Protective effects of mesenchymal stem cells-derived extracellular vesicles against ischemia-reperfusion injury of hearts donated after circulatory death: preliminary study in a pig model.

Supplementary materials and methods

Materials and Methods

2.1. Animals

Six female pigs of commercial hybrid breed (Large White x Landrance x Duroc) weighing between 40 and 55 kilograms were enrolled in the study. The animals were housed at the Department of Veterinary Medical Sciences of the Alma Mater Studiorum University of Bologna, Italy. From a microbiological point of view, the structure is free of Aujeszky's disease and accredited for swine vesicular disease. The animals were housed in multiple boxes with a light: darkness cycle of 12/12 hours, and a temperature of 18±1° C, water ad libitum and appropriate food to the age of the animal.

2.2. Animal preparation

On the day of the experimental test, the animals, fasted for 12 hours, were sedated by intramuscular injection of 5 mg/kg of tiletamine/zolazepam (Zoletil 50/50, Virbac) and left in a quiet place until they reached lateral recumbency. At this time, venous access was achieved by introducing a 20G venous catheter into the lateral auricular vein. The catheter was used to administer drugs and fluids (Lactated Ringer's 5 ml/kg/h). General anesthesia was induced using Sevoflurane (Sevoflo 6-8% in a 1:1 mixture of medical air and oxygen) via a breathing mask and then maintained, after orotracheal intubation, always with Sevoflurane (2-4%). Respiration was mechanically assisted in PCV (pressure-controlled ventilation) mode set at 12 mmHg with 5 mmHg PEEP, respiratory rate was adjusted to maintain normocapnia. Anesthesiological monitoring consisted of evaluation of pulse oximetry (SpO2), capnography and capnometry, respiratory rate, electrocardiogram, and heart rate. A single intravenous bolus of heparin (300 IU/kg; Eparina Vister, TEVA) was administered to anticoagulate the animals.

2.3. Normothermic perfusion protocol

Animals were placed in dorsal *recumbency,* and, upon surgical preparation of the field, the left jugular vein was isolated and cannulated with a 13F double lumen cannula for blood collection and central venous pressure (CVP) monitoring. A catheter was inserted percutaneously in the femoral artery to monitor arterial pressure (AP) Euthanasia was performed by administering thiopental 30 mg/kg (Pentothal Sodium, MSD, Italy); equalization of central venous pressure (CVP) and arterial pressure (AP) was used to establish the time of death of the animal. After the declaration of death, we waited 20 minutes, in accordance with Italian jurisprudence for DCD organ donation. No cardioplegia or other preconditioning was administered to the animal since Italian Law does not allow premedication. During this period ventilation was interrupted. After 20 minutes, a median sternotomy was performed to access the heart. The pericardial sac was then opened, and the heart was exposed and observed for signs of macroscopic damage. The animal's heart was then removed and prepared for perfusion. Arterial cannulation was performed with a standard raccord, the pulmonary artery was cannulated with a 22F straight cannula, and ventilation was performed with a 20F ventilation cannula. The perfusion system was composed of four peristaltic pumps, one for the arterial cannula (inserted in the aortic root), one for the venous cannula (inserted in the pulmonary artery) and two for ventilation. Additionally, we used a heat exchanger to perform hot heart perfusion (the initial perfusate temperature was set at 32°C and then heated to 37°C) and a D100 membrane oxygenator to oxygenate the perfusate. Perfusion was performed at a pressure of 40 mmHg; this value was chosen since it is the average of the values used in other experimental settings [1–3].

At the time of starting the perfusion, a priming solution mixed 1:1 with the animal's blood, which had been leukodepleted, was used. The final composition of the priming solution is described in Table 1. In addition, 250 mg of methylprednisolone, 1 IU of multivitamin and 100 ml of human albumin were added to the priming solution, following the standard protocol for organ harvesting in the clinical practice of ex-vivo cardiac preservation. The control group received 2mL of PBS+ 500mM NaCl, while the group treated with MSC-EV received 1x1011 EV, in a final volume of 1-2mL, depending on the concentration of the preparation. The vesicles were thawed at room temperature before being used and added to the priming solution. A standard maintenance solution for ex vivo cardiac perfusion was used for perfusion maintenance (2 hours). The composition of the maintenance solution is reported in Table 2. The solution maintenance was administered directly into the perfusion circuit at 20 cc/h. If necessary, Thamesol was added to the solution as a pH buffer. During the entire perfusion period, blood samples were taken from the circuit every 30 minutes and biochemical parameters were measured: glucose and lactate, to estimate the metabolic activity of the organ; ions (Na+, K+, Cl-); troponin C (TnC), AST, ALT and creatinine phosphokinase (CK) to evaluate cardiac damage. pH values were measured using iSTAT every 10 minutes of perfusion.

