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Stem cell Researches in Brazil: Present and Future Challenges

Mayana Zatz

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Abstract A bill allowing researches with human embryonic stem cells has been approved by the Brazilian Congress, originally in 2005 and definitively by the Supreme Court in 2008. However, several years before, investigations in Brazil with adult stem cells in vitro in animal models as well as clinical trials, were started and are currently underway. Here, we will summarize the main findings and the challenges of going from bench to bed, focusing on heart, diabetes, cancer, craniofacial, and neuromuscular disorders. We also call attention to the importance of publishing negative results on experimental trials in scientific journals and websites. They are of great value to investigators in the field and may avoid the repeating of unsuccessful experiments. In addition, they could be referred to patients seeking information, aiming to protect them against financial and psychological harm.

Keywords Embryonic stem cells · Brazilian legislation · Adult stem-cells · Heart conditions · Craniofacial and neuromuscular disorders

The Brazilian Legislation on Stem Cells

In 2005, the Brazilian congress approved a bill allowing researches with embryonic stem cells (ESC), with a great majority of votes: 96% of the senators and 85% of the congressmen were in favor of such researches. However, right after its approval, the former Brazilian general attorney, based on his religious beliefs, declared the bill was unconstitutional and the final decision had to be taken by the Brazilian Supreme Court. In May 2008, after many debates, and with the active participation of scientists pro and against the use of human embryos, the bill approving researches with ESC was definitively approved by the Brazilian supreme court. Brazil and Mexico are currently the only countries in Latin America allowing such researches. The Brazilian bill requires that ESC are obtained from surplus embryos kept frozen for at least 3 years in fertilization clinics, and they can only be donated for research after informed consent from the donors. The bill does not approve the production of human embryos for research purposes or therapeutic cloning. All researches must be approved by the Institution ethical committee.

The possibility of using human ESC will open new areas of investigation in Brazil. However, before 2005, researches with adult stem cells, both in vitro and in vivo, were already being undertaken by different Brazilian groups. Here, we will summarize the main findings and the challenges to go from bench to bed, focusing on heart, diabetes, cancer, craniofacial and neuromuscular disorders as well as the ethical issues related to the use of stem cells in the clinic.

Autologous Stem Cells Transplantation in Heart Conditions

Clinical and Pre-clinical Trials

Perin et al. [1] evaluated if autologous transplantation of mononuclear autologous bone marrow (ABMM) cells could promote neovascularization and improve perfusion and myocardial contractility in patients with end-stage ischemic heart disease. A group of 21 patients, who were

M. Zatz (✉)
Human Genome Research Center, Biosciences Institute,
University of São Paulo,
Rua do Matão 277, Cidade Universitária,
caixa Postal 05508-090 São Paulo, Brazil
e-mail: mayazatz@usp.br

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followed for 6–12 months, were enrolled in this open label nonrandomized study

Analysis of the sites of ABMM cell injection, in one patient, 11 months after treatment, showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle [2].

In short, the study demonstrated the relative safety of ABMM cells myocardial injections in patients with severe heart failure and the potential use of this procedure for improving myocardial blood flow. According to the authors, autologous bone marrow mononuclear cells may produce a durable therapeutic effect and improve myocardial perfusion and exercice capacity.

On the other hand, pre-clinical studies in animal models showed some positive but also some negative or intriguing results.

Bone marrow cell transplantation was apparently effective for the treatment of chronic chagasic myocarditis in a mice model [3]. According to the authors, the beneficial effects were observed up to 6 months after bone marrow transplantation.

On the other hand, Ribeiro et al. [4] reported that the occurrence of bone-like-formation in the left ventricular wall of infarcted rats did not differ between those treated with bone marrow cells as compared to the untreated ones.

More recently, two studies [5, 6] compared the effect of in situ versus systemic delivery of bone marrow (BMSC) and mesenchymal stem cells (MSCs) derived from male rats in two independent groups: hypertensive female rats submitted to myocardial infarction and hypertensive female rats submitted to coronary occlusion.

In the first group, in situ injections of both MSCs and BMCs resulted in improved cardiac morphology, reduced lesion tissue, increased capillarity density and a higher survival rate. However, only treatment with MSCs, ameliorated left ventricle dysfunction, suggesting a positive role of these cells in heart remodeling in infarcted hypertensive subjects. On the other hand, in the second study, systemic administration of BMC reduced mortality, improved functional capacity, and also left ventricular function. The authors speculate that the cells transiently home to the myocardium, releasing paracrine factors that recruit host cells to repair the lesion. Based on these observations, it would be of interest to evaluate the effect of simultaneous in situ and systemic injections in the same animals.

