Swine experimental model to evaluate stem cells implant post myocardial infarction by perfusion gated-SPET

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Abstract

Autologous bone marrow stromal cells (BMSC) implant after swine experimental myocardial infarct (MI) was investigated by serial technetium-99m (^{99m}Tc)-tetrofosmin gated single photon emission tomography (G-SPET) and compared with immuno-histochemical findings. *The aim* was to evaluate if intramyocardial BMSC implant produces any prolonged effect in the left ventricle (LV) perfusion and function. *Eleven pigs underwent* left anterior descending artery (LAD) ligature; in seven of them BMSC were injected in the border zone of the MI, while in the remaining four saline solution was injected at the same site. After LAD ligature G-SPET scans at 48h and at 5 and 10 weeks (w) after the implant were performed. Uptake defect size and LV function analysis were performed comparing 48h to 5w and 10w studies. Statistical evaluation was performed with Friedman test and unpaired Wilcoxon test. The comparison between a progressive reduction of Perfusion Image Score was observed from 48h to 5w and to 10w in the treated group (Friedman test: χ^2= 13.56; P=0.01). *No variation was observed* in the control group (Friedman test: χ^2=3; P= 0.223). Comparison of the absolute variation (Δ) between treated and control group resulted significant (Wilcoxon test W=10; P=0.007). Similar positive results were also observed for the relative extension of the uptake defect, wall motion and LVEF analysis. Histological data of our swine model demonstrated that autologous BMSC implanted in the damaged myocardium area had survived and differentiated into cells with typical features of myocardiocytes. Gated SPET is a reliable tool to evaluate prolonged positive effects of autologous BMSC implant in swine experimental MI model. *In conclusion*, autologous BMSC implanted can improve perfusion, induce cell regeneration, reduce wall motion abnormalities and prevent severe LV dysfunction in swines.

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Introduction

Congestive heart failure (CHF) remains the leading cause of morbidity and mortality in western countries. This medical epidemic is growing continuously due to general increase in aging population and in the number of patients surviving an initial myocardial infarction (MI). Although heart transplantation remains the optimal treatment for eligible patients, the shortage of donor hearts limits this option, which, together with the prevalence of CHF, has promoted the concept of repairing or regenerating, lost myocardium via cell-based treatments.

Swine experimental models have been used to evaluate acute ischemic disease and heart dysfunction since their heart anatomy and function is more similar to that of humans compared to other animals used in laboratory investigations [1-4].

To date, different species of pigs have been used to evaluate pharmacological and innovative stem cell based treatments [5, 6]. Advanced diagnostic imaging techniques are useful tools to evaluate viability, perfusion and function of myocardium in experimental animals and man, in particular, ECG gated single photon emission tomography (G-SPET) associated to standardized software images analysis are reliable in performing serial evaluation of these techniques.

The aim of the present study was to explore the feasibility of implantation of autologous bone marrow stem cells (BMSC) in porcine hearts after MI, in order to investigate the effects on regional perfusion, viability, wall motion and global left ventricular (LV) function by serial myocardial technetium-99m (^{99m}Tc)-tetrofosmin perfusion ECG G-SPET. Furthermore the scintigraphic data of intramyocardial administration of BMSC have been compared with histological and immuno-histochemical findings 10 weeks after implantation to explore differentiation of stromal cells and possible myogenesis and angiogenesis.

Animals, material and methods

Experimental animals

All animal experiments were carried out after approval by the local animal ethics committee and in accordance with the guidelines for care and use of laboratory animals. The study was performed with 11 Landrace conventional pigs aged 4±0.3 months. Prior to inclusion in the study, all pigs were physically examined and confirmed to be healthy and with normal blood pressure. The animals were housed individually in cages, indoors fed dry pig food, and provided with water *"ad libitum"*. The mean body weight of animals was 22.650kg (min 20.100 max 24.950) at the beginning of the study and 43.4kg (min 39.700 max 47.250) at the end.

The study design consisted in dividing the total of 11 pigs in 2 groups: in 7 pigs MI was followed by intramyocardial injection of BMSC, while in 4 pigs (control group) MI was followed by injection of saline solution.

