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ORIGINAL ARTICLE

Therapeutic Effects of Mesenchymal Stem Cells Derived From Bone Marrow, Umbilical Cord Blood, and Pluripotent Stem Cells in a Mouse Model of Chemically Induced Inflammatory Bowel Disease

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Abstract—Acute inflammatory bowel disease (AIBD) is a wide clinical entity including severe gastrointestinal pathologies with common histopathological basis. Epidemiologically increasing diseases, such as necrotizing enterocolitis (NEC), gastrointestinal graft *versus* host disease (GVHD), and the primary acute phase of chronic inflammatory bowel disease (CIBD), exhibit a high necessity for new therapeutic strategies. Mesenchymal stem cell (MSC) cellular therapy represents a promising option for the treatment of these diseases. In our study, we comparatively assess the efficacy of human MSCs derived from bone marrow (BM), umbilical cord blood (UCB), human embryonic stem cells (ESCs), or human-induced pluripotent stem cells (iPSCs) in a mouse model of chemically induced acute enterocolitis.

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The laboratory animals were provided *ad libitum* potable dextrane sulfate sodium solution (DSS) in order to reproduce an AIBD model and then individually exposed intraperitoneally to MSCs derived from BM (BM-MSCs), UCB (UCB-MSCs), ESCs (ESC-MSCs), or iPSCs (iPSC-MSCs). The parameters used to evaluate the cellular treatment efficacy were the animal survival prolongation and the histopathological-macroscopic picture of bowel sections. Although all categories of mesenchymal stem cells led to statistically significant survival prolongation compared to the control group, significant clinical and histopathological improvement was observed only in mice receiving BM-MSCs and UCB-MSCs. Our results demonstrated that the *in vivo* anti-inflammatory effect of ESC-MSCs and iPSC-MSCs was inferior to that of UCB-MSCs and BM-MSCs. Further investigation will clarify the potential of ESCs and iPSC-derived MSCs in AIBD treatment.

KEY WORDS: bowel disease; mesenchymal stem cells; animal model; intraperitoneal; pluripotent.

INTRODUCTION, MATERIALS, AND METHODS

Acute inflammatory bowel disease (AIBD) and its variations can be a topic of interest as it affects all different age categories, from neonates to deep adolescence. Necrotizing enterocolitis [1, 2], gastrointestinal graft *versus* host disease [3–5] and the acute phase of IBD [6–9] share common clinical features and have severe impact on the quality of life of patients. Several factors have led to an increasing rate of AIBD variations. No medical therapies have proven to be so far of clinical benefit, and most of the patients are led to severe amputating surgeries with high morbidity. The quality of life of the survived patients remains poor due to the "small bowel disease" which is induced by the massive surgical resection of the bowel. The above raise the need for alternative approaches for the treatment of AIBD.

Cellular therapies are turning into a promising challenge for the future treatment of these conditions. Mesenchymal stem cells (MSCs) are currently under investigation for the treatment of various inflammatory diseases. MSCs originate from perivascular cells *in vivo* [10] and can be isolated from almost all types of tissues including bone marrow (BM), umbilical cord blood (UCB), amniotic fluid, placenta, adipose tissue, and dental pulp [11]. They have the ability to proliferate *in vitro* and their major properties of clinical interest are their differentiation potential to several different cell types, such as adipocytes, chondrocytes, osteoblasts, myocytes, and neurons and their immunomodulatory effects that have been observed in many pre-clinical and clinical studies concerning different inflammatory conditions [12, 13]. Bone marrow and adipose tissue have so far been the most commonly used sources of MSCs due to the simple deriving technique and their ability to be obtained in a sufficient quantity. However, multiple infusions of MSCs may be necessary for cellular therapy protocols requiring multiple harvesting of those tissue samples [14, 15] .

During the last years, the rapid development in pluripotent stem cell research has allowed their usage as a new source for the derivation of MSCs. Both embryonic stem cells (ESCs) [16] and induced pluripotent stem cells (iPSCs) [17] can be an unlimited source of differentiated cells for usage in cellular therapy. Their therapeutic applications still remain on the field of research.

