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REVIEW ARTICLE

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Vascular regenerative therapies for the treatment of erectile dysfunction: current approaches

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SUMMARY

The pharmacological treatment of erectile dysfunction (ED) is mainly represented by the administration of inhibitors of phosphodiesterase-5 (PDE5). However, in the clinical practice many patients do not benefit from such a treatment, hence the scientific interest extends to other therapeutic strategies; in particular, to the vascular regenerative therapy. This review describes the main acquisitions related to this approach represented by the mesenchymal stem cell or adipose tissue stem cell transplantation and endothelial nitric oxide synthase or vascular endothelial growth factor gene therapy. Moreover, there are other two aspects of wide interest represented by the potential vascular regenerative effects exerted by the PDE5 inhibitors and the therapeutic strategies for a category of patients who more frequently do not respond to the conventional treatment for ED, the patients with diabetes mellitus.

INTRODUCTION

In addition to its diagnosis, the treatment of erectile dysfunction (ED) has received much attention in recent years. The pharmacological approach using phosphodiesterase-5 (PDE5) inhibitors is considered the first-line treatment for patients with ED. This treatment is highly effective; however, some patients require a second-line therapy primarily consisting of intracavernous injections (Vicari *et al.*, 2010). Therefore, it is useful to evaluate other potential treatments.

In particular, new therapeutic strategies offering more efficacious treatments are being studied, especially for patients who fail to respond to PDE5 inhibitors. These strategies include intracavernous injections of therapeutic genes, such as the endothelial nitric oxide synthase (eNOS) gene or totipotent mesenchymal cell (MSC) vector-mediated gene transfer, usually using an adenovirus (Deng *et al.*, 2005). MSCs derived from bone marrow cells are capable of transforming into various cell types; therefore, they are useful in tissue repair and regeneration. Furthermore, they do not induce local immune reactions and show good longevity; thus, because of these characteristics, they can be used as vehicles for gene therapy.

The aim of this article is to review recently studied options in the field of vascular rehabilitation of patients with ED. In this article, a discussion of the following topics will be presented: the role of PDE5 inhibitors in the release and differentiation of endothelial progenitor cells (EPCs), potential applications of MSC and eNOS gene therapy, the role of vascular endothelial growth factor (VEGF) gene therapy, the significance of adipose tissue stem cell transplantation and some considerations will be given to other stem cell transplantations. Finally, therapeutic strategies for ED in diabetes mellitus will be examined.

PDE5 inhibitors and EPCs: An indirect example of vascular regenerative treatment of ED

EPCs are bone marrow-derived endothelial cells with the capacity to circulate, proliferate and differentiate into mature endothelial cells (Asahara *et al.*, 1997), which represent main functional elements of postnatal vasculogenesis. The first detailed descriptions of EPCs (Asahara *et al.*, 1997) have established that markers that characterize these cells at the earliest stages of differentiation are also observed in haematopoietic stem cells, $CD34_{pos}$ cells, $CD133_{pos}$ cells, and vascular endothelial growth factor receptor 2 (VEGFR-2) positive cells. Initially, the presence of EPCs was believed to be limited to the embryonic stage; subsequently it was shown that their differentiation can also occur in situ in adults. These cells represent the product

of trans-differentiation of mononuclear cells in peripheral blood; however, they are also isolated in the bone marrow and in the vessel wall (Du *et al.*, 2012; Zhang & Huang, 2012).

We have recently shown an increase in serum concentrations of late phenotype of ECPs ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) in patients with arterial ED, most likely attributable to a more advanced phase of cell differentiation, compared with early phenotypes ($CD34_{pos}$, $CD133_{pos}$, VEGFR-2_{pos} cells) (La Vignera *et al.*, 2012) whose concentration is reduced in patients with the main cardiovascular risk factors (diabetes, hypertension, etc.). In our opinion, the use of these markers is valuable because these changes precede the structural alterations resulting from vascular damage in these patients, such as carotid atherosclerosis, which is commonly evaluated by ultrasound scan (Condorelli *et al.*, 2012).

EPCs have an increased regenerative capacity that is why they could represent a good therapeutic target. Gou *et al.* (2011) have shown that intracavernosal injection of EPCs transfected with the *VEGF165* gene can restore erectile function in an experimental model. In particular, the authors suggest the following mechanisms to explain this result: (i) an increase in the expression of the VEGF165 protein, (ii) synergy between *VEGF165* and EPCs that enhances neovascularization, and (iii) improved function of ECs supported by the incorporation of EPCs (Gou *et al.*, 2011).

PDE5 inhibitors play an indirect role in the vascular regenerative therapy of ED by facilitating EPCs release from bone marrow and through their trans-differentiation in peripheral blood (Foresta *et al.*, 2005; Dussault *et al.*, 2009; La Vignera *et al.*, 2012).