Bone marrow stromal cells preparation

Bone marrow was withdrawn from the iliac wing. A 14G bore biopsy needle and a heparinized needle to collect 40/50mL of bone marrow were used. The cells were washed twice with serum free Iscove's modified Dulbecco's medium (IMDM) by centrifugation, the supernatant was discharged, and the cells were resuspended in the serum free media, in which 4'.6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to DNA was added. A final concentration of 50mg/mL was added to label the obtained BMSC. The numbers of cells were counted using a cell counter and an average 50 million cells were prepared in 1mL of serum for transplantation. For evaluation of survival rate, 100mL of cell suspension was transferred into 2 cell culture dishes and cultured in IMDM containing 2% fetal bovine serum. Twenty-four hours later the number of cells in each dish was counted.

Creation of acute myocardial infarction and cell injection

All animals were medicated with Telazol® (tiletamine-zolazepam; 4 mg/kg) by intramuscular injection. Once an adequate level of sedation was obtained, a catheter was placed in their auricular vein. General anesthesia was induced by intravenous injection of fentanyl (4μg/kg) and propofol (3-4mg/kg) and, after orotracheal intubation, maintained with a mixture of sevoflurane and oxygen, and a constant rate infusion (CRI) of fentanyl (5μg/kg/h).

At the beginning of surgery lidocaine was administered as a bolus of 1mg/kg followed by a CRI of 50μg/kg/min until the end of the surgical procedure. All animals were mechanically ventilated in order to maintain normocapnia ($PETCO_2$ 35-45mmHg). After induction of anesthesia the left femoral vein and left external carotid arteries were incannulated following surgical cut-down in order to measure both central venous and systemic arterial pressures. Through a longitudinal sternotomy, the heart was exposed and the left anterior descending coronary artery (LAD) was tied 1.5cm from the end.

Two hours later, 1mL of solution containing on average 50 million BMSC labeled with DAPI was injected in the border zone of the ischemic area next to the healthy myocardial tissue. Only saline solution was injected into pigs of the control group with the same procedure (Fig. 1).

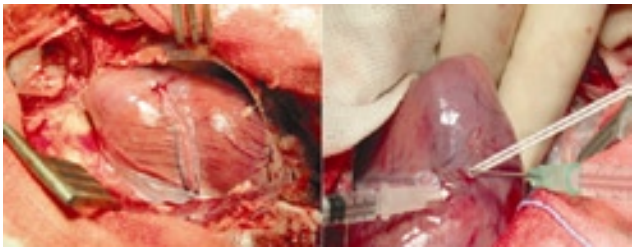

Figure 1. LAD ligature at 1.5cm from the vessel end (left). Intramyocardial injection of 5.0 million of DAPI-labeled autologous BMSC in the border zone of the ischeamic area (right).

At the end of the surgical procedure the animals were transferred to the intensive care unit where they were kept sedated for at least 48h by a CRI containing a combination of propofol, midazolam, fentanyl and lidocaine.

All animals were breathing spontaneously at room air (FiO_2:0.21), with oxygen supplemented via a nasal tube in case the PaO_2 decreased to <70mmHg. The CRI of the drug combination was started at the time of extubation (return of the swallowing reflex) using a fixed rate for each drug component (propofol 80μg/kg/min; midazolam 5μg/kg/min; fentanyl 0.03μg/kg/min; lidocaine 50μg/kg/min) that was later titrated to effect.

The following parameters were monitored during the entire sedation period: systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressures, central venous pressure (CVP), hemoglobin oxygen saturation (SpO_2), electrocardiogram (ECG), heart rate (HR) and rectal temperature (T in °C). Every 8h a sample of arterial blood was analyzed for the following parameters: PaO_2 (mmHg), $PaCO_2$ (mmHg), $P(A\text{-}a)O_2$ (mmHg), HCO_3 (mM), Base excess (BE; mM), [Na+] (mM), [K+] (mM) and [Ca2+] (mM). All the animals survived the surgery.

Myocardial perfusion and left ventricle function evaluation by G-SPET

In order to assess the myocardial perfusion and functional consequences of experimental MI all pigs underwent G-SPET 48h after LAD ligature. To evaluate the effects of BMSC implantation scintigraphic evaluation was repeated 5 and 10 weeks (w) after treatment.

Each time the same scintigraphic technique was used. A dose of 15MBq/kg of ^{99m}Tc-tetrofosmin (Myoview®, GE Healthcare, West Milwaukee, Wisconsin, USA) was injected intravenously while the animals were sedated and 60min later, G-SPET was performed with the animal in the right lateral decubitus position.

