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## Secretome effect of adipose tissue-derived stem cells cultured two-dimensionally and three-dimensionally in mice with streptozocin induced type 1 diabetes



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### ABSTRACT

**Aims:** To analyze therapeutic potential of the conditioned medium from adipose tissue-derived stem cells (ASC) cultivated in 2D (CM-2D) and 3D (CM-3D) models, in mice with Type 1 diabetes (T1D) induced by streptozotocin. **Main methods:** Viability and CD105 expression of 2D and 3D ASC were analyzed by flow cytometry. T1D was induced in mice by multiple injections of streptozocin. On the 28th and 29th days after the first injection of streptozocin, diabetic animals received CM-2D or CM-3D. Pancreatic, CM-2D, and CM-3D cytokines were analyzed by cytometric bead array (CBA) and insulin and PDX-1 were observed and quantified by immunohistochemistry. Apoptosis-related proteins were quantified by Western Blotting.

**Key findings:** ASC in three-dimensional culture released increased levels of IL-6 and IL-2, while IL-4 was decreased. CM-2D induced pancreatic PDX-1 expression and was able to reduce glycemia in diabetic mice one week after injections but not CM-3D. On the other hand, CM-2D and CM-3D were not able to reverse apoptosis of pancreatic cells in diabetic mice nor to increase insulin expression.

**Significance:** Together, these results demonstrate that the 3D cell culture secretome was not able to improve diabetes type 1 symptoms at the times observed, while 2D cell secretome improved glycemic levels in T1D mice.

### 1. Introduction

Type 1 diabetes is an autoimmune disorder in which pancreas  $\beta$  cells are attacked and destroyed. Periodic insulin administration is required and often, this intervention is not enough to prevent long-term complications such as vascular degeneration, blindness, and kidney failure. Pancreatic islet transplantation is one of the alternatives to improve symptoms for some years, but the use of immunosuppressants to minimize the possibility of rejection and the scarcity of available organs are limiting factors for this procedure (Gruessner and Gruessner, 2016).

Stem cell therapies have been recently explored in a variety of clinical and therapeutic studies for the treatment of various diseases. Mesenchymal stem cells obtained from stromal vascular fraction (SVF) of adipose tissue are easily accessible and have been used in various models of tissue repair, ischemic injuries, myocardial infarction, bone regeneration,

immune disorders (Crohn's disease, multiple sclerosis, lupus, arthritis) among other diseases (Abudusaimi et al., 2011; Garimella et al., 2015; Lee et al., 2013; Prockop, 2017).

Adipose tissue stem cells (ASC) have a promising therapeutic potential due to their ability to osteogenic and adipogenic differentiation and secrete a wide variety of cytokines, chemokines, and growth factors involved in tissue regeneration. Tissue regeneration observed after mesenchymal cell transplantation was mainly attributed to the differentiation of these cells, but recent studies indicate that secretome also plays an important role in improving symptoms of some disorders (Kapur and Katz, 2013; Quet et al., 2017; Sevivaset et al., 2017). ASC have been recognized for having great immunomodulatory potential, in which its secretome plays an important role in the suppression of inflammatory responses triggered by diseases (Holan et al., 2019; Vasilevet et al., 2019).

Preliminary studies on therapeutic potential of these cells in the

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treatment of type 1 diabetes are being developed. Systemic administration of ASC in diabetic mice prevents hyperglycemia and promotes  $\beta$ -cell survival in the pancreas (Ammaret al., 2015; Konoet al., 2014). However, there is a lack of standardization regarding the administration routes used and the number of applications required to produce a beneficial effect. Furthermore, cell therapy has some limitations, as there is a need for in vitro expansion to have an adequate cell quantity, and this can cause genomic instability and cell senescence. Malignant transformation in situ and immunological rejection can also occur (Baglio et al., 2012). An alternative would be to use the cells' secretome instead of the stem cells themselves since the secretion produced by these cells is responsible for most of the observed beneficial effects.

Mesenchymal stem cells constitutively secrete immunoregulatory factors such as PGE-2 and NO (Dugast and Vanhove, 2009), but they also secrete some factors in response to external stimuli. One of the methods to stimulate the secretion of immunomodulatory factors in stem cells is the three-dimensional (3D) culture system. This type of culture reportedly increases the secretion of anti-inflammatory cytokines compared to monolayer (2D) cultures (Madrigal et al., 2014).

Therefore, this study aimed to characterize the effect of ASC secretome under different culture conditions (2D and 3D), in an experimental model of diabetes induction using streptozotocin in mice. The immunoregulatory capacity of these cells was investigated through cytokine profiling, as well as its potential effects in pancreatic cells. Thus, this work expects to contribute to the elucidation of the therapeutic potential of ASC and its secretome in an experimental model of T1D.

## 2. Material and methods

### 2.1. Location and ethical principles of research

The present study was in accordance with the Ethical Principles in animal experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA). The project involves the production, maintenance, and use of animals belonging to the phylum Chordata, subphylum Vertebrata (except humans), and was approved by the Ethics Committee for the Care and Use of Experimental Animals (CEUA) of the Institute of Biology Roberto Alcântara Gomes of the University of the State of Rio de Janeiro (IBRAG/UERJ) according to number CEUA/028/2017. The research is in accordance with the precepts of Law No. 11,794 of October 8, 2008, of Decree No. 6889 of July 15, 2009.

### 2.2. Isolation of mesenchymal stem cells from adipose tissue

Healthy male Swiss mice (2–3 months old) were euthanized according to the approved protocol, and after asepsis, an incision in the abdominal region was made to remove the subcutaneous adipose tissue. After mechanical digestion, adipose tissue was incubated with collagenase type II (Sigma-Aldrich) diluted in DMEM (Sigma-Aldrich) at 0.2% at 37 °C under agitation for 50 min. After that time, the suspension was filtered with a 100  $\mu$ m cell filter (BD Bioscience), 10 mL of 20% fetal bovine serum (FBS) DMEM (Cultilab, Brazil) was added to the filtered content and centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was discarded and the pellet was resuspended with 5 mL DMEM with 20% FBS and antibiotics (100U/mL penicillin, 0.1 mg/mL streptomycin, 0.05 mg/mL gentamycin, and 0.006 mg/mL amphotericin B), and transferred to a 25 cm<sup>2</sup> culture bottle that was kept at 37 °C, with an atmosphere of 5% CO<sub>2</sub>. The culture medium was changed every 2 or 3 days. When cells reached about 80% of confluence, passage to other bottles with trypsin/0.25% EDTA was performed, and for the following procedures, cells in the third or fourth passage were used.