Recently, several groups reported that reduced levels of EPCs could be reversed with the administration of PDE5 inhibitors in ED patients, showing an efficacious vasculoprotection and prevention of the progression of the endothelial damage. Another possible hypothesis for the mechanism involves the inhibition of PDE5 in bone marrow that may enhance the local effects of nitric oxide, thereby leading to the mobilization of EPCs (Foresta *et al.*, 2007).

Foresta *et al.* (2006) evaluated the changes in the number of circulating EPCs in patients with ED after treatment with tadalafil, a PDE5 inhibitor. The study was conducted on 26 patients (age range 35–58 years) whose ED was diagnosed on the basis of an international index of erectile function (IIEF-5) score <21, as well as nocturnal penile tumescence and rigidity monitoring (NPTRM), and in a control group made up of 23 healthy subjects. In both groups, brachial artery flow-mediated dilatation (FMD) was evaluated, and a sample of peripheral blood was withdrawn for circulating EPC count. All patients with ED were then treated with tadalafil (20 mg three times a week for 3 months). The evaluation of brachial FMD and EPC counts were performed again 3 days after tadalafil discontinuation. The controls were re-evaluated for brachial FMD and EPC counts after 3 months of treatment with placebo. At the end of 3 months of therapy, patients with ED with or without cardiovascular risk factors showed a significant increase in the number of EPCs and an increase in FMD values, whereas the number of circulating EPCs did not change significantly in the controls. Although the study was limited by a lack of randomization, tadalafil treatment seems to improve endothelial function with vasculo-protective effects.

The mechanism by which the drug is able to increase EPCs and to restore endothelial function is not known, although at least two hypotheses can be proffered. The first relies on the assumption that PDE5 is present in bone marrow where, once inhibited, may amplify the effects of local NO, which in turn mobilizes stem and progenitor cells. Alternatively, a hypothetical peripheral action of PDE5 inhibitors on the endothelial cells of the vascular tree is modulation of the signalling pathway that leads to EPC activation and/or mobilization. It has also been observed that after treatment with tadalafil, patients with reduced baseline FMD show a less significant increase in EPC counts. This finding suggests that low FMD indicates reduced competence in the mobilization and/or production of EPCs, likely ascribable to alterations of NO-mediated mechanisms that lead to the activation of EPCs.

Although the precise mechanism is not certain, one study (Foresta *et al.*, 2006) suggests that PDE5 inhibitors may play a central role in the prevention and progression of endothelial dysfunction not only in patients with ED but also in patients with cardiovascular disease (CVD). To confirm this hypothesis, a study was conducted on 68 patients with ED and carotid artery damage of various degrees, with 25 men as controls (Foresta *et al.*, 2007). Patients were divided into three groups according to intima-media thickness (IMT) (i.e. normal, slightly increased, or atherosclerotic plaque). All controls received 20 mg of vardenafil (another PDE5 inhibitor) and underwent EPC count before and 4 sec after the administration of vardenafil. At basal conditions, a significant reduction of circulating EPCs was found in patients with ED compared with the controls. Furthermore, a reduced EPC count was found in ED patients with mild IMT increase or with plaque, but not in patients with normal IMT. Four hours after vardenafil administration, the EPC counts increased in both patients and controls. Therefore, patients with ED and a reduced number of circulating EPCs should be considered at risk for endothelial dysfunction, and an altered response to vardenafil administration may be a marker of an impaired endothelial regenerative capacity of the patient.

Recently, we examined an original immunophenotype of EPCs ($CD45_{neg}/CD34_{pos}/CD144_{pos}$) as well as of endothelial microparticles (EMPs) ($CD45_{neg}/CD144_{pos}$/Annexin V_{pos}), which were chosen as markers of endothelial apoptosis. The aim of the study was to evaluate the serum concentrations of EPCs and EMPs in patients with arterial ED and metabolic syndrome (MetS). To accomplish this, we evaluated 100 patients (aged 45–60 years) with ED and MetS, and 17 healthy men (aged 44–57 years) were used as a control group. EPC and EMP blood concentrations were evaluated before and after the administration of tadalafil (20 mg) on demand for 3 months. The results of our study showed that EPC levels increased significantly after tadalafil administration, whereas EMP levels did not differ significantly. Because tadalafil administration increased the levels of EPCs but not EMPs, this compound may play a pivotal role in the endothelial repair response (La Vignera *et al.*, 2012). In our opinion, the use of PDE5 inhibitors is very important in the clinical practice for the potential diagnostic and therapeutic implications. As far as diagnosis, it represents a further tool for the evaluation of endothelial function (we have shown a more severe degree of endothelial dysfunction in the non-responders) (Condorelli *et al.*, 2012; Condorelli *et al.*, 2013), while therapeutically, it is associated with a higher bioavailability of nitric oxide (Foresta *et al.*, 2006) and promotes the release of EPCs (Gou *et al.*, 2011) in patients with ED.