In this study, we aimed to investigate the possible therapeutic effects of different sources of MSCs of adult, fetal, and embryonic origin and compare their potential for the treatment of an AIBD mouse model.

MATERIALS AND METHODS

The animal experiments were conducted under protocols reviewed and approved by Institutional Animal Care and Use Committee (Athens, Protocol Number K/5449). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

AIBD Animal Model

To induce severe colitis, the mice received *ad libitum* (according to their drinking needs) a dextrane sulfate sodium solution (DSS, molecular weight 40,000 dt ordered from Alfa Aesar GmbH & Co KG, Karlsruhe/Germany) according to standard protocols [18, 19]. Although AIBD is a wide entity including life-threatening conditions, the histopathological and clinical picture reproduced by this chemically induced model was aimed to reach the highest level of cellular and tissue destruction, mostly compatible

to acute enterocolitis (All the standardization experiments for the exact conditions that reproduce the AIBD animal model are described in the Supplemental data).

Mesenchymal Stem Cell Derivation

BM and UCB-derived MSCs. Derivation of MSCs from BM (BM-MSCs) and UCB (UCB-MSCs) was implemented according to international standardized models [20].

ESC and iPSC-derived MSCs. For the derivation of MSCs from ESC (ESC-MSCs) and iPSCs (iPSC-MSCs), ESCs and iPSCs were transferred into ultra-low attachment plates and cultured in Knockout DMEM supplemented with 20% Knockout serum replacement (KSR), 1 mM L-glutamine, 1× non-essential amino acids, 0.1 mM 2-mercaptethanol (all from Gibco, BRL), and 50 ng/ml BMP-4 (R&D System, Inc., Minneapolis, MN), in order to form embryoid bodies (EBs). After 7 days, the EBs were transferred into gelatin-coated flasks containing Knockout DMEM supplemented with 10% KSR, 1 mM L-glutamine, 1× non-essential amino acids, and 10 ng/ml bFGF (R&D System). Cells were passaged with 0.05% trypsin (Gibco) when they reached confluency and after 2 passages, the derived MSC populations were kept in cultures containing typical MSC medium consisting of DMEM GlutaMax (Gibco, BRL) supplemented with 10% fetal bovine serum (FBS; Stem Cell Technologies, Vancouver, BC, Canada).

Flow Cytometric Analysis

For flow cytometry analysis, BM-MSCs, UCB-MSCs, ESC-MSCs, and iPSC-MSCs were harvested, washed, and incubated with specific MSC marker antibodies CD90-FITC, CD44-PE, CD105-FITC, CD73-PE, CD34-PE, and CD45-FITC. A Cytomics FC500 was used by Beckman Coulter with CXP software for the Cytomics FC500 flow cytometry system version 2, 2. We used 400 μL from the total volume of 1 mL MSCs in order to count the total number of cells and their viability, by adding 10 μl of 7-AAD for viable cells, using flow cytometry. Sample analysis was completed typically within 10 min.

Trilineage Differentiation of MSCs

MSCs derived from BM, UCB, ESCs, and iPSCs were harvested, reseeded at appropriate concentrations in plates containing MesenCult Osteogenic Stimulatory Kit (Stem Cell Technologies, Vancouver, BC, Canada), StemPro Chondrogenesis Differentiation Kit (Gibco, BRL), and StemPro Adipogenesis Differentiation Kit (Gibco, BRL). For osteogenesis and adipogenesis, respectively, they were cultured as monolayer, according to manufacturer's instructions. For chondrogenesis, 3D cultures were used for differentiation, according to the manufacturer's instructions. Assessment of the differentiated cells was carried out with staining techniques. Alizarin Red was used to stain osteocytes, Oil Red for adipocytes, and H&E for chondrocytes.

