ORIGINAL ARTICLE

Implant of mesenchymal cells decreases acute cellular rejection in small bowel transplantation

El implante de células mesenquimales disminuye el rechazo celular agudo en el trasplante de intestino

Marta Navarro-Zorraquino[1], Cristina Pastor[2], Pablo Stringa[3], Joaquín Soria[4], Francisco Hernández[5], Manuel López-Santamaría[5], and Felícito García-Alvarez[1]*

[1]Department of Surgery, School of Medicine, Universidad de Zaragoza, Zaragoza, Spain; [2] Service of Experimental Surgery, Instituto Aragonés de Ciencias de la Salud, Zaragoza, Spain; [3]School of Exact Sciences, Instituto de Estudios Inmunológicos y Fisiopatológicos, La Plata, Argentina; [4]Service of Pathological Anatomy, School of Medicine, Universidad de Zaragoza, Zaragoza, Spain; [5]Department of Pediatric Surgery, Hospital Universitario La Paz, Madrid, Spain

Abstract

Objective: *The objective of the study was to show adipose tissue-derived mesenchymal stem cells (AD-MSCs) immunomodulatory effects in small bowel transplantation (SBTx).* **Materials and methods:** *Forty Wistar Han rats (age: 10-12 weeks): were allogenic receptor rats and were allotted in 2 groups. Control group: rats undergoing orthopic SBTx ; AD-MSCs group: rats undergoing orthotopic SBTx plus AD-MSCs. Male Lewis rats were allogeneic small bowel donors. Rejection was confirmed by histological study of the explanted intestine, enterocyte apoptosis was determined in crypts and the lamina propria of the small bowel. Cytokine concentration levels (enzyme-linked immunosorbent assay) (interleukin [IL]-4, IL-10, IL-12, IL-17, IL-21, IL-23, tumor necrosis factor-alpha, and transforming growth factor [TGF]-b1) and cell percentages (flow cytometry) (CD3+ CD4+, CD8+, CD4+/25+, CD8+/25+, CD4+/25+/Foxp3+, and CD8+/25+/Foxp3+) were assessed in peripheral blood preoperatively and after death.* **Results:** *Treatment with AD-MSCs produced a significantly lower risk of rejection in the first 7 postoperative days (five rejection cases among 20 rats in the control group and only one case in the AD-MSCs group). T_{reg} cells and TGFb1 levels showed a significant increase in the AD-MSCs group.* **Conclusions:** *The local implantation of AD-MSC in the anastomosis and the intestinal lumen can induce a regulatory immune response, by increasing the percentages of Treg cells and TGb-1 levels, leading to a lower risk of acute rejection by cell mediation, in the first 7 days of the intestinal transplant. We think that the implantation of AD-MSCs, in the anastomoses and in the lumen of the donor intestine, could give rise to a chimera of donor-recipient cells.*

Key words: *Bowel transplantation. Rejection. Mesenchymal stem cells.*

Resumen

Objetivo: *Mostrar el efecto inmunomodulador de las células madre mesenquimales (AD-MSCs) en el trasplante de intestino delgado (SBTx).* **Método:** *40 ratas Wistar Han (edad: 10-12 semanas): grupo control (SBTx) y grupo AD-MSCs (SBTx + AD-MSCs implantadas en las anastomosis distal y proximal del intestino delgado y en la luz intestinal). El intestino delgado provino de ratas Lewis. El rechazo se confirmó histológicamente. Se estudió la apoptosis de los enterocitos en las criptas y en*

Correspondence:
*Marta Navarro-Zorraquino
Antonio Val-Carreres, 7, 2ºB
C.P. 50004, Zaragoza, Spain
E-mail: martana@unizar.es