Mesenchymal stem cells and eNOS gene therapy in erectile dysfunction

MSCs were first isolated from the stromal component of bone marrow, where they represent approximately 0.01% of all nucleated cells (Jones & McGonagle, 2008). The bone marrow is the biological 'niche' of MSCs. In fact, MSCs show a callback function in haematopoietic stem cells circulating in the bone marrow (homing) and support haematopoiesis. In particular, they interact with haematopoietic stem cells, which are also resident in the bone, through cell-cell contact and the secretion of growth factors and promote their differentiation into peripheral circulatory cells (Jones & McGonagle, 2008). Specifically, it has been observed that MSCs have the following characteristics, they are: (i) easily isolated because of their bonding capacity, (ii) easily separable from other cell types through the expression of a set of specific membrane markers ($CD44_{pos}$, $CD90_{pos}$, $CD105_{pos}$, $CD166_{pos}$, $CD73_{pos}$, $CD34_{neg}$, $CD45_{neg}$, $CD31_{neg}$, $CD14_{neg}$), (c) easily expanded in vitro for their high replicative potential, (d) able to exert immunosuppressive and immunomodulatory functions, and (e) able to migrate spontaneously in tissues and selectively in damaged tissues (Chamberlain *et al.*, 2007; Jones & McGonagle, 2008).

The penis is a potential target tissue for gene therapy because of its accessibility and the ubiquity of endothelial lined spaces. Gene therapy is a promising therapeutic strategy for the treatment of ED. In fact, many gene therapy strategies have focused on the NO/cyclic guanosine monophosphate (cGMP) pathway. All three NOS isoforms, including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), have been used for gene therapy to improve erectile response. Different viral and non-viral vectors have been used for the transfer of genetic material to the target cell or tissues, with heterogeneous responses (Kendirci *et al.*, 2005).

Deng *et al.* (2005) conducted a series of experiments which showed the feasibility of successfully transferring the eNOS gene. The gene was inserted in an adenovirus in ex vivo MSCs and induced subsequent protein production (confirmed by immunohistochemistry and Western blot) without interfering with the totipotency of MSCs. Similarly, the calcitonin gene-related peptide (CGRP) gene was also transferred (Deng *et al.*, 2005). These MSCs that were infected with adenoviral vectors expressing specific genes were then injected into the corpora cavernosa of old rats. Seven days after the injection, there was an improvement in ED and a reduction of the inflammatory reaction, which occurred with the direct administration of the modified virus. Finally, even an intracavernous injection of wildtype MSCs after 21 days increased eNOS expression and improved erectile function in aged rats, through a poorly understood mechanism.

Bivalacqua *et al.* (2007) confirmed that intracavernous injections of unmodified wildtype MSCs improved erectile function 21 days after injection. Putative mechanisms involved improved endothelium-derived NO/cGMP signalling as well as MSC differentiation into penile cells expressing endothelial and smooth muscle markers (Bivalacqua *et al.*, 2007). The results of this study further support the contention that erectile response is associated with increased levels of eNOS protein, increased activity of calcium-dependent NOS and increased levels of cGMP in aged tissues. These molecular changes at the penile level are able to improve neuronal-ediated erectile function, and they support the fact the MSCs injected into the corpora cavernosa can differentiate into new endothelial cells and smooth muscle cells (as shown by tissue histological analysis), thereby improving the erectile response.

Immunohistochemical analysis also confirmed that MSCs undergo phenotypic changes after 21 days, acquiring surface markers of smooth muscle and endothelial cells that were absent prior to injection. This data, in keeping with previous findings, confirm the usefulness of MSCs for therapeutic purposes in patients with ED. In contrast, MSCs have the advantage of showing all the characteristics of stem cells: self-renewal capacity, totipotency and the possibility of tissue in vivo regeneration. In addition, they may be obtained easily in large numbers by a single bone marrow aspirate. However, because MSCs show limited survival, a 2007 study attempted to immortalize these cells using a viral vector encoding the *myc* gene and assessed whether they maintain the ability to mutate into endothelial or smooth muscle cells (Song *et al.*, 2007). In particular, bone marrow cells taken from the foetal spine were isolated and then infected with a retrovirus encoding v-myc to immortalize them. Thereafter, the cells were placed in culture, and after 10 weeks, they were transplanted into the cavernous tissue of 20 rats. The cell line studied, line B10, has been shown to express specific markers of endothelial cells and smooth muscle cells only after transplantation, whereas in culture, the line only differentiates into osteoblasts, chondroblasts and fat cells. This result suggests that the integrity of the corpora cavernosa is essential for the differentiation of MSCs into EPCs and, therefore, in endothelial cells. This finding is particularly important for the successful treatment of ED with MSCs. However, v-myc is an oncogene and although it has been pre-clinically tested to immortalize cells transplanted in rats, it is not suitable for further clinical evaluation because of its tumorigenic capability. Despite its potential therapeutic applications, we think that this therapy is currently limited by ethical problems, the risk of disease and the unpredictable host response. Another critical aspect is represented by the viral vectors (i.e. naked plasmid DNA, adenovirus, herpes simplex virus), given that each possesses potential disadvantages (i.e. low transfection efficiency and risk of immunogenicity and chromosomal integration).