Animal Experiments

The experiments were repeated twice with five groups of mice every time. On the first round, the groups consisted of 5 mice and on the second round, they consisted of 10 mice each. All the experimental animals were provided with a 5% DSS (*w*/*v*) solution which they drunk *ad libitum* from day 1 to day 4 (0-96 h) resulting in severe colitis (Supplemental data). Group I was the control group, group II received BM-MSCs, group III received UCB-MSCs, group IV received ESCs-MSCs, and group V iPSC-MSCs. On the beginning of day 3 (48 h) when the expected histopathological picture was established, the laboratory animals were injected intraperitoneally once with mesenchymal stem cells according to their groups.

Every experimental animal received intraperitoneally 1 ml of the programmed solution. Group I received the control solution (solution A: normal saline 0, 9% with albumin 2.5%). Group II received with the same technique solution B (1 ml of N/S 0.9% with albumin including 5×10^6 BM-MSCs) and group III received solution C (1 ml of N/S 0.9% with albumin including 5×10^6 UCB-MSCs). Group IV intraperitoneally was injected with solution D (1 ml of N/S 0, 9% with albumin including 5×10^6 ESC-MSCs) and finally group V received solution E (1 ml of N/S 0, 9% with albumin and 5×10^6 iPSC-MSCs). The stem cell solution was injected intraperitoneally to the mice after short-term volatile anesthesia (ether given in an airchamper) with a 23 Ganz venous catheter. DSS solution was given to the injected mice until the fourth day (96 h) and then it was replaced with plain water. The animals were not treated with intravenous liquids and antibiotics.

Evaluation Criteria

The clinical condition of the mice was evaluated based on the weight, the macroscopic condition of the colon, the histopathological result, and the survival prolongation compared to the control group. The choice of proper evaluation criteria is a key aspect of any medical laboratory animal experiment. The efficacy and safety of a treatment can be evaluated by assessing clinical symptoms signs

(survival prolongation). In case of life-threatening diseases, the most commonly used relevant evaluation criterion is morbi-mortality especially when the laboratory experiment does not require very long-term studies. In addition, evaluation criteria such as the histopathological picture and the surgical macroscopic evaluation of colon (colon index) are considered to be of great sensitivity and speciality under circumstances. Consistency relates to the reproducibility of measurements made by the same well-trained observer and to the concordance of measurements made by different observers (as held in this experiment). Numerous researches use biomarkers (COX-2, IL-6) or other intermediate criteria for the evaluation of investigational cellular therapy. It was a common observation however that not always the biochemical results of those difficult measurements were aligned to the true picture of the research outcome. After their death or at standardized day 15 [day 1 is the day the mice started drinking 5% DSS solution and day 15 was set as the upper limit day (13 days of survival prolongation)], the laboratory animals were euthanized and induced to a total colon extraction for histopathological analysis and confirmation. Colon samples were fixed in 10% solution right after their extraction, embedded in paraffin and sliced into sections before staining with hematoxylin and eosin. Histological evaluation was based on the alteration of the histological architecture of the epithelium scoring from 0 to 4. 0: epithelium without alteration, 1: regional limited loss of goblet cells, 2: loss of goblet cells on large areas, 3: regional limited loss of colon crypts, 4: extended loss of colon crypts-pan colitis (Fig. 1). During the extraction, the colon was evaluated macroscopically and given a score from 0 to 4. 0: macroscopically healthy colon, 1: edema regionally found, 2: extensive edema, 3: regional ischemia and stenosis 4: extensive ischemia, stenosis, hemorrhage, and regionally megacolon (Fig. 2). The macroscopic evaluation was added to the histopathological evaluation and formed a Colon Index Evaluation (Colon Index Evaluation = Macroscopical Index Evaluation + Histopathological Index Evaluation.). The survival prolongation was evaluated from the day the mice received intraperitoneally the solutions A, B, C, D, and E.

Statistical Analysis

Data was analyzed using Microsoft Excel 2010. To compare control group (group I) with each stem cell group (group II, III, IV, and V), analysis was performed using a Mann Whitney *U* test. p values < 0.05 were considered statistically significant .