### 2.3. Obtaining the ASC conditioned medium in 2D (CM-2D) and 3D (CM-3D) cultures

For 3D cultures, ASC in the third or fourth passage were seeded in 96-

well plates with a round bottom. The bottom of the wells was previously covered with a thin film of agarose (Kasvi) at 1% diluted in saline. In each well, different concentrations of cells ( $2.5 \times 10^4$  and  $5.0 \times 10^4$ ) were plated in order to analyze which spheroid size would be the most suitable for obtaining the supernatants. The spheroids were maintained in culture with DMEM +10% FBS and antibiotics for 6 days with a change of medium on the third day of culture. On the 6th day, spheroids were washed with PBS and 200  $\mu$ L of DMEM + gentamycin was added to each well. To investigate the best conditioning time, spheroids were kept for more 24 or 48 h in culture, and then, at these two points, spheroid conditioned medium was collected, centrifuged at 2000 rpm for 10 min, filtered with a 0.22  $\mu$ m syringe filter, aliquoted in microtubes and stored at –80 °C for further use.

To obtain the secretome of cells in 2D culture,  $3.0 \times 10^5$  ASC in the third or fourth passage were plated in 6-well plates with DMEM +10% FBS and antibiotics. The next day, 2.4 mL of DMEM + gentamycin was added to the wells to maintain the same cell/medium ratio used in the spheroids ( $2.5 \times 10^4$  cells/0.2 mL). After 24 or 48 h, the conditioned medium of 2D culture was collected and processed likewise.

### 2.4. Analysis of spheroid morphology

Photomicrographs of the spheroids were taken on days 1 and 3 of culture and 24 h and 48 h after changing the medium on day 6. An Olympus IX71 microscope coupled with a CCD camera (Olympus TH4100) and Olympus DP Controller software were used for the acquisition of the images. The volume of the spheroids was measured using the Spheroid Sizer software developed by Chen et al. (2014).

### 2.5. Analysis of viability and CD105 expression in ASC 2D and 3D cultures by flow cytometry

2D ASC were dissociated from 6-well plates using Accutase for 2 min at 37 °C. The suspended cells were collected, transferred to tubes, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in PBS. After cell counting, ASC were distributed in tubes and centrifuged at 1500 rpm for 5 min. Pellets were resuspended with 100  $\mu$ L of binding buffer and 2  $\mu$ L of Annexin V and 2  $\mu$ L of 7AAD were added (Biolegend cell viability kit) to detect apoptotic and necrotic cells. After incubation for 30 min, cells were analyzed using a flow cytometer (BD C6 Accuri).

3D ASC were washed with PBS and incubated with Accutase in a proportion of 300  $\mu$ L for every 8 spheroids for 5 min at 37 °C in a water bath under orbital shaking. Subsequently, the solution was pipetted by flow and reflow for 2 min. This cycle was repeated 4 more times for complete dissociation. Cells in suspension were centrifuged at 2340 rpm for 10 min. Pellet was resuspended in PBS and after counting, cells were distributed in tubes and were centrifuged at 2340 rpm for 10 min for resuspension with 100  $\mu$ L of binding buffer, 2  $\mu$ L of Annexin V and 2  $\mu$ L of 7AAD (Biolegend cell viability kit) to detect apoptotic and necrotic cells. After incubation for 30 min, 400  $\mu$ L of binding buffer was added for analysis on the flow cytometer. Annexin V was detected in the FL1 channel and 7-AAD in the FL3 channel.

For CD105 expression analysis, after detachment or dissociation, the cells in suspension were centrifuged at 1500 rpm for 5 min (2D) or 2340 rpm for 10 min (3D). The supernatant was discarded, and the pellet resuspended in 20  $\mu$ L of PBS, and incubated with 2  $\mu$ L of CD105 antibody conjugated with Phycoerythrin (PE) (BD Biosciences) for 30 min in the dark. After this time, the cells were centrifuged at 1500 rpm for 5 min (2D) or 2340 rpm for 10 min (3D) and resuspended with 500  $\mu$ L of PBS for reading on the flow cytometer (BD Accuri C6). The FL2 channel was used to detect the CD105 antibody. For all experiments, cells were used without any markers as control and analysis were repeated twice.

## 2.6. Induction of type 1 diabetes and treatment with conditioned medium

For the induction of T1D, male Swiss mice with approximately 2 months old were used to carry out the experiments. Animals were kept in boxes (maximum of 4 animals per box), under standard 12-h light/dark cycle condition, with a temperature of 23 °C, receiving filtered water and standard commercial food suitable for rodents ad libitum. From day 0, the animals' glycaemia was measured weekly after a 6-h fast, using a monitor and glycemic strips (Accu-Chek Active) until the time of euthanasia. Animals received an intraperitoneal injection (40 mg/kg) of streptozocin (Sigma-Aldrich) diluted in citrate buffer (0.05 M) or only citrate buffer (in control animals) for 4 consecutive days. 28 days after the first streptozocin injection, animals with blood glucose  $\geq 200$  or statistically higher than the control group were subdivided into the following groups: diabetic mice (STZ), diabetic mice treated with 2D conditioned medium (CM-2D) and diabetic mice treated with 3D conditioned medium (CM-3D). The animals received an intraperitoneal injection (400  $\mu$ L) of 2D culture conditioned medium (CM-2D) or 3D culture conditioned medium (CM-3D) at days 28 and 29. 7 days after the 1st injection of supernatant (day 35), the animals were euthanized, and pancreas were collected for subsequent analysis. Experimental design is shown in Fig. 1.

## 2.7. Pancreatic and CM-2D and CM-3D cytokine profiling using cytometric bead array (CBA)

Pancreatic tissue from experimental groups was crushed with scissors and then processed with ultrasonic sonicator (Virsonic 60) in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM NaF, 0.2 mM Sodium orthovanadate, 200 mM Sodium Pirophosphate, 0.1% SDS, 1% TritonX-100) plus protease inhibitor cocktail. Cytokines IL-10, IL-17 A, TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-2 and IL-4 were measured in pancreas homogenates and 2D and 3D ASC conditioned medium (CM-2D and CM-3D) using the kit CBA mouse Th1/Th2/Th17 (BD cat. No. 560484). Samples were analyzed according to manual user guide and results were analyzed by software FCAP Array 3.0 (BD Biosciences) after collection of events in the flow cytometer (BD C6 Accuri).

## 2.8. Pancreas immunohistochemistry

After histologic preparation in silane covered slides, immunohistochemistry was performed with VECTASTAIN® Universal Quick HRP Kit (PK7800). Slides were deparaffinized with xylol and with decreasing

alcohol concentration. Then, slides were incubated for 15 min with 2% hydrogen peroxide solution and in Citrate Buffer pH = 6.0 for 20 min at 60 °C. The blocking solution was incubated for 10 min, the excess was removed and then the sections were incubated with primary antibody anti-PDX-1 (Santa Cruz Biotechnology-sc-14664) or anti-insulin (Santa Cruz Biotechnology-sc-9168) diluted in PBS/BSA 1% in the proportion of 1: 100 for 24 h. The slides were washed, incubated with biotinylated secondary antibody and after washing, incubated with streptavidin/peroxidase solution for 5 min. After washing and incubation with DAB solution (ImmPACT® DAB Substrate, Peroxidase HRP), slides were counterstained with hematoxylin, and mounted with coverslips.