Vascular endothelial growth factor gene therapy in erectile dysfunction

VEGF is one of several polypeptides that show significant angiogenic activity in vitro and in vivo. Extensive characterization of the VEGF gene and its products has shown that several different mature mRNA transcripts exist, and all originate from alternative splicing of the basic VEGF transcript. A number of VEGF mRNA isoforms are expressed in the rat and human penis, and the splice variant encoding a 164-amino acid protein is present in the greatest abundance (Burchardt *et al.*, 1999). VEGF polymorphisms affect the responsiveness of clinical erectile dysfunction patients to PDE5 inhibitors. In particular, the AAG haplotype is more common ED patients who are poor responders than those that are good responders (Lacchini *et al.*, 2012).

In an experimental model, Rogers *et al.* (2003) showed that VEGF treatment reversed the cavernosometric findings of leakage (venogenic erectile dysfunction), suggesting that

intracavernous injection of the VEGF gene may be a preferred therapy to preserve erectile function in patients in whom testosterone therapy is contraindicated. In another experimental model, VEGF seemed to alleviate the neurogenic and vasculogenic erectile dysfunction associated with hypercholesterolaemia (Gholami *et al.*, 2003).

In another study, the importance of VEGF in the aetiopathogenesis of ED, a condition in which VEGF receptors are downregulated (Strong *et al.*, 2008), was highlighted. Based on this observation, experiments were conducted in rats that showed that the intracavernosal administration of VEGF protects the endothelium of penile vessels from the damaging effects of hypercholesterolaemia. In experimental models of ED induced by hypercholesterolaemia, the intracavernosal administration of VEGF leads to hypertrophic and hyperplastic phenomena indicative of remodelling of the vascular structures. Furthermore, VEGF may also exert anti-apoptotic actions, protect the endothelium in response to acetylcholine receptors, restore the levels of sex hormones, increase the expression of eNOS and direct and stimulate phosphorylation. In the same study, the beneficial effect of superoxide dismutase (SOD) overexpression in MSCs using a viral vector was noted. SOD is the enzyme that catalyses the conversion of superoxide into hydrogen peroxide and water, thus leading to a net reduction of reactive oxygen species (ROS), whose harmful actions against the endothelial tissue are well established.

In our opinion, VEGF therapy shows great potential in ED treatment and is based on solid clinical evidence. For example, the recent study by Tomada *et al.* (2010) evaluating the expression of VEGF in the young and aged human cavernosum corpus showed the abundant expression of VEGF in smooth muscle cells and its decreased expression in aged tissue.

Adipose tissue stem cell therapy for erectile dysfunction

Adipose tissue stem cell (ADSCs) isolated from the stromal vascular fraction of adipose tissue have been investigated concerning their multiple differentiation characteristics. They share properties of other stem cells, such as the ability to divide and renew themselves over long periods of time and to differentiate into specialized cells. They are easily obtained in large quantities; therefore, they appear to be a promising option as a clinical application for ED regeneration medicine.

In 2009, an interesting study was conducted in ADSCs to evaluate whether they could differentiate into endothelial cells in the penis, with the aim of identifying the underlying mechanisms of endothelial differentiation of ADSCs (Ning *et al.*, 2009). To evaluate their endothelial differentiation in vivo, ADSCs were marked with bromodeoxyuridine (BrdU), injected into mouse corpora cavernosa and localized by immunofluorescence microscopy in various phases. For endothelial differentiation in vitro, ADSCs were cultured in the appropriate endothelial growth media (EGM2), stained for the endothelial markers CD31, vWF, and eNOS, and then evaluated for the ability to form tubular structures in matrigel and phagocytose acetylated LDL (LDL-Ac). To identify factors that promote endothelial differentiation, ADSCs were cultured in various media, each one containing a specific combination of additional factors in EGM2, and the uptake of LDL was evaluated. PD173074, a selective inhibitor of the fibroblast growth factor 2 (FGF2) receptor, was used to confirm the importance of FGF2 in the signalling pathway for endothelial differentiation for ADSCs. In vivo, 4 weeks after injection at the penile level, ADSCs were positive for BrdU and for the antigen endothelial cell of rats 1 (RECA-1). In contrast, it was observed in vitro that ADSCs that express endothelial markers multiplied more rapidly in the middle EGM2, rather than in the traditional culture medium, DMEM (Dulbecco's Modified Eagle's Medium). These properties are reduced in populations of ADSCs grown in the absence of FGF2, and endothelial differentiation is not observed with PD173074 treatment. This finding underscores the importance of the FGF2 growth factor in the repair of penile endothelial damage, and it opens new horizons in the field of ED treatment. Lin and collaborators have shown that ADSCs in culture medium supplemented with FGF2 can differentiate into endothelial cells, which can be injected into the corpora cavernosa of rats with ED, thereby enabling the recovery of poor erectile function (Lin *et al.*, 2009).