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la lámina propia del intestino delgado. Se determinaron por ELISA las citocinas (IL-4, IL-10, IL-12, IL-17, IL-21, IL-23, TNF-α, TGF-b1) en sangre periférica y por citometría de flujo los porcentajes celulares (CD3+ CD4+, CD8+, CD4+/25+, CD8+/25+, CD4+/25+/Foxp3+, CD8+/25+/Foxp3+) en el preoperatorio y después de la muerte. **Resultados:** *El empleo de AD-MSCs se asoció a una disminución significativa del riesgo de rechazo en los primeros 7 días posoperatorios (cinco casos de rechazo de 20 ratas en el grupo control y un solo caso en el grupo AD-MSCs). Las células T_{reg} y los valores de TGFb1 mostraron un incremento significativo en el grupo AD-MSCs.* **Conclusiones:** *El implante local de AD-MSCs en las anastomosis del trasplante de intestino delgado podría disminuir el rechazo celular agudo. Pensamos que la implantación de AD-MSCs, en las anastomosis y en el lumen del intestino donante, podría dar lugar a un quimera de células donante-receptor.*

Palabras clave: *Trasplante de intestino. Rechazo. Células madre mesenquimales.*

Introduction

Patients with intestinal failure due to different causes who fail total parenteral nutrition require small bowel transplantation (SBTx) as the only possible treatment. SBTx is increasing in clinical procedures, due to advances in surgical techniques and immunosuppressant treatments. The specific immune response from SBTx induces a major risk of rejection and infection as compared to other solid organ transplantation. Acute and chronic rejection and post-operative infections after SBTx remain the most feared complications. Some authors have reported that control of the acute cellular rejection (ACR) improves early graft survival and may enhance long-time survival[1]. In addition, subclinical allogeneic rejection in the initial post-operative period of SBTx shows a negative influence on graft survival[2].

After achieving the differentiation, obtaining, and cultivation of mesenchymal stem cells (MSCs), there have been early clinical studies on tissue regeneration, to achieve a cure for various diseases through the implant[3-6]. In this decade, there have been some studies that refer to the possible role of MSCs in the transplantation of organs and tissues[7-10] and more recently intestinal transplantation[11-14]. The first results are encouraging, but the biological aspects are still unknown at the cellular and molecular level, the regenerative process obtained, and especially the immune response involved in tissue regeneration, inflammatory process, and possible immunomodulation of these cells to prevent or mitigate rejection. Experiments "*in vitro*" and "*ex vivo*" show a large immunomodulator potential of MSCs and the absence of adverse effects in clinical application, in particular in many aspects of surgery: acute inflammatory reaction after surgical trauma, healing of wounds, vascular and visceral anastomoses, and the rejection of organ and tissue transplants. Intestinal transplantation in human clinical practice may be benefited by MSCs use, because it currently has a higher percentage of failures, due to the increased incidence and severity of rejection and the occurrence of serious infections. Nevertheless, experimental results are far from being able to be transferred to human clinical practice.

In experimental studies, the majority of authors infuse MSCs in arterial or venous systems and conclude that the immunomodulatory effects of inoculation of mesenchymal cells systemically (in the bloodstream) are similar to those obtained by local administration (in this case, the transplanted organ); these authors think that immunomodulatory effects produced by mesenchymal cells are independent of the mode of administration and delivery of cell implant, in animal models. Following Gao et al.[14] and Lam et al.[15], we think that most infused MSCs are trapped by the lung; in addition, systemic arterial infusion could induce embolism or occlusion. Some authors have studied immunological response in SBTx since 1992[16,17]. In the present study, we obtained AD-MSCs from adipose tissue of a syngeneic animal, therefore in human clinical practice, it could be obtained from the recipient. We tried to avoid rejection by means of local implantation of AD-MSCs in anastomoses and intestinal lumen during SBTx surgery. Regarding the immune response, this study seeks to obtain a donor-recipient cell immunological chimera by means of AD-MSCs activating Treg cells function and moderating immune response through the Th1 pathway.