Gated SPET data were obtained over an 180° orbit from the right anterior oblique 45° view to the left posterior oblique 45° view, using a large field-of-view gamma camera Infinia (GE Medical Systems, West Milwaukee, Wisconsin, USA). For data acquisition the gamma camera was equipped with low energy high-resolution collimators and a 140keV photopeak with a 20% acceptance window was used (32 views, 30s/view, 64x64 matrix, zoom 1.0, 16 frames/cardiac cycles). The data were stored in a Xeleris workstation (GE Medical Systems, West Milwaukee, Wisconsin, USA). Image reconstruction, display and analysis were performed by standard software package. Filtered back projection was performed with a low-resolution Butterworth filter with a cut-off frequency of 0.5 cycles/pixel, order 5.0. No attenuation or scatter correction was applied.

The LV transaxial tomographic slices were reoriented into the short, horizontal and vertical long-axis views.

Polar maps for all sets of images were also obtained.

The LV analysis of regional myocardial perfusion and function based on the 17 myocardial segments model of ACC/AHA/ASNC Guidelines was performed (Fig. 2).

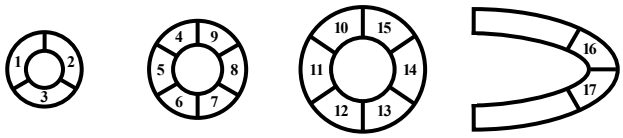

Figure 2. Myocardial seventeen segments model used to evaluate regional LV perfusion and function.

Qualitative perfusion images analysis were carried out blindly by two experienced nuclear physicians using a semi-quantitative four-point grading score method: normal =0, mildly reduced =1, severe reduced =2, and absent ^{99m}Tc-tetrofosmin uptake =3). Segments 1, 3-5, 9-11 and 15-17 were considered LAD artery territory.

Visual classification was done by 2 indipendent observers blinded to the injection of BMSC or saline solution. Discrepancies were resolved by consensus.

The sum of all LV segments score was the expression of the severity of perfusion abnormality, as combination of the extension and the degree of perfusion reduction.

The relative extension of ^{99m}Tc-tetrofosmin myocardial uptake defect was performed by automatic counts based analysis. The voxels with a count value lower than 50% of maximum voxel counts were considered abnormal.

The extension of ^{99m}Tc-tetrofosmin myocardial uptake defect was also expressed as a percentage of the entire LV myocardial mass by automatic dedicated software.

The LV function analysis based on wall motion abnormalities (WMA) and ejection fraction (EF) was performed. The LV end-diastolic and end-systolic volumes were measured with a count-based technique and LVEF was calculated according to cavity size by automatic software.

The WMA analysis was carried out using a semi-quantitative five-points grading score method: normal =0, mildly reduced =1, severely reduced =2, and absent motion =3, dyskinesia =4. This score was obtained by the consensus agreement of two experienced nuclear physicians in blinded fashion.

The sum of all LV segments score was the expression of dysfunction severity, as combination of the extension and the degree of WM abnormalities.

Histology and immunohistochemistry

The pigs were put down 10w after cell implantation. The heart weight was 287.6±5.6gr. The excised heart was fixed with 2% paraformaldehyde phosphate-buffered saline solution. Sections 6mm thick were mounted on a set of gelatin-coated glass slides to ensure different stains could be used on successive sections of tissue cut through the implantation area. One of the sections was mounted without stain so the DAPI-labeled donor cells could be located and viewed with fluorescence microscopy. Other sections were selected for immunostaining of connexin 43, desmin and troponin C with rabbit or mouse polyclonal antibodies.

Statistical analysis

Comparison among measures at different times (48h, 5w and 10w) was performed by the Friedman test, and a P value <0.05 was considered significant.

The absolute variation (Δ) among first and last evaluation was compared between treated and control groups by unpaired Wilcoxon test.

Results

All animals survived at 10w. Statistical evaluation was performed in all of the eleven pigs according to the pre-defined protocol.

In all series of images performed at 48h, 5w and 10w, severe ^{99m}Tc-tetrofosmin myocardial uptake defects were found only in LAD artery territory and involved antero-lateral, anterior, antero-septal and apical segments of the left ventricular walls (Fig. 3).