In 2010, Garcia and his team once again studied the therapeutic use of ADSCs in subjects with diabetes mellitus type 2, in which ED is a frequent and important complication (Garcia *et al.*, 2010). The study was conducted on 22 diabetic, fat, impotent rats. At 22 weeks of age, all animals were subjected to unilateral stimulation of the cavernous nerve, and the intracavernous pressures (ICP) and blood glucose levels were measured. Subsequently, the adipose perigonadal tissue was collected to obtain the ADSCs, and at 23 weeks of age, one million ADSCs were injected into the penis of the treatment group rodents, whereas the control group received only the vehicle. Erectile function was re-evaluated after 26 weeks, and a sample of tissue was taken for histological analysis. The results showed that after treatment, the stimulation of the cavernous nerve induced a greater increase in ICP compared with the controls, and an increase in the ICP/mean arterial pressure (MAP) ratio was also observed. Moreover, in the removed tissue, greater production of nNOS and a greater number of endothelial cells in the corpora cavernosa was observed. However, given that only a small contingent of ADSCs was found in the corpora cavernosa, it seems that the improvement in erectile function and the microarchitecture of the corpus cavernosum itself can be attributed to the paracrine action of NO rather than ADSC endothelial differentiation.

Hyperlipidaemia is also widely associated with ED; thus, ADSCs may be of therapeutic utility in this condition. A study was conducted on male rats that were induced to develop hyperlipidaemia through a high-fat diet (hyperlipidemic rats, HR), while a group that was fed with a normal diet served as the control (normal rats, NR) (Huang *et al.*, 2010). Five months later, ADSCs were isolated from the perigonadal fat of all rats. The cells were cultured for 1 week, marked with 5-ethynyl-2′-deoxyuridine (EDU) and then injected in the corpora cavernosa of 18 HR, whereas 10 HR were injected phosphate buffer saline (PBS). At two and 14 days post-transplantation, four ADSC-injected HR were sacrificed to monitor the transplanted cells. Twenty-eight days after transplantation, the remaining HR underwent blood testing, evaluation of erectile function, and penile histological examination. Erectile function was estimated by measuring the ICP during electro-stimulation of the cavernous nerve, whereas the cavernous nerves and endothelial and smooth muscle cells were assessed by immunohistochemistry. Total serum cholesterol and LDL were significantly higher in both NR and HR rats, whereas HDL was significantly lower in HR than in NR. The ICP/

MAP ratio was reduced in PBS-injected HR or NR, in contrast to ADSC-injected HR. The levels of nNOS-positive nerve fibres and the number of endothelial cells were lower in PBS-injected HR, compared with ADSC-injected HR. Smooth muscle cells were significantly more represented in both HR and NR rats. This suggests that treatment with ADSCs improves the adverse effects of hyperlipidaemia; therefore, this study confirms their potential therapeutic role in ED.

Very recently, another study was carried out on ADSCs to assess their ability to differentiate into smooth muscle and endothelial cells (Orabi *et al.*, 2012). ADSCs were isolated from rats and differentiated into smooth muscle cells and endothelial cells, appropriately labelled, and then used to construct the cavernous tissue. This tissue was subsequently implanted into the penis of normal rats. After 1 and 2 months, the rats were sacrificed, and penile tissue and bone marrow samples were taken to assess cell survival. Using immunohistochemistry, the tissue was stained with haematoxylin-eosin and Masson's trichrome, The cells had retained the ability for differentiation into smooth muscle cells and endothelial cells, and 2 months later, a significant number survived and were well integrated into penile tissues, supporting the potential for use of ADSCs in the therapeutic treatment of ED.