Materials and methods

Animals

Male Wistar Han rats (280-340 g) (age: 10-12 weeks) were employed as SBTx orthotopic receptors and to obtain AD-MSCs, and male Lewis rats (200-220 g) were employed as allogeneic SBTx donors. Animals were obtained from the laboratory (IFFA Inc., Lyon, France) and were housed individually in standard

facilities, maintained on a 12 h light/dark cycle, at temperature 22-24°C and provided with commercially available chow and water *ad libitum*. Food was withheld from both donor and recipient animals for 24 h before surgery. All experimental procedures were approved by the Animal Care and Research Committee and were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Forty Wistar Han rats were allotted to two groups: (1) control group, rats undergoing orthotopic SBTx (rats receiving placebo: normal saline solution) and (2) AD-MSCs group, rats undergoing orthotopic SBTx receiving adipose derivate mesenchymal cells.

Surgery procedure

Rats were anesthetized by means of a sevoflurane inhalation (4% to induction and 3% to maintain doses). After anesthesia, rats received ceftriaxone intramuscularly (50 mg/Kg) and meloxicam (Metacam®, Boehringer Ingelheim, Barcelona, Spain) subcutaneously (0.2 mg/Kg) and underwent surgery using the orthotopic SBTx model based on Kort's orthotopic transplantation technique[18], with modifications[16,17]. At the moment of carrying out both distal and proximal donor-receptor anastomoses, 1 mL of normal serum solution (placebo group) or 1 mL normal serum solution containing 1×10^6 MSCs (AD-MSCs group) were injected in intestinal donor subserosal by means of an 8G needle and 2×10^6 MSCs in intestinal lumen. One day previous to surgery and after surgery, rats received subcutaneously a 0.2 mg/day dose of cyclosporin (Sandimmun®, Novartis) until the end of the experiment.

Isolation and characterization of AD-MSCs

Cells were isolated from the abdominal adipose tissue of male Wistar Han rats. MSCs were obtained using the Zuk et al. technique[19]. The cells isolated from adipose tissue rats were confirmed as AD-MSCs based on their morphology, adherence to plastic, and ability to differentiate into chondrocytes, adipocytes, osteocytes, and hepatocytes *in vitro*. Flow cytometry showed that the AD-MSC preparations were 95% pure and that 98% of these cells were positive for CD29, CD90, and RT1A and negative for CD34, CD45, and RT1B.

Clinical manifestations

Rats underwent euthanasia when showing clinical manifestations of rejection, anastomosis dehiscence, infection, or severe post-operative complications. Deaths occurring within the first 72 post-operative hours were attributed to surgical failures and these animals were excluded from the study. Allograft rejection was diagnosed clinically according to the criteria of Schraut and Lee[20].

Histopathological analysis

The presence of rejection was confirmed by histological study of the explanted intestine. Two fragments of 1 cm of length containing the proximal and distal anastomoses, respectively, plus a portion of intestine (1 cm) equidistant from the anastomoses were taken. Intestinal tissues were fixed in 10% formalin, embedded in paraffin, and cut into 5 mm sections, which were stained with hematoxylin and eosin. The slides were blindly reviewed and rejection was graded as (a) moderate (great mononuclear cell infiltration of the intestinal wall but moderate destruction of villi), (b) intense (great mononuclear cell infiltration and destruction of villi), and (c) massive (massive mononuclear cell infiltration and total destruction of villi). Enterocyte apoptosis was determined in crypts and the lamina propria of the small bowel by means of the Gavrieli et al. technique[21].

Flow cytometry

Serum was obtained from peripheral blood of recipient rats, 10 days before surgery and when euthanasia was performed or death was observed. CD3+ CD4+, CD8+, CD4+/25+, CD8+/25+ CD4+/25+/Foxp3+, and CD8+/25+/Foxp3+ cell percentages were assessed in peripheral blood at pre-operative situation and after euthanasia procedures or spontaneous death. Cell percentages were determined by flow cytometry (Gallios™ Flow Cytometer, Beckman Coulter) using monoclonal antibodies (Anti-rat CD25 APC 17-0390 [eBioscience]; Anti-Mouse/rat Foxp3 PE 12-5773 [eBioscience]; Anti-Rat CD8a FITC 11-0084 [eBioscience]; Anti-Rat CD4 FITC 11-0040 [eBioscience]; Anti-RAT CD45 V450 561587 [BD Horizon]; and RAT T lymphocyte Cocktail 558493 [BD Pharmingen]).