The slides were photographed under an Olympus light microscope with attached camera. An average of 25,7 islets per group were photographed at 400 $\times$  magnification. The intensity of the staining was quantified with Image-Pro Plus 6.0 software. The images of the islets were segmented into black and white images, where the positive regions for DAB were designated as white. The percentage of the area occupied by the white color was defined by the histogram tool.

## 2.9. Protein analysis by Western Blotting

To detect the amount of protein in pancreas of experimental groups, organs were homogenized, sonicated and lysed with RIPA lysis buffer on the day of euthanasia. A protease inhibitor cocktail was also added to the lysis buffer. Total proteins were measured with the BCA kit (ThermoFisher) to obtain the standard curve, and 20  $\mu$ g of protein was used for each well in the electrophoresis run on 12% polyacrylamide gel. Proteins were transferred to a PVDF membrane where immunostaining was performed. Non-specific bonds were blocked with 5% non-fat milk diluted in TBS-T and incubated with the antibodies against insulin (Santa Cruz Biotechnology, sc-9168) 1: 1000, Bax (Santa Cruz Biotechnology, sc-6236) and Bcl-xl (Santa Cruz Biotechnology, sc-7195). Membranes were washed 3 times with 1% T-TBS and incubated with anti-rabbit antibody conjugated with HRP (Invitrogen #31460) (1: 5000) for 1 h. After washing, membranes were incubated with ECL (ThermoFisher) for 5 min and then captured by Chemidoc (Biorad). The band intensities were quantified in Image J software (<https://imagej.nih.gov/>) and normalized to actin as the relative target protein expression levels, expressed in arbitrary units (A.U.).

## 2.10. Statistical analysis

Results were expressed as mean  $\pm$  standard deviation of the mean.

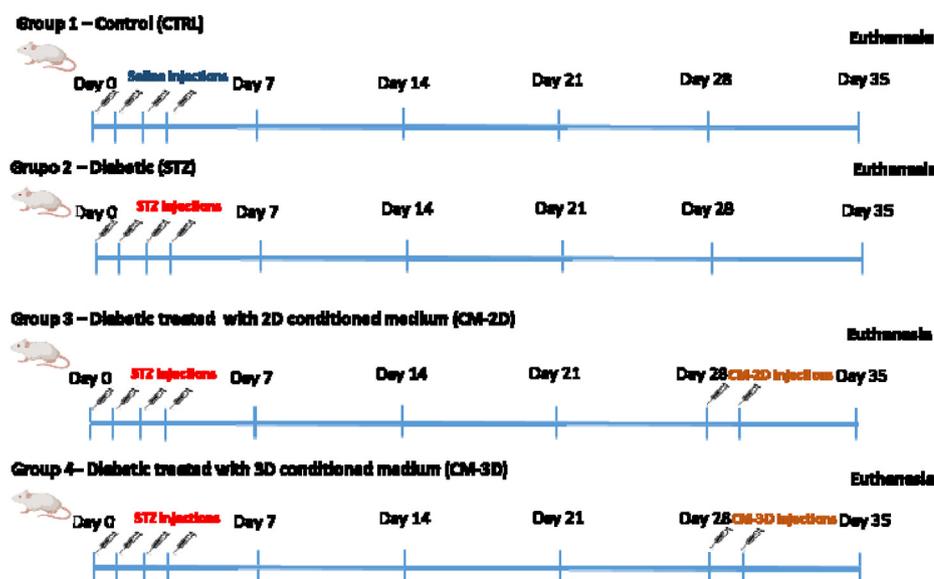


Fig. 1. *In vivo* experimental design. Group 1 (Control group) received saline injections during 4 consecutive days. Group 2 (Diabetic group) received streptozocin injections during 4 consecutive days. Group 3 (Diabetic group treated with 2D conditioned medium) received streptozocin injections during 4 consecutive days and 2D conditioned medium injection at days 28 and 29 after first streptozocin injection. Group 4 (Diabetic group treated with 3D conditioned medium) received streptozocin injections during 4 consecutive days and 3D conditioned medium injection at day 28 and 29 after first streptozocin injection. Animal were euthanized at day 35. n = 4.

Cytokine levels, expression of apoptotic proteins and immunohistochemical quantification of the pancreas were analyzed by One-Way ANOVA with Tukey post-test. The blood glucose of fasting animals was analyzed by Two-way ANOVA with Tukey post test. The other analyzes were determined by the unpaired Student's *t*-test. The statistically significant difference between groups was determined with values of  $p < 0.05$ . All analyzes were performed using GraphPad Prism 6.0 software.

### 3. Results

#### 3.1. 2D and 3D cell culture morphology

Morphology of the 2D and 3D cultures grown for 24 and 48 h without serum is shown in Fig. 2. We observe the typical fibroblastic morphology of ASC adhered to plastic surfaces in 2D cell culture. In 3D cell culture, obtained using a suspension method with a non-adhesive surface (agarose), the round-shaped morphology is observed in  $2.5 \times 10^4$  cell seeding concentration.

After one day of 3D cell culture plating, spheroid formation was already observed in the two seeding concentrations and its rounded morphology was maintained during the 8 days of culture. With the software Spheroid Sizer, spheroid volume ( $\text{mm}^3$ ) was measured. Spheroids with initial seeding of  $5.0 \times 10^4$  had higher volume than  $2.5 \times 10^4$  spheroids during 8 culture days (Supplementary Fig. 1). Smaller spheroids had more homogeneity within its size as demonstrated by lower standard deviation. For statistical analysis, 15 spheroids from each group were measured. Spheroids with initial seeding of  $2.5 \times 10^4$  had a mean volume of  $0.121 \pm 0.005$  at day 1;  $0.071 \pm 0.005$  at day 3;  $0.040 \pm 0.004$  at serum-free day 7 and  $0.039 \pm 0.006$  at serum-free day 8. Spheroids with initial seeding of  $5.0 \times 10^4$  had a mean volume of  $0.244 \pm 0.013$  at day 1;  $0.135 \pm 0.006$  at day 3;  $0.094 \pm 0.011$  at serum-free day 7 and  $0.083 \pm 0.009$  at serum-free day 8 (Fig. 3).