Another potential use of ADSCs may pertain to the treatment of ED resulting from radiotherapy for prostate cancer (Qiu *et al.*, 2012a). Thirty male rats were divided into the following three groups: a control group that received only an injection of PBS, a group subjected to pelvic irradiation + injection of PBS and the treatment group, which received prostatic irradiation and a subsequent injection of ADSCs marked with EDU. After 17 weeks, erectile function was evaluated by measuring ICP during electrical stimulation of the cavernous nerves, and the penile tissue and major pelvic ganglia were examined by immunofluorescence staining for EDU. The study showed that irradiation significantly alters the mechanisms of erection, most likely as a result of damage to the cavernous nerve. Irradiation also reduced the expression of nNOS and the content of smooth muscle cells at the penile level, however, it did not influence the number of endothelial cells. The injection of ADSCs would regenerate the nerve, allowing for the recovery of erectile function, the expression of nNOS and restoring the content of smooth muscle cells.

ED is often a consequence of prostatectomy, and in such cases, the aetiopathogenetic mechanism is caused by an iatrogenic lesion of the cavernous nerves. Therefore, neuroprotective therapy and the use of stem cells could be promising forms of treatment. In particular, in an experimental model, Piao *et al.* (2012) evaluated the therapeutic efficacy of ADSCs and brain-derived neurotrophic factor (BDNF). Rats were divided into the following five groups: control group, group with a bilateral lesion of the cavernous nerves, the group receiving ADSCs, the group receiving the BDNF membrane and the group receiving both ADSCs and BDNF. After 4 weeks, the ICP/MAP ratio and the contents of smooth muscle cells and collagen at the penile level were evaluated by Masson's trichrome staining, whereas the expression of eNOS and cGMP was assessed by immunohistochemistry. In the group of rats treated with ADSCs and BDNF, erectile function and the muscle cells/collagen ratio improved significantly. Furthermore, the quantities of nNOS, eNOS and cGMP significantly increased compared with those in the other four groups. These results suggest new possibilities for the treatment of post-prostatectomy ED.

In conclusion, in our opinion, adipose-derived stem cells are potential candidates for the treatment of ED for many reasons. In fact, these cells can be easily obtained in large quantities and possess the potential to undergo long-term proliferation, self-renewal and multipotent differentiation. In addition, they may find use in other applications such as the reconstruction of penile tissue, enlargement of the penis and in strengthening of the vaginal muscle.

Therapeutic strategies for erectile dysfunction in diabetes mellitus

DM is frequently associated with ED, and these patients often use PDE5 inhibitors. These patients are difficult to treat because, with time, they are unresponsive to PDE5 inhibitors administration highlighting the relevance of establishing novel therapeutic strategies. Accordingly, Qiu *et al.* (2011) investigated the effects of bone marrow-derived MSC transplantation on erectile function in an experimental model. In this study, male rats were injected with streptozocin to induce diabetes, and other rats were injected with a citrate buffer (vehicle) and served as control rats. Rat MSCs were transplanted into diabetic rat corpora cavernosa. Four weeks after transplantation, all rats were evaluated for erectile function and penile histology. Erectile function was assessed using the ratio between ICP and MAP during electrical stimulation of the cavernous nerve. The transplanted MSCs were identified by immunofluorescence, and the smooth muscle cells of the corpora cavernosa and the endothelium were assessed by immunohistochemistry. After transplantation, the ICP/MAP ratio was significantly increased compared with control rats. The contents in smooth muscle and endothelial cells in the corpus cavernosum of transplanted rats were significantly increased. Immunofluorescence showed that MSCs labelled with CM-Dil persist in the corpus cavernosum for at least 4 weeks, and some of the MSCs expressed vWF, CD31, calponin, or α-smooth muscle actin, which are markers of endothelial cells and smooth muscle cells. Thus, the intracavernous transplantation of MSCs confirmed its beneficial effects on the erectile function of patients and increased the content of the endothelium and smooth muscle of the corpora cavernosa.

A similar study was conducted by Gou *et al.* (2011) who examined the effects of transplantation of EPCs that were transfected with the VEGF165 viral gene in the corpus cavernosum of diabetic rats with ED. Diabetes was induced in the rats using an intraperitoneal injection of STZ. Subsequently, previously extracted EPCs were divided into the following three groups and transplanted into rats: group 1 received EPCs transfected with DNA containing the VEGF165 gene; group 2 received EPCs transfected with empty DNA; and group 3, the 'normal' test subject group, received wild-type EPCs. After transplantation, the ICP was monitored and it was found that a significant increase was present only in rats in group 1. In these rats, the histological examinations revealed the extensive induction of cell survival in the corpus cavernosum. The microscopic examination of fluorescence showed that many of the EPCs transplanted in the study group survived and differentiated into endothelial cells integrated at sites of neovascularization. Based on these results, it is possible to conclude that transplantation of EPCs transfected with VEGF165 in the corpus cavernosum of diabetic rats with