RESULTS

Characterization of MSCs

BM-MSCs, UCB-MSCs, iPSC-MSCs, and ESC-MSCs expressed all specific MSC markers CD73, CD105, CD90, CD44 and lacked expression of the hematopoietic markers CD34 and CD45. Differentiation to adipocytes, chondrocytes, and osteocytes was observed in all sources of MSCs.

In Vivo Anti-Inflammatory Effects

In C57BL/6 mice, administration of a 5% DSS solution for 4 days resulted in severe histological inflammation of the entire colon. All DSS-treated mice developed clinical signs similar to necrotizing enterocolitis (NEC), gastrointestinal graft *versus* host disease (GVHD) and acute phase of inflammatory bowel disease (IBD). The control group (group I) which received intraperitoneally only albumin solution had a survival prolongation of 1 to 2 days, while the prolongation of groups II, III, IV, and V varied (Table 1). On day 15 or at the day of their death, the entire colon of the mice was removed and evaluated macroscopically and histopathologically. A significantly important difference in the prolongation ($p < 0.05$) of survival was noticed between 5% DSS + 0.9% N/S and 5% DSS + UCB-MSCs group (group I and III) ($U_{stat.\ CB} = 0$ and $U_{CRIT} = 64$ $p < 0.05$) (Table 1; Fig. 3). Treatment with UCB-MSCs reduced the extent of the inflamed area. The crypt damage was partially restored. In the UCB-MSCs group, the Colon Index was significantly ($p < 0.05$) reduced compared to the control group (Ustat. CB = 58.5 and $U_{CRIT} = 64$ $p < 0.05$) (Table 2; Fig. 3).

The survival prolongation and the Colon Index were comparatively assessed in the control group and the BM-MSCs (group I and II). A significantly important difference in the survival prolongation was noticed between groups I and II (Ustat. $_{BM} = 19.5$ and $U_{CRIT} = 64$, $p < 0.05$) (Table 1; Fig. 3). Treatment with BM-MSCs reduced the extent of the inflamed area in some animals. In the BM-MSC group, the Colon Index was significantly ($p < 0.05$) reduced compared to the control group (Ustat. $_{BM} = 57$ and $U_{CRIT} = 64$, $p < 0.05$) (Table 2; Fig. 3).

The survival prolongation and the Colon Index were comparatively assessed in the control group and the ESC-MSCs group (groups I and IV). A significantly important difference in the survival prolongation was noticed between groups I and IV (Ustat. $_{ES} = 46.5$ and $U_{CRIT} = 64$, $p < 0.05$) (Table 1; Fig. 3). Treatment with ESC-MSCs, however, did not reduce the extent of the inflamed area.

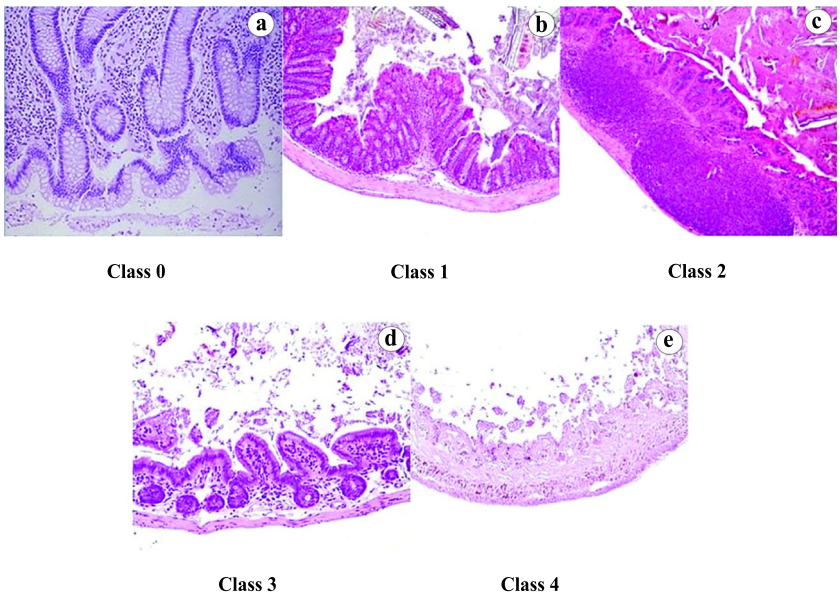

Fig. 1. Histopathological scoring of bowel sections from 0 to 4 (**a** 0 = normal, **b** 1 = regional limited loss of goblet cells, **c** 2 = loss of goblet cells on large areas, **d** 3 = regional limited loss of colon crypts, **e** 4 = extended loss of colon crypt / pan-colitis) (× 100, scale bar 100 μm).