Enzymoimmunoanalysis (enzyme-linked immunosorbent assay [ELISA])

Recipient serum was obtained from peripheral blood 10 days before surgery and when euthanasia was performed or death observed. Cytokine concentrations

Table 1. Cause of death and rejection grade in placebo and MSC groups during the first 7 post-operative days

Day	Placebo group (n = 20)				AD-MSCs group (n = 20)			
	nº. of deaths	Cause of death	Rejection grade	Necropsy findings	nº. of deaths	Cause of death	Rejection grade	Necropsy findings
Day of surgery	6	Surgery			6	Surgery		
1st	1	Surgery			1	Surgery		
4th	1	Rejection	Massive	Rejection	1	Euthanasia	None	Anastomoses dehiscence
5th	1	Rejection	Massive	Rejection	1	Euthanasia	None	Anastomoses dehiscence
	1	Euthanasia	Moderate	Rejection	1	Euthanasia	None	Anastomoses dehiscence
	1	Euthanasia		Lumen hemorrhage				
6th	1	Rejection	Intense	Rejection				
	1	Euthanasia		Anastomoses dehiscence				
7th	1	Rejection	Massive	Rejection	1	Euthanasia	Intense	Rejection
	1	Euthanasia		Anastomoses dehiscence				
Remaining rats	5				9			

were measured by ELISA kits as described by the manufacturers, including comparisons with standard curves. Kits used and minimum detectable concentrations of interleukins (IL): IL-4: 1.5 pg/ml (RayBiotech Inc., Norcross, GA, USA); IL-10: 10 pg/ml (RayBiotech Inc., Norcross, GA, USA); IL-12: 9.375 (Elabscience, Bethesda, MD, USA); IL-17: 23.43 pg/ml (Cusabio, Hubei, China); IL-21: 3.3 pg/ml (Merck-Millipore, Billerica, MA, USA); IL-23: 0.196 pg/ml (Merck-Millipore, Billerica, MA, USA); tumor necrosis factor-alpha (TNF-a): 15 pm/ml (Diaclone, Besancon Cedex, France); and transforming growth factor (TGF)-b1: 11.4 pg/ml (Milliplex®, Merck-Millipore, Billerica, MA, USA).

Statistical analysis

Appearance date, grade of rejection, and spontaneous death due to rejection were confirmed by histopathological analysis and apoptosis grade. Data were analyzed by means of nonparametric tests: the Mann–Whitney U-test to study differences between means and rates was compared between groups using the Kaplan–Meier analysis. Log-rank (Mantel-COX) testing was used to ascertain the significance of survival differences between groups[22,23]. CD3+ CD4+, CD8+, CD4+/25+, CD8+/25+ CD4+/25+/Foxp3+, and CD8+/25+/foxp3+ cell percentages and cytokine concentrations data were analyzed by means of ANOVA tests. Differences between groups were compared using paired Student's t-test. $p < 0.05$ was considered statistically significant. Results are expressed as mean ± standard deviation.

Results

Table 1 shows the number of rats undergoing euthanasia or suffering death due to cell acute allograft rejection or surgical causes and date and also the grade of rejection. In AD-MSCs group, one rat died due to rejection. In the AD-MSCs group, nine rats survived the first 7 post-operative days, four rats of these underwent euthanasia at the 7th day to obtain necropsy, and none of them presented rejection signs. For the late post-operative period (more than a week after surgery), we studied five rats in each group: we observed one rejection case 12 days after surgery in the control group, and one rejection case 16 days after surgery in the AD-MSCs group. The remaining rats (three rats in each group) did not show signs or symptoms of rejection from the 18th post-operative day until euthanasia 4 months after surgery. Figure 1 shows the Kaplan–Meier recipient survival curve and the statistical study of differences between groups with regard to survival and acute rejection. AD-MSCs improved the recipient survival (death due to rejection) ($p = 0.002$) (Regressión coefficient: placebo vs. AD-MSCs $p = 0.017$; COX regression:

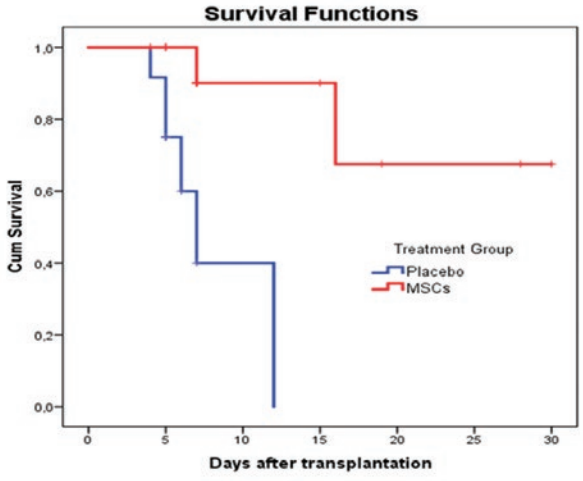

Figure 1. *Kaplan–Meier recipient survival curve.*

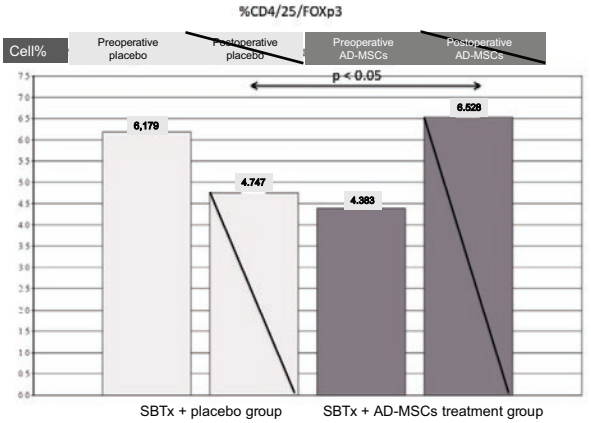

Figure 3. *%CD4/25/FOXp3 variations in peripheral blood, at pre- and post-operative period.*

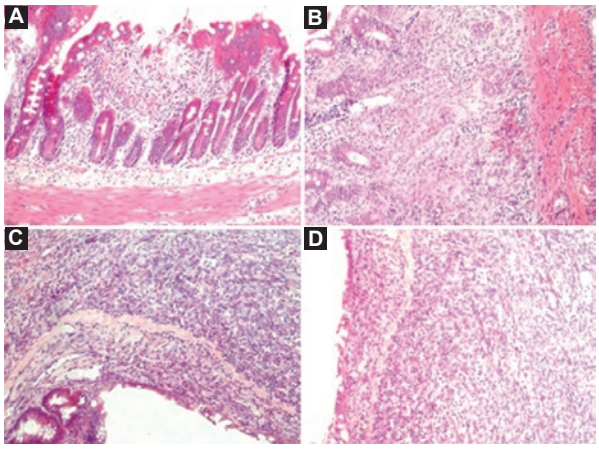

Figure 2. *Degrees of rejection assessed morphologically.* **A:** *mild-moderate rejection with incipient villous decapitation and predominantly mucosal lymphoid infiltration.* **B:** *intense rejection with high lymphoid infiltrative component in mucosa and intestinal submucosa with cryptovillous architectural destructuring.* **C and D:** *massive rejection with architectural abolition of the intestinal wall and intense lymphoid infiltrates.*

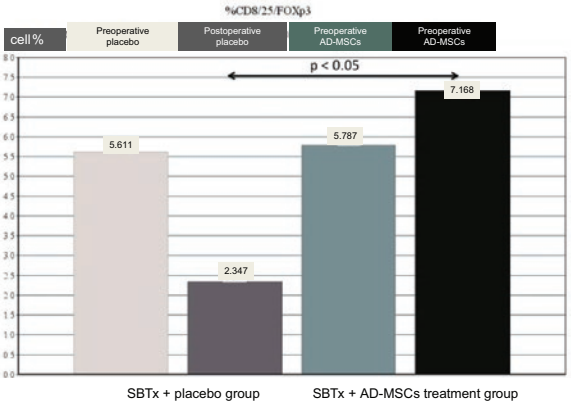

Figure 4. *%CD8/25/FOXp3 variations in peripheral blood, at pre- and post-operative period.*