ED can restore erectile function. The same group of authors evaluated the effects of MSC transplantation when transfected with the *VEGF164* gene in an adenovirus (Ad-Vegf164) in diabetic mice with ED (Qiu *et al.*, 2012b). Diabetes was induced in 45 rats by injection with STZ, and they were divided into three groups that received the following: (i) PBS intracavernosally (PBS + DM); (ii) unmodified MSCs (DM+); and (iii) MSCs transfected by Ad-Vegf164 (DM + VMSC). Ten rats served as the controls and were intracavernously injected with PBS. Four weeks after the injection, erectile function was measured by electrostimulation of the cavernous nerve, and penile tissue was harvested for histological examination. Erectile function and the contents of smooth muscle and endothelial cells in the corpus cavernosum increased significantly in MSC-injected rats compared with DM + PBS group. Furthermore, there was a more significant improvement in ED, the contents of smooth muscle and endothelial cells, and the concentration of corpus cavernosum VEGF in the group DM + VMSCs, compared with the other two groups. Therefore, this study validates the potential of a combined injection of MSCs and VEGF for the treatment of ED associated with diabetes.

An experimental model based on diabetic rats with ED has been used by Nishimatsu and collaborators to evaluate the effect of ADSC transplantation on the role of cytokines produced by these cells (Nishimatsu *et al.*, 2012). Diabetic male rats were injected with ADSCs or adenovirus at the baseline and after 4 weeks, and the following parameters were evaluated: ICP, MAP and the penile expression of specific proteins for vascular endothelial cells by Western blot. The results confirmed that ADSCs restored erectile function. This effect is particularly apparent when cells are cultured in media containing growth factors for the vascular endothelium, and this effect was associated with an improvement in corpora cavernosa histology and increased expression of endothelial markers such as E-cadherin and eNOS. The most interesting aspect of this study is that reduced expression of adrenomedullin (AM), a vasoactive peptide originally isolated from human pheochromocytoma, was observed. The effect of ADSCs on ED was significantly lower, and reduced expression of E-cadherin and eNOS was observed. In contrast, the overexpression of AM, induced by adenoviral infection, significantly improved erectile function in diabetic rats and was associated with increased expression of E-cadherin and eNOS. These results suggest that AM produced by ADSCs could play an important role in the treatment of ED. Regarding MSCs and EPCs, the ADSCs transfected by a viral vector with gene coding for the VEGF165 have been shown to stably overexpress VEGF in vitro, therefore this could justify their therapeutic use (Sun *et al.*, 2011).

Very recently, the possibility that the administration of melatonin may mobilize EPCs from the bone marrow to the peripheral circulation has been explored (Qiu *et al.*, 2012c). An analysis was performed on STZ-induced diabetic rats, and the concentration of EPCs was measured by flow cytometry, while the extent of oxidative stress in the bone marrow was evaluated by measuring SOD and malondialdehyde. Erectile function was assessed by measuring ICP during electrostimulation of the cavernous nerve, and the density of endothelium and the proportions of smooth muscle and collagen in the corpus cavernosum were assessed by immunohistochemistry. The administration of melatonin resulted in an increased level of SOD and a reduction in the level of malondialdehyde in the bone marrow, as well as an increased level of circulating EPCs. Furthermore, the ICP/MAP ratio in rats in the treated group was significantly higher compared with healthy test subject rats. The histological analysis showed an increase of the endothelial density of the corpora cavernosa after the administration of melatonin, but there were no changes in the proportions of smooth muscle and collagen. Therefore, the chronic administration of melatonin may have a beneficial effect on the prevention of ED in diabetic rats, by promoting the mobilization of EPCs.

Among recent studies carried out to identify new therapies to treat ED, Sun and co-workers evaluated whether bone marrow MSCs (MSC-BM) showed beneficial effects on erectile function in rats with type I diabetes (Sun *et al.*, 2012). In particular, their main objective was to analyse the potential neurotrophic effects of BM-MSCs in STZ-induced diabetic rats. Rats were randomly divided into the following three groups: a control group, a BM-MSC-treated group and a BM-MSC conditioned medium-treated group. Four weeks after intracavernous injection of BM-MSCs or BM-MSC conditioned medium, the ICP was measured to evaluate erectile function, and immunohistochemistry was used to mark the presence of BM-MSCs in the penile tissue and to identify the dorsal nerve fibre of the penis and nNOS-positive neurofilaments (NF). Finally, the concentrations of VEGF, nerve growth factor (NGF) and BDNF were measured in the BM-MSC conditioned medium. BM-MSCs produced detectable levels of VEGF, NGF and BDNF, and the intracavernosal administration of these cells resulted in an improvement of erectile function in diabetic rats. However, after the injection, a time-dependent reduction in BM-MSCs occurred. The method has also proven effective in improving nerve regeneration in diabetic rats, most likely through a mechanism that involves paracrine factors produced by the BM-MSC.