In the ESC-MSCs group, the Colon Index was comparable to the control group ($Ustat._{ES} = 103$ and UCRIT = 64, $p < 0.05$ (Table 2; Fig. 3).

Finally, the control group and the iPSC-MSCs group (groups I and V) were compared for the survival prolongation and the Colon Index. A significantly important difference in the prolongation of survival was noticed between groups I and V ($Ustat._{iPS} = 35$ and $U_{CRIT} = 64$ $p < 0.05$) (Table 1; Fig. 3). Treatment with iPSC-MSCs, as with ESC-MSCs, did not reduce the extent of the inflamed

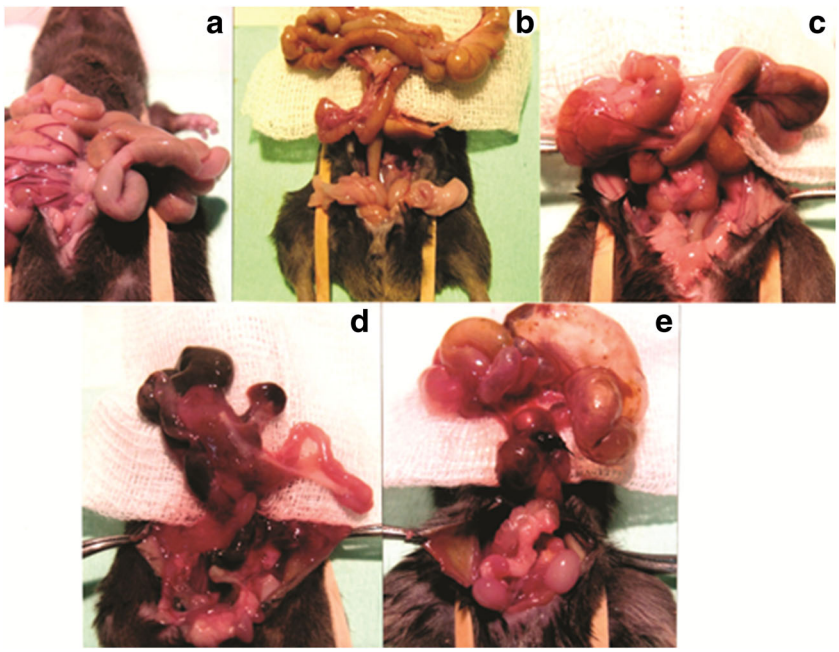

Fig. 2. Macroscopic evaluation of the colon. **a** Macroscopically healthy colon. **b** Oedema regionally found. **c** Extensive oedema. **d** Regional ischemia and stenosis. e Extensive ischemia, stenosis, haemorrhage and regionally megacolon.

Table 1. Prolongation of Survival of Experimental Animals After Providing the Control Solution, UCB-MSCs, BM-MSCs, ESC-MSCs and iPS-MSCs. Results for 15 Animals per Group. The Experiment Was Held Twice

	Prolongation of survival (days)				
S/N	Control	CB	BM	ES	IPS
1	1	3	4	3	1
2	2	13	13	1	3
3	1	8	2	3	4
4	1	4	5	2	3
5	2	6	3	4	3
6	2	8	13	5	13
7	2	13	13	2	4
8	2	13	4	5	2
9	1	8	4	1	13
10	1	13	6	3	2
11	2	13	2	13	3
12	1	13	13	1	3
13	2	7	7	2	4
14	1	13	1	3	1
15	2	13	3	4	2
	Control	CB	BM	ES	IPS
Mean value	1.53	9.9	6.2	3.5	4.1

area. In the iPSCs group, the Colon Index was comparable to the control group ($Ustat._{iPS} = 111$ and $U_{CRIT} = 64$, $p < 0.05$) (Table 2; Fig. 3).