Other international studies on the potential of ABMM in heart conditions showed variable results ranging from validation to disproof. According to recent reviews [7, 8], the conclusion is that the two camps may not be as far apart, and the potential of one day achieving regenerative therapy is indeed a vision that both parties enthusiastically share. However, adequately powered trials using optimal dosing, well characterized stem cells, longer term outcome assessments, and more patients including placebo groups are required.

Clinical trials to test the efficacy of autologous bone marrow derived mononuclear cells are currently underway in Brazil in four cardiopathies: acute and chronic ischemic heart disease, chagasic and dilated cardiomyopathy [9]. Results of these multicenter trials, which are double-blind and placebo controlled will be very important to assess the effectiveness of this therapeutic approach in heart conditions. In any case, it is already anticipated that other types of stem cells will have to be used since it is not expected that mononuclear stem cells will regenerate cardiomyocytes.

Induced Liver Injury: Pre-clinical Studies

Autologous Bone Marrow Cells Delivered to Wistar Rats

Two recent studies, evaluating the effect of autologous bone marrow transplant in female wistar rats with severe induced liver chronic injury, showed no beneficial effect.

In the first study, the authors tested the effect of bone marrow cell (BMC) transplantation in either preventing or reversing cirrhosis on an experimental model of chronic liver disease [10]. Ten animals (cell-treated group) received five injections of BMCs during the cirrhosis induction protocol and four animals received the cells after liver injury was established through a tail vein. However, the experiment seemed to be ineffective in improving morphofunctional parameters of the liver when applied to chronic cases either during or after establishment of the hepatic lesion.

In the second study, the authors evaluated the therapeutic potential of bone marrow mesenchymal stromal cells (MSC), injected in the portal vein, in another group of 14 female Wistar rats with severe chronic liver injury [11]. Two months after cell or placebo injection, no differences were observed between MSC and placebo groups. In short, under these experimental conditions, MSC were unable to reduce fibrosis or improve liver function in a rat model of severe chronic liver injury.

Clinical Therapeutic Trials for Diabetes

Autologous Hematopoietic Stem Cell Transplantation (AHST) for Early Type 1 Diabetes Mellitus

Voltarelli et al. [12] were the first to perform a cellular therapy trial in an attempt to interdict type 1 diabetes

mellitus (T1DM). This form of diabetes is the result of the autoimmune response against pancreatic insulin producing beta cells. This response begins months or even years before the first presentation of signs and symptoms of hyperglycemia. At the time of clinical diagnosis near 30% of beta-cell mass still remains.

To determine the safety and metabolic effects of high-dose immunosuppression followed by autologous nonmyeloablative hematopoietic stem cell transplantation (AHST), 15 newly diagnosed type 1 DM patients (aged 14–31 years) were enrolled in a prospective study. During a 7- to 36-month follow-up (mean 18.8), 14 patients became insulin-free. There was no mortality. The only acute severe adverse effect was culture-negative bilateral pneumonia in 1 patient and late endocrine dysfunction in 2 others. According to the authors, high-dose immunosuppression and AHST showed acceptable toxicity in a small number of patients with newly diagnosed type 1 DM. Beta cell function was increased in all but 1 patient and induced prolonged insulin independence in most patients.

Although the study revealed a significant improvement in beta-cell function, it received several criticisms right after its publication [13, 14] such as: "There was no randomized control group; It is not known whether the beneficial effects of AHSCT are due to immune reconstitution; There is a well known honeymoon period of relative remission after the onset of type 1 DM that complicates interpretation of results. The duration of follow-up was insufficient to determine if the improvement was sustained".

In order to address such criticisms, an update of this first study was recently published by Voltarelli et al. [15] including 5 additional patients. Fourteen patients are continuously insulin-free since insulin suspension after a medium follow-up of almost 2 years (ranging from 3 months to 4 years). However, the authors agree that longer follow-up and controlled studies are certainly needed to evaluate the full potential of this procedure in the reversal of type 1 diabetes.