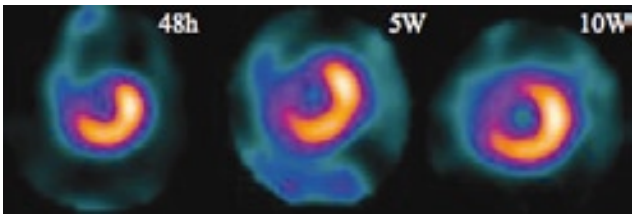

Figure 3. ^{99m}Tc-tetrofosmin G-SPET of pig number 2. Short axis images at 48h post LAD ligature (top) and 5 (middle) and 10 weeks (bottom) post-BMSC intramyocardial implant. Radiopharmaceutical uptake defect of medial and distal segments of antero-septal, anterior wall and apex was observed at first evaluation. In 5 and 10 weeks evaluation a progressive improvement of radiopharmaceuticals uptake in the border zone of ischaemic area as well as the reduction of uptake defect size were observed. Left ventricular growth is evident by comparing the 48h to the 10w studies.

Qualitative images analysis of radiopharmaceutical uptake severity showed a slight improvement of myocardial perfusion radiopharmaceutical after 5w, and a further improvement at 10w. A significant progressive reduction of Qualitative Perfusion Image Analysis Score was observed from 48h to 5w and to 10w in the treated group (Friedman test: χ^2=13.56; P=0.01). No significant variation was observed in the control group (Friedman test: χ^2=3; P=0.223). Comparison of the absolute variation (Δ) between treated and control group resulted significant (Wilcoxon test W=10; P=0.007) (Fig. 4A). The score decreased in the myocardial segments, which were localized in the marginal infarct areas.

A significative reduction of Relative Extension of the Uptake Defect at 48h, 5w and after 10w was observed only in the treated group (Friedman test: χ^2=14; P=0.001). No significant variation was observed in the control group (Friedman test: χ^2=0; P=1). Comparison of the absolute variation (Δ) between treated and control group resulted significant (Wilcoxon test W= 10; P=0.008) (Fig. 4B).

Significative reduction of WMA analysis using a five points scoring scale and a progressive decrease of LV wall motion abnormalities at 48h post LAD ligature and at 5 and 10 weeks post BMSC implant was observed only in the treated group (Friedman test in the treated group: χ^2=13.56; P=0.001. Friedman test in the control group: χ^2=1.86; P=0.395).

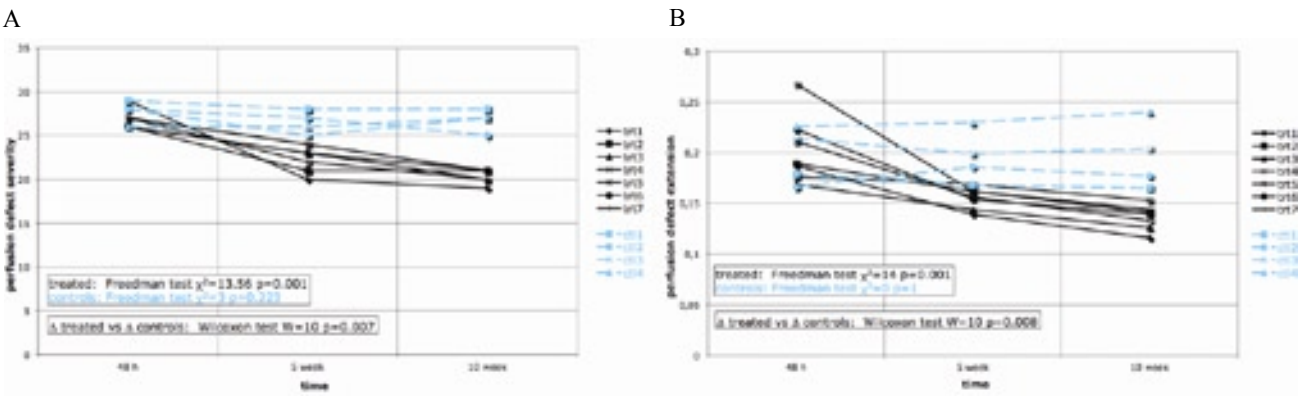

Figure 4. (A) Modification of the LV severity of ^{99m}Tc-tetrofosmin uptake defect at 48h post LAD ligature and at 5 and 10 weeks post-BMSC intra-myocardial implant expressed by 4 points scoring scale qualitative images analysis. A progressive reduction of radiopharmaceuticals uptake defect was observed. A progressive reduction of Qualitative Perfusion Image Analysis Score was observed from 48h to 5w and to 10w only in the treated group. **(B)** Modification of relative extension of LV myocardial ^{99m}Tc-tetrofosmin uptake defect. A significative reduction of Relative Extension of the Uptake Defect at 48h, 5w and after 10w was observed only in treated group.