placebo vs. AD-MSCs HR: 13.08). Treatment with AD-MSCs produced a significantly lower risk of rejection in the first 7 post-operative days (five rejection cases in the control group and only one case in the AD-MSCs group). Treatment with AD-MSCs associated with 92.8% reduction of the risk of death due to rejection. Figure 2 presents degrees of rejection assessed morphologically.

We did not find any differences in %CD3, %CD4, %CD8, %CD4/25, and %CD8/25 between placebo and AD-MSC groups in the pre-operative or in the post-operative studies in peripheral blood. Figures 3 and 4 show %CD4/25/FOXp3 and %CD8/25/FOXp3 variations in peripheral blood, respectively, at pre- and post-operative period; at post-operative period, we observed significantly higher cell percentages of CD4/25/FOXp3 and CD8/25/FOXp3 in the AD-MSCs group with regard to the control group. Studied cytokine levels (IL-4; IL-10, IL-12, IL-21, IL-23, and TNF-a) in peripheral blood did not show significant variations between groups. Nevertheless, AD-MSCs group showed lower IL-17 levels (not statistically significant) in the post-operative period (placebo group IL-17 4.384 ± 3.093; MSC group 2.586 ± 1.436 pg/ml). On the other hand, the placebo group presented lower post-operative TGFb-1 levels ($p<0.05$) than the AD-MSCs group in peripheral blood (placebo group 33.947 ± 14.312 vs. AD-MSCs group 49.504 ± 5.933 pg/ml).

Discussion

In 1993, Thomas Starzl and colleagues discussed how many of the enigmas of transplantation

immunology can be explained by chimerism[24]. In 2004, Starzl[25] related that: *"with the discovery in 1992 of small numbers of donor leukocytes in the tissues or blood of long-surviving organ recipients (microchimerism), we concluded that organ engraftment was a form of leukocyte chimerism-dependent partial tolerance. In this initially controversial paradigm, alloengraftment after both kinds of transplantation is the product of a double immune reaction in which responses, each to the other, of coexisting donor and recipient immune systems results in variable reciprocal clonal exhaustion, followed by peripheral clonal deletion."* When in 2008, we began to study the action of MSCs on SBTx immune response, we considered the possibility of achieving a "microchimera" between the cells of the donor and the recipient through the AD-MSCs immunomodulation potential.

In 2015, Grant et al.[26], on behalf of the Intestinal Transplant Association, reported an "intestinal transplant registry report" since 1985, with 2887 transplants reported in 2699 patients. This study shows that current actuarial patient survival rates are 76%, 56%, and 43% at 1, 5, and 10 years, respectively, with no improving in rates of graft loss beyond 1 year. Data suggested that grafts including colon segment had better function, inclusion of a liver component, and maintenance therapy with rapamycin were associated with better graft survival[26]. Grant et al.[26] referred that results of clinical intestinal transplantation had modestly improved over the past decade[26]. Clinical indications to carry out the SBTx are increasing[27]. In this decade, a few clinical studies have been reported using MSCs as immunoregulators in SBT; but until now, studies have not obtained a conclusive clinical approach[28,29]. Recently, in clinical liver transplantation, immune treatment by means of Th_1/Th_2 pathway response modification with Treg expanded *"ex vivo"* application has been reported and shows a much better future in solid organ transplantation to prevent rejection[30].