Leukemia and Cancer Stem Cells

In vitro Studies

According to Papenucci et al. [16], umbilical cord blood (UCB) transplants, compared with bone marrow (BM), in leukemic patients result in delayed engraftment, better reconstitution of progenitors, higher thymic function, and a lower incidence of the graft-versus-host disease. In an attempt to understand the molecular mechanisms causing these intrinsic differences, these authors analyzed the differentially expressed genes between BM and UCB hematopoietic stem and progenitor cells (HSPCs). The expressions of approximately 10,000 genes were compared by serial analysis of gene expression of magnetically sorted CD34(+) cells from BM and UCB. A significantly higher number of NF-kappaB cis-regulatory elements (present in 22 genes) than would be expected by chance was found, pointing to an important role of the NF-kappaB pathway on the molecular and functional differences observed between BM and UCB HSPCs. According to the authors, these results form the basis for future studies and potentially for new strategies to stem cell graft manipulation, by specific NF-kappaB pathway modulation on stem cells, prior to transplant.

In a comparative analysis of cancer stem cells with their neoplastic and non-neoplastic counterparts, Okamoto et al. [17] reported subpopulation of tumorigenic stem-like cells, isolated from human glioblastomas. Microarrays covering 55,000 transcripts showed that the up-regulation of two genes, E2F2 and HOXC9, were associated with malignancy. Due to their distinctive expression, the use of E2F2 and HOXC9, as therapeutic targets for tumor eradication, is suggested by these investigators.

In another study, these authors [18] identified several genes and signaling pathways not previously associated with ex vivo expansion of CD133+/CD34+ cells, most of which associated with cancer. Regulation of MEK/ERK and Hedgehog signaling genes in addition to numerous proto-oncogenes was detected during conditions of enhanced progenitor cell expansion, revealing potential molecular targets for oncogenic transformation in CD133+/CD34+ cells. According to the authors, these findings strengthen the link between deregulation of stem/progenitor cell expansion and the malignant process.

Bone Regeneration

In Vitro and Pre-clinical Studies

Studies aiming bone regeneration in affected patients by malformation syndromes has been one of the focuses of research at the Human Genome research center, at the University of São Paulo.

Based on the study of patients with Apert syndrome, a severe form of craniosynostosis, caused by dominant gain-of-function mutations in the FGFR2 gene, a mutation in this gene was shown [19] to alter osteogenic potential of periosteal cells turning them more committed toward the osteoblast lineage. This observation reinforces the importance of studying genetic disorders in an attempt to identify and enhance our comprehension on genes involved in cell differentiation.

Human Dental Pulp Stem Cells (hDPSC) to Reconstruct Cranial Bone Defect in Rats

De Mendonça Costa et al. [20] evaluated the capacity of human dental pulp stem cells (hDPSC), isolated from

deciduous teeth, to reconstruct large-sized cranial bone defects in nonimmunosuppressed (NIS) rats. The hDPSC cells, previously isolated and characterized by Kerkis et al. [21] were shown to have the potential in vitro for differentiation into muscle, neurons, cartilage and bone under defined culture conditions. Two symmetric full-thickness cranial defects were done on each parietal region of eight NIS rats. In six of them, the left side was supplied with collagen membrane only and the right side with collagen membrane and hDPSC. Bone formation was observed 1 month after surgery but a more mature bone was present in the right side supplied by a collagen membrane and hDPSC cells, as compared to the opposite side. The use of hDPSC in NIS rats did not cause any graft rejection, and the presence of human cells in the new bone was confirmed by molecular analysis. These findings suggest that hDPSC is an additional cell resource for correcting large cranial defects in rats and constitutes a promising model for reconstruction of human large cranial defects in craniofacial surgery.

Stem Cells Obtained from Orbicular Oris Muscle (OOM): a New Source of Stem Cells to Reconstruct Bone Defects