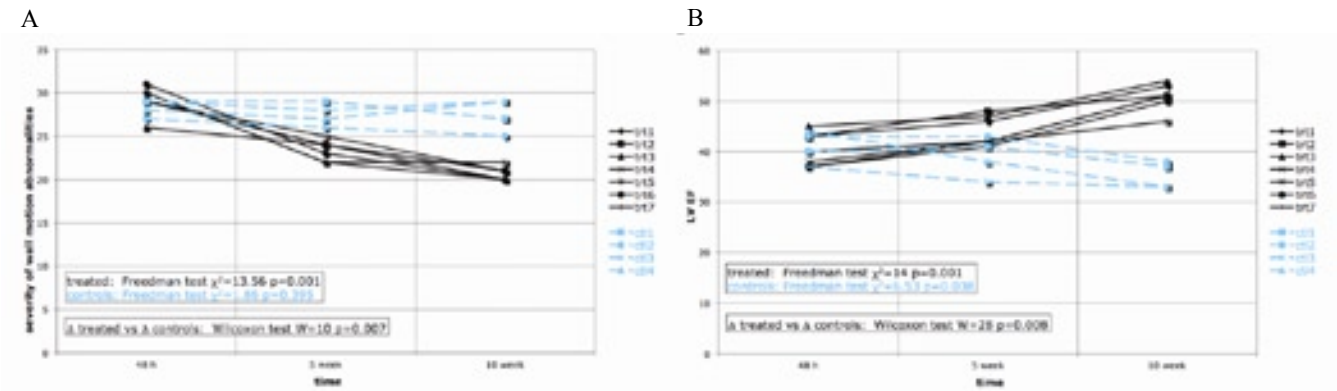

Figure 5. (A) Significative reduction of WMA analysis using a 5 points scoring scale and a progressive decrease of LV wall motion abnormalities at 48h post LAD ligature and at 5 and 10w post BMSC implant was observed only in treated group. **(B)** A progressive increase of LVEF was found in serial evaluation of LVEF at 48h post LAD ligature and at 5 and 10w post BMSC implant only in treated group.

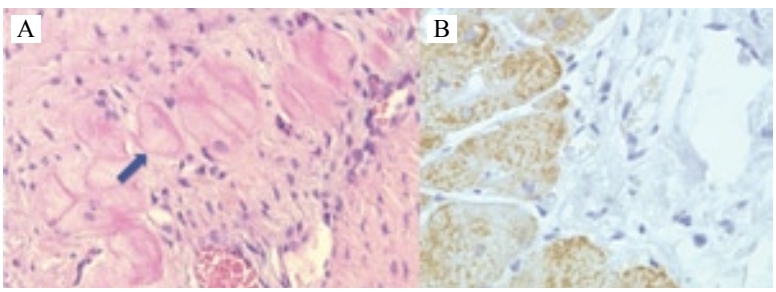

Figure 6. A. Histological-immunochemistry myocardial cells image at 10w after BMSC implant: DAPI labeled cells incorporated in the myocardium were detected (blue arrow). These cells tend to align themselves, have abundant cytoplasm and are larger than normal myocardiocytes. **B:** Histological-immuno chemistry myocardial cells image at 10w after BMSC implant: troponin C (dark brown) expressed by BMSC derived cells was observed.

Comparison of the absolute variation (Δ) between treated and control group resulted significant (Wilcoxon test W=10; P=0.007) (Fig. 5A). A progressive increase of LVEF was found in the serial evaluation at 48h post LAD ligature and at 5 and 10 weeks post BMSC implant only in the treated group (Friedman test: χ^2=14; P=0.001) while in the control group a little worsening of LVEF was observed (Friedman test: χ^2=6.53; P=0.038).

Absolute variation among first and last observations were significantly different between treated and control groups (Wilcoxon test W=28; P=0.008) (Fig. 5B).

No LV dilation or segmental dyskinesia was observed in the treated group.