SBTx, in the present study, was orthotopic, similar to that performed in human SBTx. AD-MSCs were obtained from the fat tissue of the recipient (isogeneic rats). AD-MSCs were implanted locally, in anastomoses and intestinal lumen at the moment of surgery. Adas et al.[31] presented that systemic transplanted bone marrow (BM)-MSCs therapy significantly accelerated the healing parameters for ischemic colonic anastomosis. Although most experimental studies employ MSCs obtained from BM (BM-MSCs), we prefer employing MSCs obtained from adipose tissue (AD-MSCs) because obtaining them is preferable in human clinical practice since they are more easily accessed. Plock et al.[32] compared immunomodulatory efficacy of AD-MSCs and BM-MSCs administered intravenously in a hindlimb vascularized composite allotransplantation model in rat. They found that AD-MSCs and BM-MSCs exhibited strong dose-dependent suppressor function "*in vitro,"* which was significantly more pronounced for adipose cells; and regulatory T-cell levels were increased with AD-MSCs and BM-MSCs, but specially in the AD-MSC groups, however, AD-MSCs group did not show any increase in the survivorship of allograft with respect to the BM-MSCs group. In the Plock et al. study,[32] all animals revealed peripheral multi-lineage chimerism at 4 weeks independently of cell type and dosage and MSC treatment resulted in long-term (> 120 days) allograft survival in 47% of the animals, which correlated with durable microchimerism in BM and spleen. Mattar and Bieback[33] carried out a review compiling the current literature regarding the similarities and differences between three sources for MSCs with a special focus on their immunomodulatory effects on T-lymphocytes subsets and monocytes, macrophages, and dendritic cells, and they found similar results to Plock et al.[32] and to data obtained from review of Gao et al. in 2016[34].

With respect to the doses of MSCs, there are notable differences in the literature, experimental studies use between 1×10^6 and 10^7 cells/animal, we performed some preliminary studies on SBTx rats to fix MSC doses and subclinical immunosuppressive treatment doses employing cyclosporine (data unpublished). Finally, we used 4×10^6 MSCs and 0.4 mg/animal/day.

Our results showed a lower number of ACRs and less severity of the rejection in AD-MSCs treated animals with regard to the control group. Although recently, some authors use BM-MSCs infused through the penile vein[35,36] with partial success, other authors have employed the arterial way without success. We think that MSCs have been extensively investigated for their potential to regenerate tissue and to modulate the immune system. Their characteristic features are adherence to tissue and plasticity. Therefore, direct application in intestinal lumen and anastomoses in SBTx could be a very important factor to prevent rejection, but this fact is related to the dose of MSCs.

From the point of view of the immune response, all authors agree that MSCs immunomodulate the immune response through the Th_2 pathway activation. We agree with Yang et al.[35], whose results in SBTx undergoing

BM-MSCs treatment showed an increase of Treg levels and upregulation mainly due to increases of TGFb-1. Our results showed a significant increase of CD4+25+Foxp3+ and CD8+25+Foxp3+ cell percentages in peripheral blood in SBTx rats treated with AD-MSCs. The increase of CD8+25+Foxp3+ cell percentage was greater than the increase of CD4+25+Foxp3+ cell percentage. The previous studies[37-39] showed that earliness of rejection correlated with the percentage of CD8+ cells and the intensity of rejection with numbers of CD8+ cells; in addition, we observed a significant correlation between apoptosis and rejection, between CD8+ and CD54+ with apoptosis and with rejection, and between CD8+ and CD54+[38]. Therefore, CD8+25+ cells are essential in rejection. CD54+ is an intercellular adhesion molecule-1 that is found on endothelial cells, it indicates that the activation of endothelial molecules and cells may play an important role in established SBTx rejection. Results of the present study highlight an increase of CD8+25+Foxp3+ cells in animals undergoing SBTx and AD-MSCs treatment. Until now, this fact has not been published. The AD-MSCs group showed lower IL-17 levels than the placebo group (not statistically significant) in the post-operative period. Besides, the placebo group presented significantly lower post-operative TGFb-1 levels than the AD-MSCs group in peripheral blood. These variations are in accordance with other authors[34,36,40].

Conclusions

We think that AD-MSCs induce regulatory immune response by increasing Treg percentages and TGFb-1 levels, thus probably leading to an immune donor-recipient chimera when AD-MSCs are implanted in anastomoses and intestinal lumen in SBTx, leading to a lower risk of acute rejection by cell mediation, in the first 7 days of the intestinal transplant.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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