#### 3.2. Cell viability and CD105 expression of 2D and 3D cell cultures

Viability analysis of 2D and 3D culture cells plated during 24 h or 48 h without serum was performed by flow cytometry with Annexin V-FITC and 7AAD markers. In order to verify which spheroid size and culture time has a greater cell viability, firstly we delimited debris in the R1 gate of forward and side scatter graphs. Then a gating (M8) was performed on the FL2 channel fluorescence intensity graph that interferes with the FL1

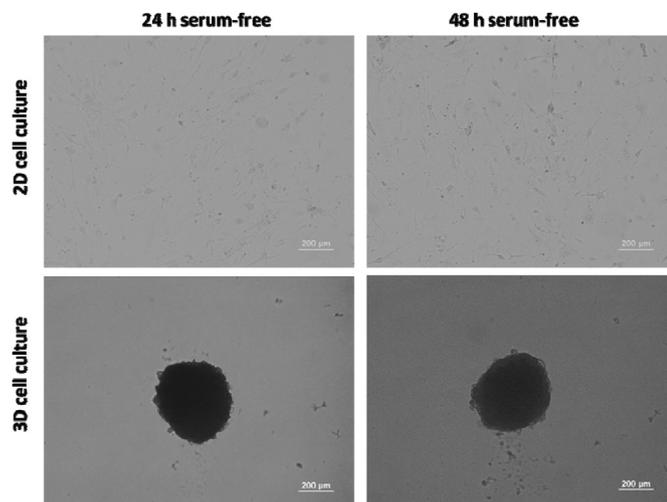


Fig. 2. Photomicrographs of 2D ASC culture and 3D ASC culture ( $2.5 \times 10^4$ ) cultivated for 24 and 48 h without serum. We observed the spindle-shaped characteristic morphology of ASC adhered to plastic surfaces in 2D culture. In 3D cell culture there is a round-shaped structure. The morphology does not change significantly over time of cultivation.

#### Volume

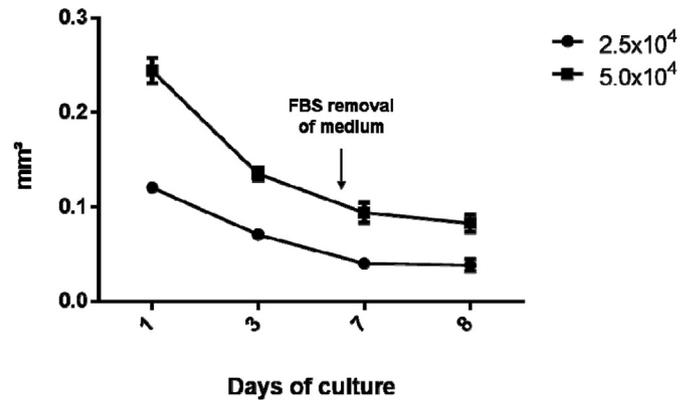


Fig. 3. Spheroids morphometry measured in volume ( $\text{mm}^3$ ). Spheroids with seeding concentration of  $2.5 \times 10^4$  cells were smaller than  $5.0 \times 10^4$  spheroids during 8 days of culture.

channel in the samples for further compensation. These delimitations were important to analyze the FL1/FL3 graph, which included all events, except the gates R1 and M8 (Gating process and viability graphs of other spheroids sizes is demonstrated in Supplementary Fig. 2). Smaller spheroids had a better cell viability ( $2.5 \times 10^4$ ) for 48 h without serum, with a mean of  $95.2\% \pm 3.8$  viable cells, whilst 2D cell culture had a mean of  $67.4\% \pm 12.3$  viable cells. (Fig. 4c).

Regarding CD105 expression by flow cytometry analysis, 2D culture grown for 48 h without serum had a mean of  $21\% \pm 13.2$  CD  $105^+$  cells while 3D culture grown for 48 h without serum had a mean of  $2.6\% \pm 0.6$  CD  $105^+$  cells (Fig. 4f).

#### 3.3. Cytokine profiling of conditioned medium of 2D and 3D cell culture

2D and 3D cultures with a concentration of  $2.5 \times 10^4$  cells cultivated for 48 h without serum were analyzed for cytokine secretion with a Th1/Th2/Th17 profile. The cytokines IL-10, IL-17, TNF, and IFN- $\gamma$  showed no difference in their expression between the 2D and 3D groups. The IL-6 and IL-2 cytokines remained increased in the 3D culture while the IL-4 cytokine was decreased in this group compared to the 2D culture (Fig. 5).

#### 3.4. Glycemic levels in experimental groups

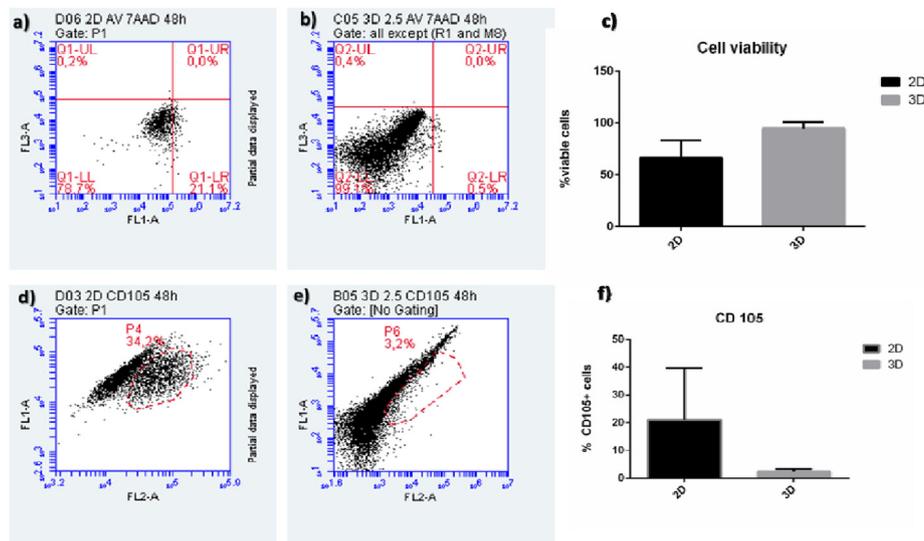
Fig. 6 demonstrates fasting glycemic levels of animals during experimental procedure. At day 14, STZ had higher glycemic levels than CTRL group. At day 21, CM-2D also had higher glycemic levels than CTRL. From the 28th day on it occurs an elevation of glycemic levels in STZ, CM-2D and CM-3D compared to CTRL group. At day 35, 7 days after the last injection of conditioned medium, CM-2D presented lower glycemic levels than STZ and CM-3D.

#### 3.5. Pancreas immunohistochemistry

The insulin and PDX-1 expression in pancreas were analyzed by immunohistochemistry as seen in Fig. 7. Quantitative analysis demonstrated that insulin expression decreased in diabetic groups although it was not restored with injections of 2D or 3D conditioned medium. On contrast, expression of PDX-1 was higher in 2D conditioned medium group.