Histology and immunohistochemistry

A number of DAPI-labeled cells were incorporated in the host myocardium (Fig. 6A). They were characterized by a large diameter (3-4 times that of a normal myocardiocyte) and had aligned to form a syncytium. Several vessels surrounded it. The cells were found to be able to express desmin and troponin-C (Fig. 6B), typical markers of a myocardial differentiation, and connexin 43, which indicates the formation of gap junctions.

Discussion

Congestive heart failure may represent a dangerous end-stage of ischemic heart disease. The onset of ischemia and the progression to infarction and dysfunction are three stages that cause high morbidity and mortality in humans [7-12].

Preclinical studies on reperfusion and regeneration of transplanted cells in the myocardium have shown to be of potential therapeutic value in preventing serious dysfunc-

tion [13-15]. These therapeutic approaches have been addressed due to the shortage of heart donors. Micropigs and pigs are steadily gaining importance as animal models in the field of regenerative treatments including stem cells research.

To translate the results obtained from small sized experimental animals it is often necessary to test larger animals. The size, anatomy, physiology and organ function of pigs resemble that of humans and so they are considered a good challenge for preclinical studies. Compared to other mammalian species, pigs have organs and systems that resemble the human anatomy, physiology, organ function and immune system features. In particular, the heart and large blood vessels are more similar to humans and offer a good model for research in this field [16]. Pigs are also easier to handle and present less ethical controversy compared to non-human primates.

The intramyocardial administration has been chosen because it is the more direct method for correct cells distribution in the myocardium and to avoid the interference caused by the passage from blood to myocardium. Furthermore with this administration is possible to inject nutritional factors which can act as a scaffold that provides a suitable microenviroment for the BMSC to adhere and perform normal cellular function, and differentiation stimulating factors for BMSC into endothelial cells and smooth muscle cells which promotes angiogenesis and prevents further apoptosis of cardiomyocytes within the infarct zone, leading to the preservation of cardiac function [17]. Nevertheless, intracoronary stem cells infusion was also proposed in various studies with different results. The most recent of these is the SCIPIO study, which suggested intracoronary infusion of autologous cardiac stem cells in humans [18].

The cardiac output of porcine hearts is similar to that of humans and any differences are due to different posture and gravity. Pigs are also considered the most suitable donors of organs and tissue for transplantation based treatments. Others also described many similarities in echocardiographic evaluation of human and pig hearts [19].

Our experience indicates that, in a swine model, it is possible to induce LAD artery infarction and to evaluate the follow up of sophisticate treatments that produce their positive effects several days after administration.

Numerous studies demonstrate that the implantation of cells into damaged myocardium could regenerate the infracted area and the accumulated evidence suggests that cells transplantation may prevent heart failure [8-12, 20, 21]. In addition, cardiac function has been shown to improve after cardiomyocyte cells transplantation [22].

Recently, literature data suggest that stem cells, such as bone marrow mesenchymal stem cells, could be used to regenerate damaged myocardium [11, 23, 24].

At the end of an observation period of 10w post-implantation we found numerous vital DAPI-labeled BMSC in the MI induced area. Histological data of our swine model demonstrate that BMSC implanted in margins of the damaged myocardium area survive, can improve perfusion, induce cells regeneration, reduce WMA and prevent severe LV dysfunction.

We preferred to use autologous cells transplantation to avoid immunosuppression treatment during the study as this can induce serious side effects.

In this study the observation was extended to 10w, which was previously done only by Jameel et al (2010) that extended the outcome to 4 months [25]. It is thus possible to evaluate the long-term effect of BMSC on myocardium and of their initial differentiation.

Technetium-99m-tetrofosmin is a lipophilic cationic diphosphine, routinely used for the diagnosis and risk stratification of coronary artery disease. It enters into viable myocardial cells through passive transport driven by the negative membrane potential of the intact cell and is localized mainly within the cytosol while only a fraction passes into the mitochondria and the degree of uptake is strictly correlated with regional perfusion [26]. Technetium-99m-tetrofosmin does not undergo significant redistribution from its initial pattern of uptake, therefore imaging can be performed for up to 4h after injection. In nuclear cardiology, G-SPET imaging by perfusion radiopharmaceuticals has provided the one step possibility of assessing cardiac function and perfusion simultaneously as well as viable myocardium [27] with a sensitivity of 92% and a specificity of 80%. Recently, others established that ^{99m}Tc-tetrofosmin G-SPET is an adequate technique for estimating the heart functions of healthy pigs [19] with an adequate dose [23].