DISCUSSION

Necrotizing enterocolitis, gastrointestinal graft *versus* host disease, and inflammatory bowel disease can be life-threatening conditions. The mechanism that leads to the expression of these diseases is not entirely known but genetic, environmental, and immunological factors seem to cooperate for the final expression of the clinical condition. The proc. research on cellular therapies the last decades has opened a new field on regenerative medicine, giving the potential of less-invasive or non-invasive strategies for the treatment of these diseases.

In our study, the colitis mouse model chemically induced by DSS displays symptoms similar to NEC, GVHD, and AIBD. To our knowledge, this is the first report that uses many different sources of derived mesenchymal stem cells including adult and fetal tissues, as well as pluripotent

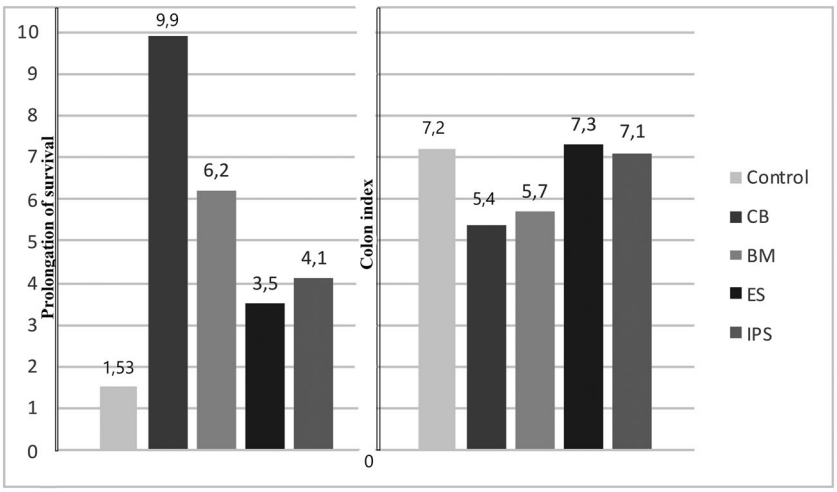

Fig. 3. Comparative results of survival prolongation and colon index between control group (5%NSS + N/S) and four groups of mesenchymal stem cells.

Table 2. Statistical Evaluation of Colon Index Between the Control Group and the Four Groups of Experimental Animals Receiving Mesenchymal Stem Cells. Results for 15 Animals per Group. The Experiment Was Held Twice

	Colon Index evaluation				
S/N	Control	CB	BM	ES	IPS
1	8	8	8	8	8
2	6	4	4	8	8
3	8	7	6	7	6
4	6	8	7	8	7
5	8	7	6	6	8
6	8	8	4	6	4
7	7	3	3	8	7
8	8	4	7	7	8
9	6	6	7	8	5
10	8	3	6	7	7
11	6	4	8	4	8
12	7	5	2	8	8
13	8	8	6	8	6
14	6	4	6	8	8
15	8	2	6	8	8
	Control	CB	BM	ES	IPS
Mean value	7.2	5.4	5.7	7.3	7.1

stem cells in order to compare their efficacy when injected intraperitoneally at a colitis mouse model. In this study, we used MSCs derived from ESCs, UCB, BM, and iPSCs. The results can be of great interest in terms of defining the efficacy of MSCs derived from pluripotent stem cells. All categories of stem cells led to a significant survival prolongation on the experimental animals. Not all categories, however, had the same amelioration of the Colon Index (Histopathological + Macroscopic Evaluation) (Fig. 3). Both UCB-MSCs and BM-MSCs led to a significant reduction of the inflammation, and some of the animals had histopathological picture close to normal (stage 1) (Fig. 4). On the other hand, although ESC-MSCs and iPSC-MSCs seem promising, their efficacy when injected intraperitoneally was not of great value. There was not at all or just a slight improvement of the inflammation of the bowel. The experimental animals may have lived significantly longer than the control group, but almost all ended up suffering from a severe inflammatory bowel disease.