#### 3.6. Cytokine profiling in pancreas of experimental groups

Pancreas lysates were analyzed for cytokine expression with Th1/Th2/Th17 profiles. The levels of TNF, INF-GAMMA, IL-4, IL-6, IL-10 and



**Fig. 4.** Cell viability and CD105 expression of 2D and 3D cell culture. Representative flow cytometry graphs of 2D (a) and 3D (b) cell viability and graph representing percentage media of viable cells in 2D and 3D culture by 48 h without serum (c). Representative flow cytometry graphs of 2D (d) and 3D (e) CD105<sup>+</sup> cells and graph representing percentage media of CD105<sup>+</sup> cells in 2D and 3D culture by 48 h without serum (f). n = 2.

IL-17 did not differ between groups. IL-2 level was increased in the STZ and CM-3D groups compared to the CTRL group (Fig. 8).

### 3.7. Pancreas apoptosis-related proteins

Quantification of pro-apoptotic (Bax) and anti-apoptotic (Bcl-xl) proteins in pancreatic lysates was performed after Western blotting. The Bax/Bcl-xl ratio was performed and the STZ group showed a higher ratio of pro-apoptotic proteins compared to the CTRL group. Treatment with CM-2D or CM-3D failed to reverse apoptosis in the pancreas of diabetic animals one week after the injections (Fig. 9).

## 4. Discussion

Lately, it has been shown that MSC performs its therapeutic capacity mainly through its secretome, with factors that promote tissue regeneration (Rahimiet al., 2021). MSC in a conventional 2D culture would not adequately mimic an in vivo environment, as cell-cell contact and extracellular matrix production would be limited. Three-dimensional culture would overcome this issue, enabling interaction between cells, production of extracellular matrix, and a gradient of paracrine factors, nutrients, and other metabolic products (Seo et al., 2019). Currently, 3D culture models have been used in experimental models to better understand diseases pathophysiology using neural stem cells, embryonic stem cells, tumor cells, among others (Duvalet et al., 2017). Also, 3D environment would serve as a stimulus for MSC to increase secretion of certain immunoregulatory cytokines, angiogenic factors and the differentiation capacity. Although, in this study, for the first time, we demonstrated that secretome of 2D ASC have more regeneration potential in diabetes type 1 than 3D secretome cell culture.

The 3D culture used in this study was the forced floating technique, which uses a non-adhesive surface that prevents cells from adhering to the round bottom of 96-well plates forming spheroids. This method has the advantage of being relatively simple and allowing the control of spheroid size by number of cells plated in each well. Standardizing spheroid size and its time under culture conditions is an important step in obtaining the secretome and planning the execution of the experiments. In 24 h, cell compaction and spheroid formation in each well was observed. As expected, spheroids with  $2.5 \times 10^4$  cells remained smaller than spheroids with  $5.0 \times 10^4$  cells during the eight days of culture. Over time, the spheroids became more compact. The size of the spheroids

maintained its uniformity at the two concentrations, showing the reproducibility of the experiments. Serum-free culture time (24 h and 48 h) did not affect spheroids morphology. In the literature, we see different results regarding spheroid compaction along cultivation time. In the 2010 study by Bartosh, spheroids cultured by hanging drop method compacted between 48 and 96 h (Bartosh et al., 2010), but other study showed that spheroids became larger with cultivation time (Lee et al., 2016).

In this work, we verified which size and for how long cells cultured without serum (2D or 3D) would maintain an adequate viability. Smaller spheroids (with  $2.5 \times 10^4$  cells) cultured serum-free for 48 h had a higher percentage of viable cells. Previous studies demonstrate that cell survival rate negatively correlates with spheroid size (Murphy et al., 2017). Some studies suggest that this low cell viability in larger spheroids would be due to a necrotic center, but these studies were conducted on cancer cell spheroids and hepatocytes (Curcio et al., 2007; Grimes, Kelly, Bloch, Partridge; Langan et al., 2016). Smaller spheroids tend to have better viability, which may be related to cell-extracellular matrix (ECM) interactions. Smaller spheroids have less ECM and thus better resistance to apoptosis. In larger spheroids, there is a lower packing density and consequently less cell-ECM interaction (Shearier et al., 2016). Regarding culture time, spheroid cultured for 48 h showed greater viability, while in the 2D culture a greater cell survival was observed in 24 h.

When analyzing CD 105<sup>+</sup> cells percentage, we observed that in 3D culture there is a decrease of this molecule compared to 2D culture. This marker, also known as endoglin, is a transmembrane glycoprotein belonging to the TGF-beta superfamily and participates in cell adhesion and cytoskeleton organization. In the cytosolic fraction, endoglin binds to adapter proteins which in turn bind to F-actin molecules (Sanz-Rodriguez et al., 2004). In 2006, the International Society of Cell Therapy determined the parameters so that a cell could be characterized as a mesenchymal stem cell. One of them would be the expression of CD105<sup>+</sup> in more than 95% of the cultivated cell population (Dominici et al., 2006). However, when the cultivated stem cell is derived from adipose tissue, there are different reports in the literature and a consensus has not yet been observed (Ong et al., 2021). When analyzing the SVF of adipose tissue which was recently isolated, a lower CD105<sup>+</sup> cells percentage is observed. After in vitro expansion, this percentage increases, suggesting that the CD105 molecule would be a culture artifact (Prieto González, 2019). Another impression that would confirm this hypothesis would be the reduction in the expression of this molecule observed in this study

