprogrammed and differentiated into several types of cardiovascular cells¹⁹: myocytes, vascular endothelial cells and smooth muscle cells corresponding to the arterial mean⁴². At the time, a revolutionary approach was the use of stem cells of the patient, depending on the genetic variability of the individual and their own genetic markers⁴³.

The hiPSC differentiation protocol in myocytes is based on the use of embryoid bodies (EB)⁴⁴. These are mouse embryonic fibroblasts, irradiated and grown in suspension culture, where they form spontaneous cell aggregates⁴⁵. The obtained cells resemble the early-stage myocytes with respect to the myofibrillar organization

and catecholamines response⁴⁶. These can be structurally and electrophysiologically differentiated⁵⁰. Subsequently, the hiPSC system has been used in diseases such as: arrhythmias, cardiomyopathies, valvulopathies, blood vessel diseases, cardiometabolic disorders⁵¹, but it cannot be used on a large scale.

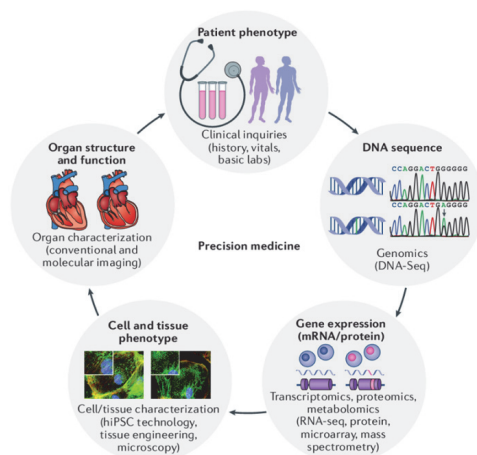


Figure 3 | The role of hiPSC systems, integrated in humans. Obtaining pluripotent stem cells specific to the individual phenotype by gene expression and DNA sequence analysis will correct the structure of the modified organs regardless of the nature of the disease.

Major issues were related to low efficiency and considerable variability¹⁸, including in terms of aggregate size. The next evolutionary step was the creation of enzyme-adapted cultures⁴⁹, selected from the point of view of size by centrifugation¹⁸.

These have resulted in uniform aggregates having a consistent cardiac differentiation⁵². The precise modulation of the mesodermal induction⁵³ was achieved by timely introducing and removing the growth factors that influenced four major pathways of development: the pathway of the bone morphogenetic protein (BMP), the beta factor or the nodal pathway, the Wnt pathway and the pathway of the Fibroblast growth factor (FGF)¹⁸. Dubois and his colleagues⁴⁷ achieved 98% purity myocardiocytes using an EB approach based on the sequential addition

of multiple growth factors (BMP4, VEGF, activin A and DKK1 protein) followed by a flow cytometry purification, against a new patient-specific cell surface⁴⁷.

The differentiation was confirmed by the position of the troponin T and cardiac markers⁴⁷. The complexity of the technique and the intensive work required by the protocol has prevented the use of the method on a large scale.

Laflamme and co-workers⁴⁸ cultivated embryonic stem cells as monolayers and induced the sequential addition of cardiac differentiation and the subsequent elimination of activin A and BMP4; the purity of cultures was only 30%⁴⁸.

Burrige and co-workers⁵³ improved and simplified the monolayer approach using Roswell basal medium (RPMI), ascorbic acid, recombinant human albumin and Wnt pathway inhibitors, thus obtaining 95% purity myocardial fibers differentiated into 11 cell lines⁵³.

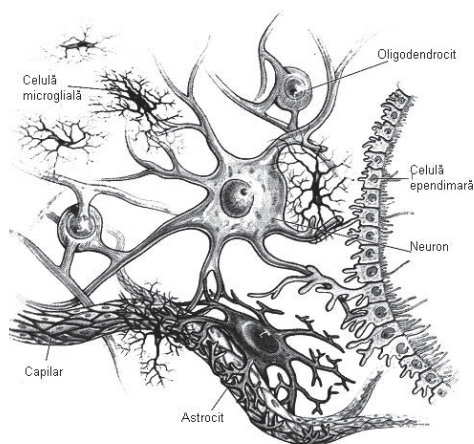


Figure 3 | Types of neuronal linkages in the structure of the nervous tissue (scheme). The neuron establishes links of the type of cell anastomoses with no electrical capacity (ependymal cells, capillary endothelium) or synapses (astrocytes, microglia, oligodendrocytes).

As far as *in vitro* neuron cultivation is concerned, Bakmand⁵⁴ has developed a microfluidic system adapted for cerebral tissue cultivation that combines conventional

systems and fluid culture systems with the possibility of integrating a sensor system⁵⁴.

Primary neurons are grown on a modified peptide substrate (PNW) mimicking *in vivo* conditions⁵⁴. It was found that tissues grown in the microfluidic system were superior in quality compared to those maintained on conventional plastic substrates⁵⁴.

As a support, biological membranes are used on which electrode sensors have been fixed to measure the nervous cell parameters in real-time⁵⁴.

Discussion

Histologically, in the myocardium affected by cardiomyopathy, the disorganization of myofibrils occurs⁷. In the cytoplasm of myocardiocytes, myofibrillar subunits and neurofilaments may occur without Z-membrane organization and regular sarcomas⁷, and in strongly dilated areas the myofibrillar disorganization and myofibrilolysis occur⁸. Different degrees of disorganization of actin and myosin filaments are observed in sarcomeres²⁰.

The nodal tissue is an essential element in the heart that initiates the contraction through the pacemaker cells from its structure²¹.

Experimenting a method to improve its function and structure in dilated cardiomyopathy is vital in order to discuss about the success of treatment.

In order to have clinical utility, cardioplasty using cardiospheres¹⁵, *in vitro* multiplied cardiac stem cells, requires a strategy that permits the activation, isolation, expansion and reintroduction into the cardiac lesion for the autologous therapy.

More research is needed on the routes of administration, the ability to differentiate and maintain a mature and functional cardiac phenotype, the mode of fusion with the host myocardium, the longevity of the grafts¹⁵, the risks and long-term side effects, especially immune responses and the rejection of the graft. Collectively, the studies previously presented on hiPSC systems¹⁸ show that chemical protocols have had notable scientific results but have been very little applied and are not suitable for large-scale use.

Among the successes, the patient-specific hiPSC derivatives allowed *in vitro* modeling

of a large number of cardiovascular diseases and allowed the cardiotoxicity testing of some drugs¹⁸. By combining several methods that have been experimented and have had notable success rates, each of them, but which could not be developed on large scale, I tend to think that the common approach presented in this study may increase the practical applicability rate of these implants.

Conclusions

The three-dimensional organization of cardiac stem cells and neurons could improve the contractile heart function and restore the main changes in dilatation cardiomyopathy.

The concept of proliferation and differentiation of cardiac stem cells requires more in-depth studies in order to control these properties *in vitro* and *in vivo*. HiPSC systems have an endless capacity for using; the challenge is to find exact specific phenotypes clinically adapted to each individual patient.

More experimental research is needed to determine the degree of adaptability of the two types of cells in the receiving myocardium, the degree of improvement in the contractile function, and last but not least, the rate of post-interventional survival of affected dogs. On the basis of the vital prognosis, a comparison can be made between the method presented in the study and the current symptomatic and adjuvant drug treatment.

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