The therapeutic effect of intraperitoneal injection of mesenchymal stem cells has in the past been tested in animal models on medical conditions of oviduct dysfunction and salpingitis, as well as in acute pangreatitis. The trials show that this route of provision can be a safe mean of administration for cell-based treatment. Most investigational models present an amelioration of conditions involving infection and small intestine injury, and their beneficial effects seem to be mainly mediated *via* indirect actions and not by their differentiation into target cells. When injected intraperitoneally, MSCs undergo chemotaxis towards the damaged and infected intraperitoneal tissues. These experimental researches establish this less-invasive way of providing MSCs, as an efficient therapeutic approach [21–23].

In the last two decades, there is an extensive research for the use of mesenchymal stem cells in bowel disease mostly for chronic degenerative mucosal medical conditions (IBD) [24–26]. The studies were held on animal models, and the cell population was provided either endoscopically or by submucosal injection on the part of the bowel with the most extended damage. There are also several trials held on acute bowel disease (especially on GVHD and necrotizing enterocolitis) using the IV injection of mesenchymal stem cells. Due to their accessibility, the most used sources of mesenchymal stem cells were bone marrow and adipose tissue. In these studies, although the markers used to compare the efficacy of mesenchymal stem cells were not similar (endoscopic intestinal picture, weight loss, lymphocyte proliferation, IL6/IL10), all of them show promising results [27, 28]. The endoscopic picture of the intestinal barrier showed partial tissue regeneration, although the biopsy has not always confirmed the macroscopic picture. The clinical condition of AIBD models has been repressed and IL6/IL10 markers were significantly reduced. The weight loss was reduced and the general clinical picture was ameliorated. Mesenchymal cells appear to have an inherent capacity to migrate into the

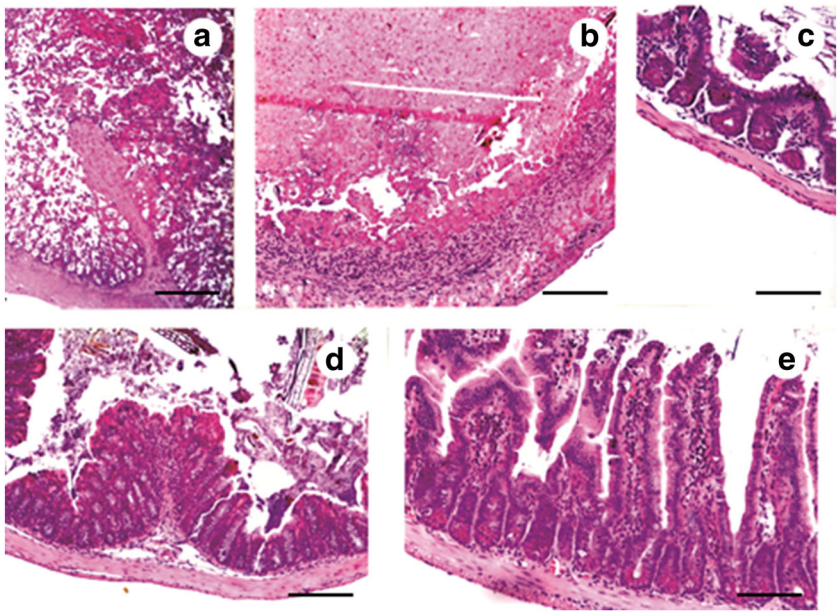

Fig. 4. Histopathological picture of the mucosa after the intraperitoneal injection of **a** control solution and albumin, **b** ES-MSCs, **c** IPS-MSCs, **d** BM-MSCs and **e** UCB-MSC (× 100, scale bar 100 μm).

inflamed bowel epithelium [29–31]. However, obstacles such as the short timescale, limited ability for longitudinal evaluation, and non-standardized clinical relevance of these models mean that there are multiple challenges to overcome before proceeding in clinical trials [32–35].