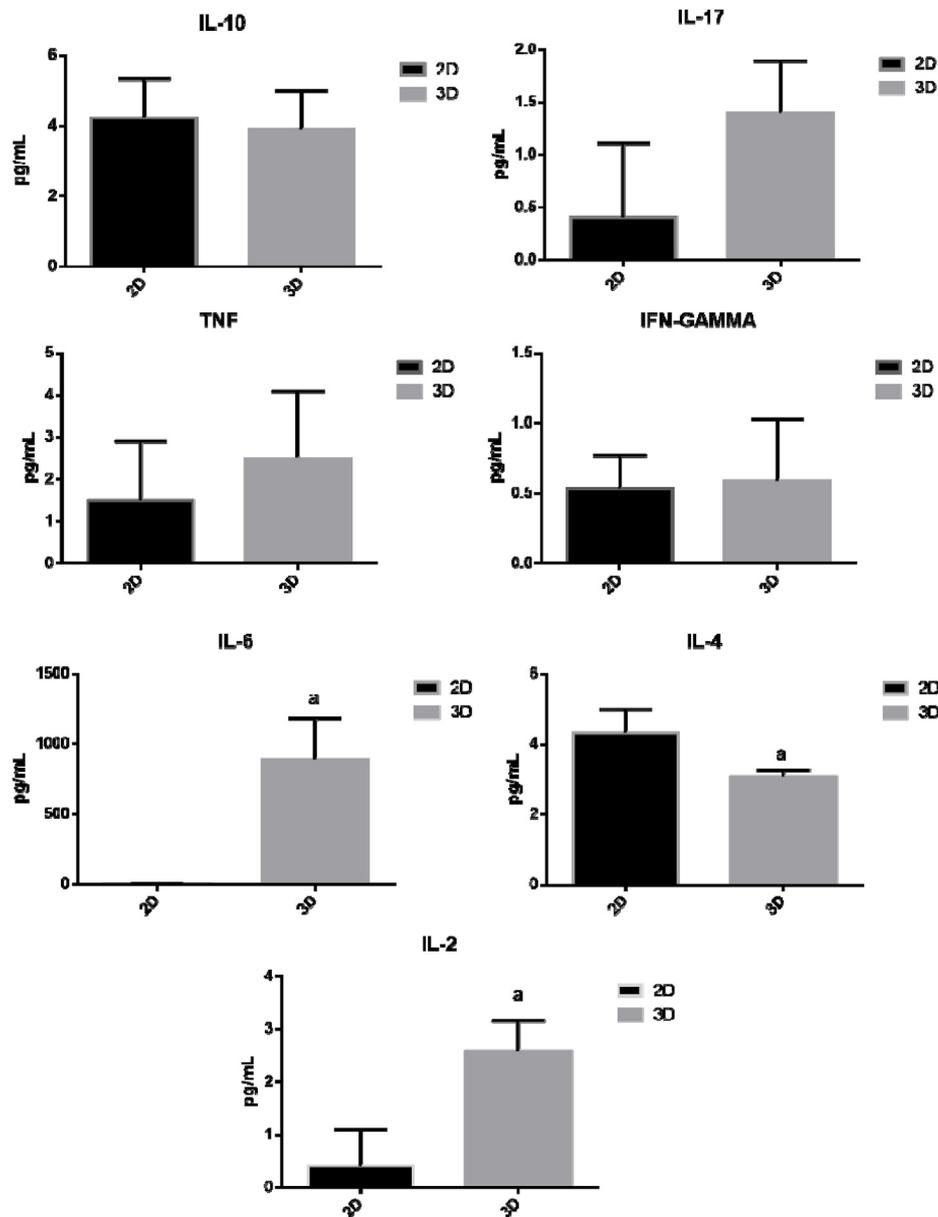


Fig. 5. Cytokines in 2D and 3D conditioned medium. Regarding IL-10, IL-17, TNF and IFN- $\gamma$  concentrations, there was no statistical difference between cultures. IL-6 and IL-2 remained increased while IL-4 showed a decrease in the 3D culture compared to the 2D culture. n = 3, p < 0.05.

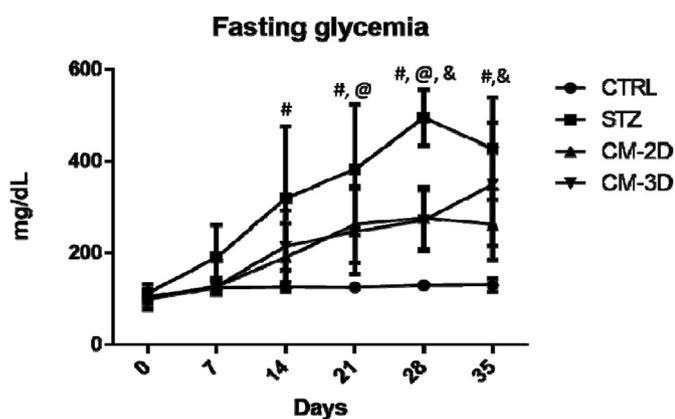


Fig. 6. Fasting glycemia in experimental groups. At day 35, CM-2D presented lower glycemia than STZ and CM-3D groups. n = 4. Significant difference: p < 0.05. # = STZ, @ = CM-2D, & = CM-3D.

when cells are cultured in 3D compared to 2D culture. Other studies have already demonstrated these results and showed that three-dimensionally cultivated cells show a decrease in the percentage of this marker and do not show any loss in their differentiation and proliferation potential. This suggests that the CD 105 molecule would be involved in cell adhesion to plastic (Cheng et al., 2012).

In 3D cell culture within 48 h of conditioning, it was observed that IL-6 and IL-2 were increased while IL-4 was decreased compared to 2D culture secretome. IL-6 was initially considered a proinflammatory cytokine, but this is a very simplistic view as this effect will depend on its concentration, distribution and synergy with other factors (Kristiansen and Mandrup-Poulsen, 2005). Studies have shown that 3D cultured MSC have shown increased secretion of IL-6, and this would be an inducible factor of angiogenesis (Parket et al., 2018; Xie et al., 2017) and improve the antifibrotic potential (Zhang et al., 2016). Specifically in relation to type 1 diabetes, studies demonstrated that this cytokine is not cytotoxic to beta cells and even shows a protective effect on beta cells lineage and mouse islets in vivo. Beta cells produce IL-6 and some data suggest that