The software package for automatic evaluation of function and perfusion is a reliable tool to detect heart impairment after MI and possible positive effects of BMSC implant without intra or inter-observer variability.

In this study, using ^{99m}Tc-tetrofosmin G-SPET, the experimental occlusion in the medial portion of LAD coronary artery induced a necrotic area of 16%-20% of the whole LV walls according with data reported by others [2, 4].

No LV remodeling, dilatation or aneurysms were observed in the acute phase, at 5w or 10w post infarction. Moreover, after BMSC intramyocardial injection, a slight but progressive improvement of perfusion in the edges of the infarcted area was observed.

Histological examination showed that DAPI-labeled BMSC implanted in the MI area tend to differentiate into elements which exhibit typical features of cardiomyocytes, notably a tendency to form a syncitium and the ability to express connexin 43, desmin and troponin C. These BMSC-derived cells are three to four times larger than normal myocardiocytes and are typically surrounded by several new vessels. The DAPI technique permits recognition of normal myocardyocites as those derived from transplanted BMSC. The presence of specific smooth myocardial muscle cells demonstrates the good differentiation and function of implanted BMSC. These data concord with the improvement of myocardial ^{99m}Tc-tetrofosmin uptake and of regional and global LV function in the 7 treated pigs, suggesting that the implant of BMSC has produced a beneficial effect on global cardiac function through a perfusion improvement of the marginal necrotic areas. Others suggested that implanted bone marrow stromal cells could contribute to preserve the ventricular function and prevent ventricular expansion [23]. In the above mentioned study the LV end diastolic volume was significantly smaller in treated versus untreated pigs.

The edge of MI area presented contemporary viable and necrotic cells. These cells can favor development and growth of BMSC and also favor differentiation of the BMSC cells to typical myocardial cells.

The engrafted cells might provide endothelial progenitor cells, which could participate in the formation of new blood vessels [28], or could release vascular endothelial growth factors to induce new vessels formation or vessels growth.

Others demonstrated that unrestricted somatic stem cells

transplanted into the infarcted myocardium in a porcine model survived in scar tissue and prevented scar thinning and LV dilation [29].

Analyzing segmental LV myocardial perfusion, we found a reduction of ^{99m}Tc-tetrofosmin myocardial Uptake Defect Size localized exactly around the injection site. The improvement of ^{99m}Tc-tetrofosmin uptake, due to better perfusion and to more viable cells, is also in concordance with improvement in segmental kinetics and LVEF. According to perfusion data, the regional WMA analysis showed that dysfunction localized in a marginal area of infarction in the treated pigs was reduced, while in the control group dysfunction was the same. Furthermore, in the control group, LVEF was rather worse.

The number of BMSC we used was 50 million, which showed a positive effect on regional myocardial perfusion, viability and function. Up to date various kinds and numbers of cells were used, so further studies are necessary to establish the minimum efficacious cell number and the injected volume.

The duration of experimental observation was limited to 10 w, after then the DAPI labeling marker washes out and is no longer recognizable.

The growth of the pigs during the observation period may have influenced our results because the increase of body size and weight corresponds to an increase in heart weight, size and myocardial mass. During the growth phase of our animals there may be endothelial growth factors that stimulate vessels formation and myocardiocytes multiplication and differentiation. Nevertheless, the control group in our study didn't show any improvement of the perfusion defects or of LV function; the comparison of the treated and control groups data allow us to exclude the possible bias that might had been induced by the growth of our animals.

The comparative analysis between treated and control groups indicates that the reduction of the necrotic area was a consequence of the BMSC implant.

Recently, others suggested " a new momentum to cardiac cell therapy" by publishing the preliminary data of the first phase 1 trial in humans with promising results on the efficacy of autologous cardiac stem cells that improve LV systolic function and reduce infarct size in patients with heart failure after MI [18, 30].

In conclusion, this study in swines suggests that autologous BMSC implanted into acute myocardial infarcted tissue are able to survive for 10w and differentiate into new cells with typical features of normal myocardiocytes. Present findings suggest that BMSC could play an important role in repairing damaged myocardium after myocardial infarction and improve cardiac function. Further studies are required to evaluate, in animal models, the survival rate of the implanted cells, their final fate and the long-term beneficial effect of BMSC on the global function of a failing heart.

The authors declare that they have no conflicts of interest.

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