Regarding pluripotent stem cell-derived MSCs, animal model research upon their therapeutic effects in inflammatory conditions is at its onset and so far, there are not many reports comparing their efficacy in comparison with MSCs derived from standard tissues. A recent study [36] showed that mouse iPSC-derived MSCs reduce the trend of inflammatory infiltration, when injected intravenously in mice implanted with sponges infiltrated with *P. gingivalis* bacteria. After local subcutaneous injection, the level of the inflammatory inhibition was statistically significant. However, only CXCL1 was significantly decreased and not other cytokines such as TNFa, TGFβ, or IL-6. Furthermore, this study presents some therapeutical effects of iPSC-MSCs without though a comparative assessment with MSCs derived from other sources.

Another recent interesting animal model research compares the efficacy of iMSC and adipose-derived stem cells on a chemically induced colitis, resembling the clinical entity of IBD. This study suggests that the use of iMSCs and adipose-derived stem cells can be effective in the treatment of IBD. The study also revealed a significant stimulatory effect of iMSC cellular therapy on epithelial proliferation and angiogenesis, and restoration of normal microbiome populations. This promising result, however, was achieved after repeated supportive i.v. injections on laboratory animals [37]. In our study, the iPSC-derived MSCs led to a survival prolongation, without a countable amelioration of the regional inflammation and the clinical scoring. All laboratory animals ended up suffering from a devastating pan-colitis before the day of euthanasia. This may be attributed to the fact that iPSC-MSCs and ESC-MSCs present cell populations which are derived through an *in vitro* differentiation process that depends on certain protocols that drive cells in certain directions. *In vitro* differentiation pathways may play a role in the properties of the derived MSCs in a manner probably similar to the fact that MSC populations derived from various sources may exhibit different characteristics. Pluripotent stem cell-derived MSCs represent, similarly to adult tissue MSCs, heterogenous populations. The heterogeneity and the purity of the cell populations play a role on the effect of those cells under an *in vivo* inflammatory environment. Different protocols of MSC derivation may give rise to populations with different heterogeneities and therefore contain cells with different characteristics. Furthermore, compared to adult

tissue MSCs, pluripotent stem cell-derived MSCs may possibly contain more immature subpopulations of cells, due to the fact that they pass through the pluripotent state and then towards the mesenchymal lineage. These different immature subpopulations may have not obtained the capability to respond *in vivo* to the inflammatory signals, to which the mature adult tissue MSCs are responsive. Although those populations appear to have many similar properties with BM-MSCs and UCB-MSCs (morphology, specific surface antigen expression, trilineage differentiation potential), the *in vitro* differentiation process may be incomplete when starting from a pluripotent cell. Further studies should focus on the protocols used to drive pluripotent stem cell differentiation. A better understanding of the mechanisms of differentiation will allow the development of differentiation protocols that lead to the derivation of MSC populations with the desirable characteristics.

The clinical trials so far are limited and the efficacy of the MSC treatment has not been clarified yet. Intraperitoneal administration of MSCs may prolong survival on AIBD conditions mostly through their anti-inflammatory effects. UCB-MSCs and BM-MSCs in this study demonstrate a noticeable therapeutic effect on AIBD. New questions rise about the inflammatory mechanisms that promote the disease and their in-depth understanding will lead to better therapeutic approaches.

Further investigation is needed to overcome all the difficulties and the scientific dead ends rising. In a medical landscape that non-invasive or less-invasive medicine gains space step by step, the need to answer those questions remains inevitable.

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COMPLIANCE WITH ETHICAL STANDARDS

The animal experiments were conducted under protocols reviewed and approved by Institutional Animal Care and Use Committee (Athens, Protocol Number K/5449). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Conflict of Interest. The authors declare that they have no conflict of interest.

Informed Consent. Informed consent was obtained from all individual participants included in the study.

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