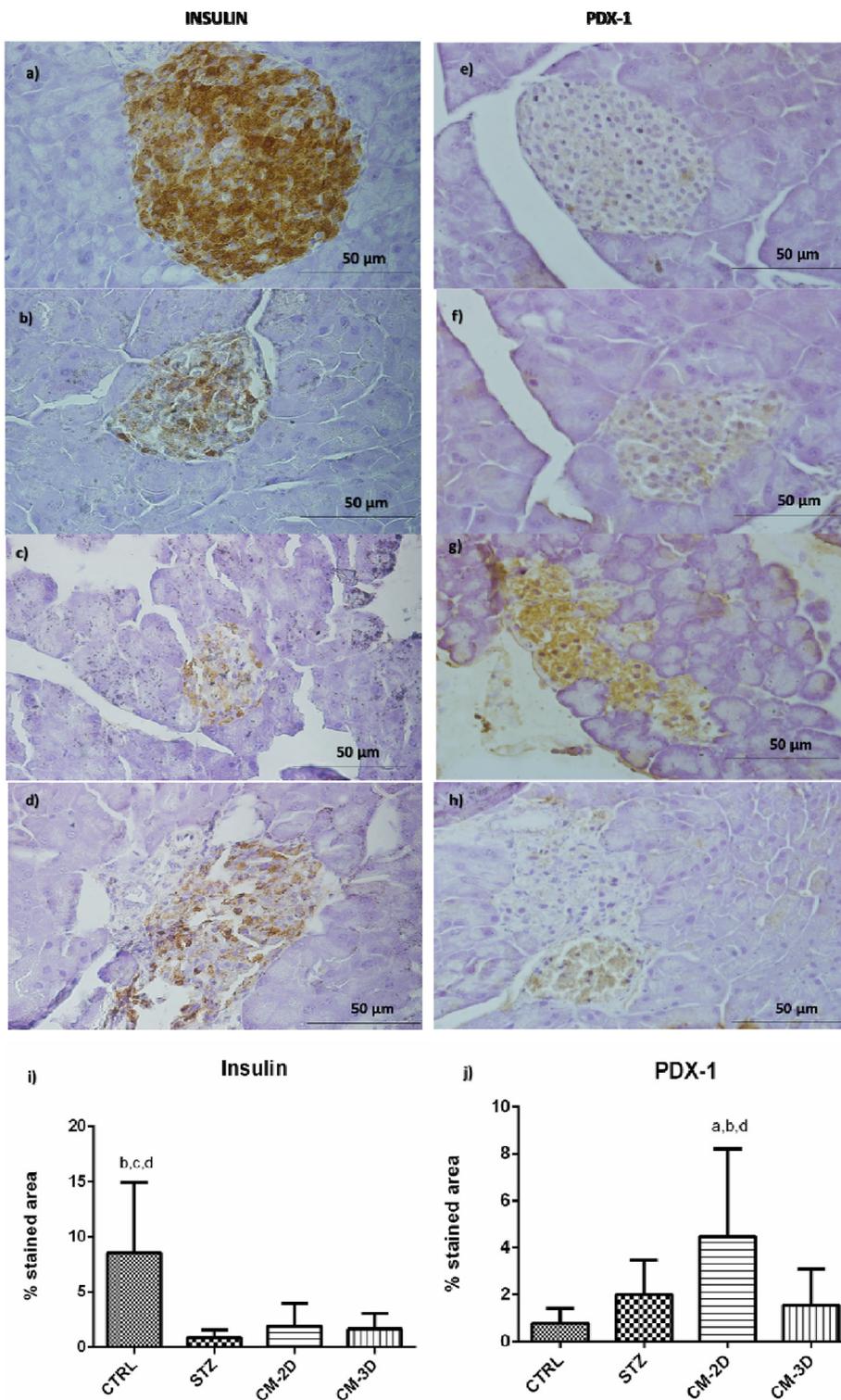
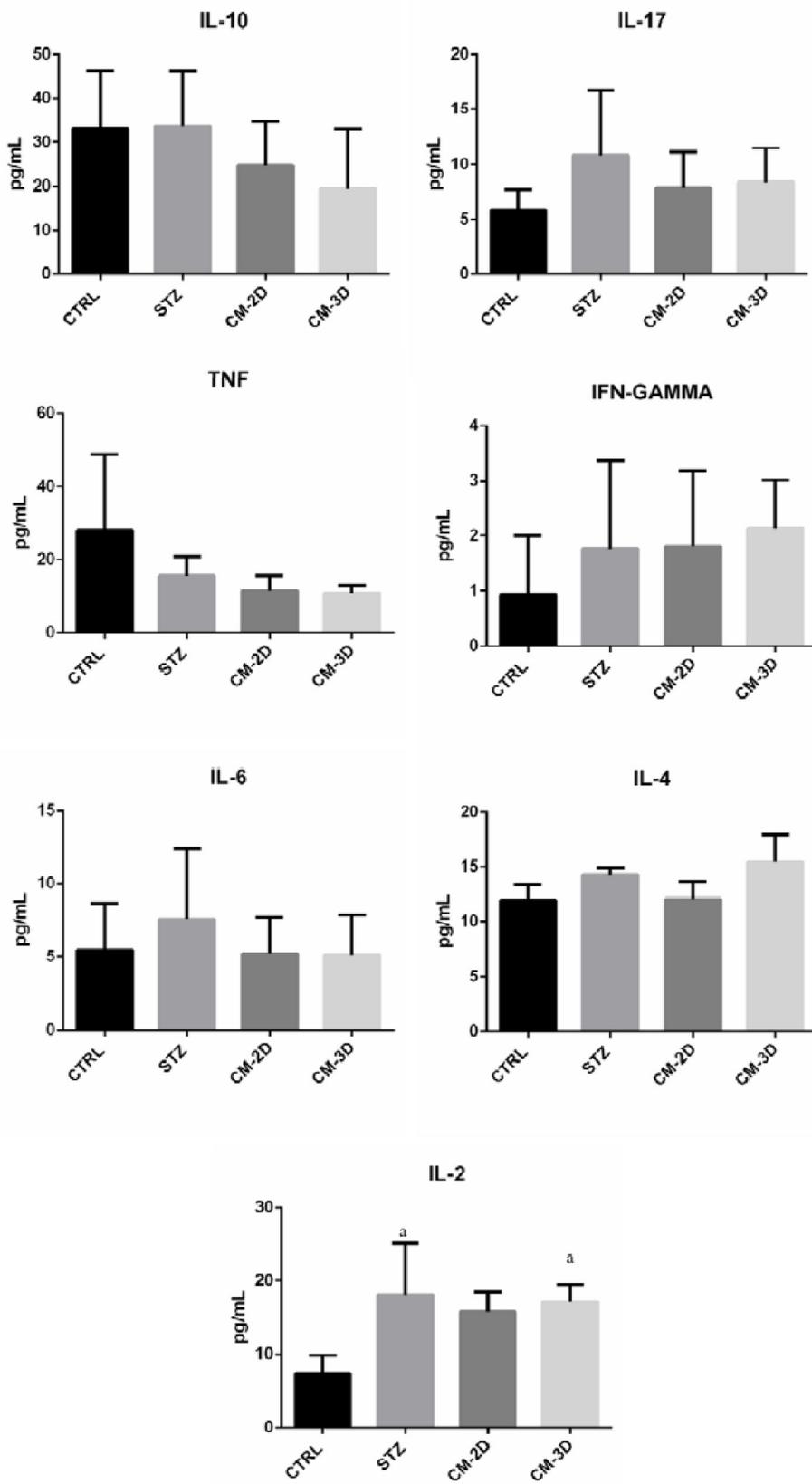


Fig. 7. Pancreas immunohistochemistry. Photomicrographs showing insulin and PDX-1 immunostaining in pancreatic islets: a) CTRL group, b) STZ group; c) CM-2D group; and d) CM-3D group. 40× objective. Quantification of the immunoperoxidase-labeled areas of slides with anti-insulin antibody (i) and anti-PDX-1 antibody (j), with results expressed in percentages. Insulin-labeled areas were lower in the STZ, CM-2D and CM-3D groups in relation to CTRL. Areas marked with PDX-1 were higher in the CM-2D group than in the CTRL, STZ and MC-3D groups. n = 4 p < 0.05.

this cytokine may contribute to the pathogenesis of T1D but its effect alone would not be able to induce or promote beta cell apoptosis (Kristiansen and Mandrup-Poulsen, 2005). IL- 2 has been evaluated for a therapeutic effect in type 1 diabetes. IL-2 is used by cells expressing intermediate-affinity receptors present on conventional and effector T cells and high-affinity receptors that are expressed only on T-reg cells. T-regs need this cytokine to maintain its development and immunoregulatory function. IL-2 deprivation causes apoptosis of these cells that act

as suppressors of effector T cells, which in turn are implied in the progression of type 1 diabetes. However, it is extremely important to stipulate the appropriate dose to avoid toxicity or stimulate effector T cells, as lower concentrations of IL- 2 can stimulate effector T cells, potentiating autoimmunity (Hulme et al., 2012).