Cleft lip and palate (CLP), one of the most frequent congenital malformations, affect the alveolar bone in the great majority of the cases, and the reconstruction of this defect still represents a challenge in the rehabilitation of these patients. One of the current most promising strategy to achieve this goal is the use of bone marrow stem cells (BMSC). However, isolation of BMSC or iliac bone confers site morbidity to the donor. In order to identify a new alternative source of stem cells with osteogenic potential, Bueno et al. [22] investigated the therapeutic potential of stem cells obtained from orbicular oris muscle (OOM) fragments, which are regularly discarded during surgery repair of CLP patients. Cells obtained from OOM fragments of four unrelated CLP patients were positively marked for five mesenchymal stem cell antigens (CD29, CD90, CD105, SH3, and SH4), while negative for hematopoietic cell markers, CD14, CD34, CD45, and CD117, and for endothelial cell marker, CD31. After induction under appropriate cell culture conditions, these cells were capable to undergo chondrogenic, adipogenic, osteogenic, and skeletal muscle cell differentiation, as evidenced by immunohistochemistry. The authors also demonstrated that these cells together with a collagen membrane lead to bone tissue reconstruction in critical-size cranial defects previously induced in nonimmunocompromised rats. The presence of human DNA in the new bone was confirmed by PCR with human-specific primers and immunohistochemistry with human nuclei antibodies. These findings suggest that these cells represent a promising source of stem cells for alveolar bone grafting treatment, particularly in young CLP patients.

Muscle Regeneration in Progressive Muscular Dystrophies

In Vitro and Pre-clinical Studies

Investigation on progressive muscular dystrophies (PMDs) has been a major interest at the Human Genome Research Center. PMDs include a group of genetic disorders characterized by a progressive muscle degeneration and weakness for which there is no treatment. Among them, the most prevalent forms are the X-linked form of Duchenne/Becker muscular dystrophy (DMD/BMD) and limb-girdle muscular dystrophies (LGMD). DMD/BMD affects only males and is caused by the absence or deficiency of muscle dystrophin. LGMDs, with an equal incidence in both sexes are a heterogeneous group, with more than 20 genes identified to date. The possibility to replace the defective muscle or improve its function through stem cell therapy, regardless of the specific gene defect, opened a new and very promising field of investigation in PMDs. However, before going to translational medicine, a great amount of research has to take place. In recent years, our group has focused on the potential of various stem cells obtained from different adult tissues to differentiate in myogenic lineages and express specific muscle proteins both in vitro and in vivo. We are currently investigating stem cells obtained from umbilical cord, adipose tissue and dental pulp, which are being injected in different animal models for neuromuscular disorders, aiming to address the following questions: What is the best source of adult stem cells for muscle regeneration? Cell transplantation: local or systemic? What is the effect of single versus multiple injections? How to direct homing? How to circumvent rejection? Here we will summarize the main findings.

Obtention of Mesenchymal Stem Cells from Umbilical Cord Units: Cord is Richer than Blood

The identification of mesenchymal stem cell (MSC) sources that are easily obtainable is of utmost importance. Several studies have shown that MSCs could be isolated from umbilical cord (UC) units. However, the presence of MSCs in human umbilical cord blood (HUCB) is controversial. We compared the efficiency in obtaining MSCs from unrelated paired of HUCB and HUC (human umbilical cord–Wharton jelly) samples harvested from the same donors [23]. The samples were processed simultaneously, under the same culture conditions. Although MSCs from blood were obtained from only 1 of the 10 samples, we were able to isolate large amounts of multipotent MSCs

from all UC samples, which were able to originate muscle, adipose, bone, and cartilage cell lineages. These results were now confirmed in a larger sample of 65 match-paired UC units (Secco et al., unpublished). Since the routine procedure in UC banks has been to store the blood and discard other tissues, such as the cord and/or placenta, these results are of immediate clinical value [24]. Furthermore, the possibility of originating different cell lines from the HUC of neonates born with genetic defects may provide new cellular research models for understanding human malformations and genetic disorders, as well as the possibility of testing the effects of different therapeutic drugs.

Mesenchymal Stem Cells from Human Adipose Tissue (hASCs) and CD134+ from Umbilical Cord Blood are Able to Express Muscle Dystrophin In Vitro

Liposuctioned human fat is available in large quantities and therefore it may be an ideal source of stem cells for therapeutic applications. It has been shown that adipose-derived stem cells have a myogenic potential [25] and are able to restore dystrophin expression in the muscles of mdx (the murine model for DMD) mice [26]. Therefore a question we wanted to address is how these cells interact with human dystrophic muscle. With this in mind, we isolated hASCs from human lipoaspirates and observed that they participate in myotube formation when cultured together with differentiating human DMD myoblasts or myotubes, resulting in the restoration of dystrophin expression. These preliminary results showed that hASCs have the potential to interact with dystrophic muscle cells, restoring dystrophin expression of DMD cells in vitro [27].