Other stem cell transplantations for the treatment of erectile dysfunction

Another possibility was considered: to repair the damage to the corpus cavernosum via the intracavernous injection of stem cells of the neural crest (NCSCs) that have been transfected by a retroviral vector with the gene v-myc (Song *et al.*, 2008). Two weeks after the injection, specific markers for endothelial cells (such as vWF and CD31) and smooth muscle cells (actin,

Figure 1 Summarizes the current options of the vascular regenerative medicine for the treatment of erectile dysfunction (eNOS: endothelial nitric oxide, VEGF: vascular endothelial growth factor, MSCs: mesenchymal stem cells, ADSCs: adipose tissue stem cells).

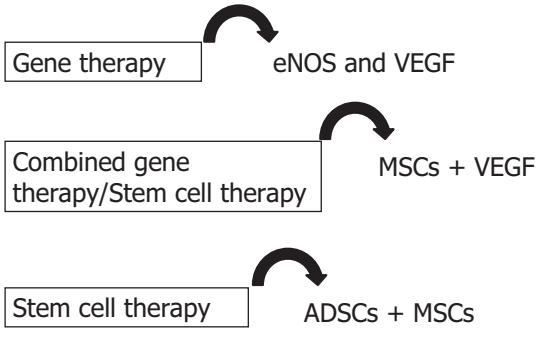

Table 1 Main advantages of stem cell based therapy for erectile dysfunction

Advantages
Enhanced endothelial nitric oxide synthase expression
Transplanted cells showing endothelial and smooth muscle cell markers
Increased content of smooth muscle and endothelium in corpora cavernosa
Enhanced neovascularisation in corpora cavernosa
Increased content of neuronal nitric oxide synthase-positive nerve fibers in penile dorsal nerves
Inhibition of apoptosis in corpora cavernosa
Inhibition of fibrosis and apoptosis
Disadvantages
Potential adverse events
Low transfection efficiency
Risk of chromosomal integration
Potential for malignant transformation

calponin and desmin) were identified via immunohistochemistry. Consequently, NCSCs are an ideal source to regenerate endothelial and smooth muscle cells in the corpus cavernosum of patients with ED. Finally, rat brain stem cells, cultured in a suitable culture medium, differentiate into penile smooth muscle cells and thus may be used for the treatment of ED (Song *et al.*, 2009).

Another potential approach is represented by hMaxi-K gene transfer in men with erectile dysfunction. hMaxi-K is a 'naked' DNA plasmid carrying the human cDNA encoding hSlo (for human slow-poke), the gene for the alpha, or pore-forming, subunit of the human smooth muscle Maxi-K channel. The results of the first human trial were published in 2005. In this study, three patients each were given 500, 1 000 and 5 000 μg, and two patients were given 7 500 μg of hMaxi-K and monitored for 24 weeks. The safety, tolerability and clinical efficacy were evaluated. The authors suggest that the therapy is safe, although the efficacy deserves further evaluation using a control group (Melman *et al.*, 2006). In another and more recent evaluation conducted on eight male cynomolgus monkeys with ED secondary to moderately severe, diet-induced atherosclerosis, the results indicate that intracorporal Maxi-K-channel gene transfer enhances erectile capacity and sexual behaviour (Christ *et al.*, 2009).

CONCLUSIONS

Stem cell therapy is a new frontier in medicine. Previously classified as a virtually recoverable condition, ED has already been shown to be an ideal target for such a treatment. The use of stem cell therapy alone or in association with other pharmacological interventions shows great potential in the care of patients with ED (Fig. 1). PDE5 inhibitors in combination with the direct effects on the mobilization of EPCs, MSCs, eNOS gene therapy, VEGF, adipose tissue stem cells and other stem cells currently represent a promising approach in the field of the vascular regenerative therapy for ED. In addition to the potential applications, the disadvantages and limitations that still remain must be addressed. In fact, these therapies have shown general limitations and specific problems, as shown by previous research in the field of ED. In particular, the primary open problems are represented by the following factors: the low transfection efficiency in most cells, the risk of immunogenicity, the potential chromosomal integration or disruption, the toxicity and the potential for malignant transformation of transplanted cells (Harraz *et al.*, 2010; Hakim *et al.*, 2012). Finally, the combined therapy using stem cells and gene therapy, although it seems to have a positive effect, actually results in the integration of very few cells into the cavernosum corpus. This effect may be primarily mediated by the local release of growth factors. Table 1 summarizes the main advantages of stem cell-based therapy for ED.

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