Animals that received CM-2D had their blood glucose level decreased one week after applications. Despite this glycemic decrease response, there was no correspondence with the expression of insulin in the



**Fig. 8.** Cytokine levels in pancreas lysates. IL-10, IL-17, TNF, IFN-GAMMA, IL-6 and IL-4 showed no significant differences between groups. IL-2 was increased in the STZ and CM-3D groups compared to the CTRL group. n = 4. P < 0.05.

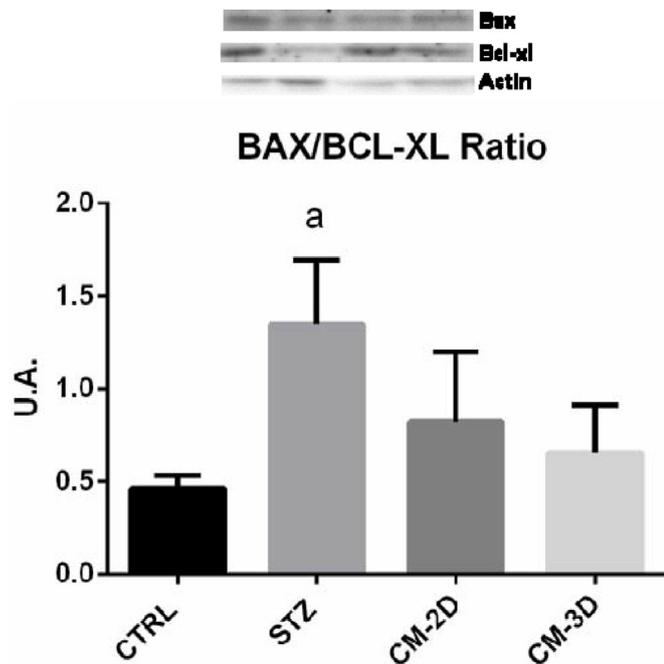


Fig. 9. Ratio of Bax/Bcl-xl proteins expressed in Arbitrary Units (A.U.). STZ group has a higher Bax/Bcl-xl ratio than other groups.  $n = 4$   $p < 0.05$ .

pancreas. Previous studies demonstrate a decrease in blood glucose in mice with type 2 diabetes treated with mesenchymal stem cells from primary teeth, however there was a correlation with increased insulin expression (Xuet et al., 2020). Animals treated with CM-3D did not have their glycaemia decreased compared to diabetic animals. When analyzing the amount of PDX-1, it was seen that there was a greater expression of this molecule in the group treated with CM-2D. This molecule acts as a transcription factor that plays a role in the regulation and maturation of pancreatic cells and acts as an important trigger of pancreatic beta cell differentiation. It binds to the TATA box domain of the GLUT-2 gene and participates in its transcription (McKenna et al., 2015; Talebi et al., 2012). The decrease in blood glucose in the MC-2D group may be related to the increase in PDX-1 in the pancreas, since the GLUT-2 transporter would be one of the target genes that are activated by PDX-1, and consequently would decrease glycemia. The non-restoration of insulin levels in the MC-2D group could be explained by an early stage of beta cell differentiation in which they would not be mature enough to produce insulin in detectable levels. Furthermore, the insulin sensitivity of organs could be increased in diabetic mice which receive 2D secretome. We observed that IL-4 was up-regulated in MC-2D secretome, and studies demonstrated that this cytokine can improve insulin sensitivity (Song et al., 2019; Shiau et al., 2020).

ASC and its conditioned environment can produce anti-apoptotic factors and reverse beta cell destruction. An in vitro study demonstrated that conditioned medium from bone marrow MSC was able to reverse cytokine-induced apoptosis in pancreatic islet cell lines by an IL-10-dependent mechanism (Al-Azzawiet al., 2020). Bhang et al. demonstrated that ASC conditioned medium contains anti-apoptotic factors, including VEGF, which could be responsible for the protective effects of islet functions (Bhanget al., 2013). However, an in vivo study demonstrated that bone marrow MSC did not decrease the level of the effector protein caspase-3 in diabetic mice treated with STZ at the times analyzed (7 and 65 days after transplantation) (Ezquer et al., 2012). In this study, the application of 2D and 3D conditioned media failed to reverse apoptosis in pancreas of diabetic animals in the time-points analyzed. This can be explained by the timing of MSC injection, as when these conditioned media are injected at a later stage of disease development, the islets could be already irreversibly damaged. We suggest further

studies are needed to elucidate the better time points for greater regenerative effects.

## 5. Conclusion

In summary, data from this project demonstrate that the conditioned medium from viable ASC cultivated by 3D method did not decrease blood glucose or reverse apoptosis of pancreatic beta cells in diabetic animals. On the other hand, after one week of injections of the conditioned medium from 2D ASC, the glycemia of diabetic animals decreased and the pancreas showed a higher expression of the transcription factor PDX-1, suggesting an increment towards pancreatic regeneration. Then, this research opens new paths in pursuing alternatives to the treatment for DT1 and brings perspectives in translational studies, although more research needs to be done to explore the molecular mechanisms of action of this regeneration.

## Declarations

Authors declare that there is no conflict of interests.

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## Credit authorship contribution statement

**Isabelle Dias:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Preparation. **Daphne Pinheiro:** Methodology, Investigation, Writing – review & editing. **Karina Ribeiro Silva:** Methodology, Investigation, Writing – review & editing. **Ana Carolina Stumbo:** Supervision, Funding acquisition. **Alessandra Thole:** Funding acquisition. **Erika Cortez:** Resources, Funding acquisition, Writing – review & editing. **Laís de Carvalho:** Funding acquisition. **Simone Nunes Carvalho:** Conceptualization, Writing – review & editing, Project administration, Resources, Funding acquisition.

## Declaration of competing interest

Authors declare that there is no financial interest that may influence the interpretation of results.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crphar.2021.100069>.

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