In vitro studies showed that CD134+ stem cells from umbilical cord blood also have the potential to differentiate in myogenic lineages and express dystrophin in vitro although the expression is very low and detectable only through immunofluorescence [28].

Pre-clinical Studies in Animal Models for Muscular Dystrophy

Human Umbilical Cord Blood Stem Cells Delivered In Situ to mdx Mouse

In situ delivery of GFP (green fluorescent protein)-transduced mononucleated cells from HUCB, which comprises both haematopoietic and mesenchymal populations, into quadriceps muscle of mdx mice resulted in the expression of human myogenic markers [29]. After recovery of these cells from mdx muscle and in vitro cultivation, they were able to fuse and form GFP-positive myotubes that expressed human dystrophin in larger amounts.

Human Stem Cells from Human Immature Dental Pulp (hIDPSC) Delivered to GRMD Dogs

In order to investigate the therapeutic potential of hIDPSCs for muscle regeneration we injected them in the golden retriever muscular dystrophy dog (GRMD). The GRMD dogs represent the best available animal model for therapeutic trials aiming at the future treatment of human DMD. We were able to obtain a rare litter of six GRMD dogs (3 males and 3 females) born from an affected male and a carrier female which were submitted to a therapeutic trial with adult human hIDPSC stem cells. Our aims were to investigate the cells' ability in respect to migrate, engraftment, and myogenic potential, and the expression of human dystrophin in affected muscles as well as the efficiency of single and consecutive injections [30]. Chimeric muscle fibers were detected by immunofluorescence and fluorescent in situ hybridization (FISH) using human antibodies and X and Y DNA probes. No signs of immune rejection were observed and these results suggested that hIDPSC cell transplantation may be done without immunosuppression. We showed that hIDPSC were able to engraft in GRMD dog muscles, although human dystrophin expression was modest and limited to several muscle fibers. However, a better clinical condition was observed in only one dog, which received monthly arterial injections and is still clinically stable at 30 months of age. Our data suggested that systemic multiple deliveries seem more effective than local injections, which opens important avenues for further researches. However, since the clinical course is highly variable in GRMD dogs, these studies must be repeated in larger samples which are followed for a longer period before drawing any conclusion.

Human Adipose Stem Cells (hASCs) Delivered Systemically to SJL Mouse

In order to evaluate the behavior of hASCs in vivo we injected them, systemically, in *SJL* mice, the murine model of limb-girdle muscular dystrophy 2B(LGMD2B). LGMD2B in humans is caused by the deficiency of muscle dysferlin [31]. The *SJL* mice, show muscle weakness that begins at 4–6 weeks and is nearly complete by 8 months of age. Adipose stromal cells (hASCs) were injected in the caudal vein of *SJL* mice, without immunosuppression, aiming to assess: their ability to engraft into recipient dystrophic muscle after systemic delivery; form chimeric human/mouse muscle fibers; express human muscle proteins in the dystrophic host and improve muscular performance. Interestingly, we obtained positive results only with undifferentiated hASCs, that is, cells that had not been previously cultivated with myogenic induction media. We showed for the first time that undifferentiated hASCs were not rejected after systemic

injection even without immunosuppression, were able to fuse with the host muscle, express a significant amount of human muscle proteins, and improve motor ability of injected animals. These results may have important applications for future therapy in patients with different forms of muscular dystrophies. A similar approach with different animal models is currently underway.

Ethical Issues

Negative Results Should be Published and Publicly Advertised

How long will it take to go from bench to bed? This is probably the most frequent question we are asked and the most difficult to answer. Although preliminary studies are very promising, a significant amount of research will have to be undertaken, particularly in animal models, before starting human therapeutic trials. Despite the fact that this is a consensus among stem cell researchers, unproven stem cell interventions are currently being offered by different clinics around the world at a high cost. Some sites offer treatment for numerous conditions such as Down syndrome, spinal cord injuries, cerebral palsy, stroke, or even baldness, taking advantage of patients and families despair when faced with still untreatable disorders. How can we protect patients from being exploited by unethical persons? In the first place, patients should know that experimental trials are not supposed to be charged for. This should be constantly advertised in the media. It is also of utmost importance to have negative results on experimental trials published in scientific journals and sites which could be referred to patients seeking information, aiming to protect them against financial and psychological harm. In addition, information on negative results are invaluable to investigators in the field and may prevent them from repeating unsuccessful experiments.

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