Blood samples taken from the circuit were analyzed at the clinical pathology service (CLINLAB) of the Department of Veterinary Medicine of the Alma Mater Studiorum University of Bologna. Biochemical analysis was performed using a Beckman Coulter AU480 clinical chemistry analyzer, using serum. Hematological analysis was performed using a SIEMES ADVIA 2120 hematology analyzer using K3EDTA whole blood samples. After 2 hours of perfusion, samples were taken for analysis. Samples for histological analysis were fixed in 4% paraformaldehyde in PBS, while the rest of the samples were frozen in liquid nitrogen until use.

Substrate	Concentration
Na+ (mEq/L)	132
K+ (mEq/L)	4,2
Ca++ (mEq/L)	1,2
Cl- (mEq/L)	84
Mg++ (mEq/L)	1
Phosphate (mEq/L)	2,9
Glucose (mg/dl)	148

Table S1. Composition of the priming solution.

Substrate	mg in 500ml H_2O
$CaCl_2 \cdot 2H_2O$	2400
NaCl	1750
Adenosine	750
L-arginine	700
$MgSO_4 \cdot 6H_2O$	400
Glycine	350
L-leucine	343
L-glutamic acid	258
L-aspartic acid	245
Lisine acetate	225
L-histidine	225
L-alanine	174
L-valine	171.5
L-proline	126
L-isoleucine	115.5
L-serine	93
L-tirosine	92
L-treonine	70
L-methionine	59
L-phenialalanine	52
L-triptophan	35
KCl	20

Table S2. Composition of the maintenance solution

2.4. MSC culture

HUCPVC-derived MSCs purchased from PromoCell (Heidelberg, Germany). HUCPVCs were cultured as previously described [4] and expanded until passage four to establish a cell stock for sEV isolation. At this point, cells were characterized according to ISCT guidelines [5]. For sEV isolation, passage four to five HUCPVCs were thawed and cultured in low-glucose Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific) at 37°C in a humidified incubator containing 5% carbon dioxide. Next, cells were collected using trypsin 0.1% in Hank's buffer (Thermo Fisher Scientific) and plated in 175 cm2 culture flasks at a density of 4000 cells/cm2 until 70% confluence. Finally, culture medium was changed to alpha minimal essential medium without phenol red (Thermo Fisher Scientific) or FBS. HUCPVCs were cultured in starvation medium for 48 h. Afterward, conditioned medium was collected and centrifuged at 2500 x g for 10 min to eliminate cellular debris and then filtered using a 0.22-mM syringe-driven filter unit (Millex-GP; MilliporeSigma, Burlington, MA, USA). The supernatant was processed immediately and the obtained sEVs were subjected to particle number assessment and protein, lipid and RNA quantification. Next, the sEV preparations were aliquoted and frozen at -80°C until use. Three separate independent isolations from HUCPVCs (passages six to eight), thawed at different times but from the same lot, were performed for each isolation method.

2.5. MSC-EV isolation

MSC-sEVs were isolated by ion exchange chromatography (IEX) or ultrafiltration (UF). Conditioned media was processed as previously described [6]. Briefly, to isolate MSC-sEVs by IEX, either fresh or thawed (at 37°C) conditioned media (150 ml) was applied directly to a column containing an anion exchange resin (4-ml bed volume, Q Sepharose Fast Flow, GE Healthcare, Chicago, IL, USA) that had been equilibrated with 50 mM NaCl in 50 mM phosphate buffer (pH 7.5). Then, the column resin was washed with 100 mM NaCl in 50 mM phosphate buffer (pH 7.5) and then eluted with 8 mL of 500 mM NaCl in 50 mM phosphate buffer (pH 7.5). Eight fractions of 1 ml each were collected. EVs were eluted in fraction 4 and only this fraction was used for the experiments. Samples were stored at -80°C until their use.

2.6. Tunable Resistive Pulse Sensing (tRPS)

Particle concentration and size distribution were analyzed using tunable-resistive-pulse-sensing (TRPS) technology with the qNano instrument (Izon Science, Christchurch, New Zealand). NP100 or NP150 membranes were used for the analysis. The concentration of particles was standardized using a CPC100 calibration solution diluted 1:1,000 (110 nm mean carboxylate polystyrene beads; stock concentration 1.00 × 1012) [4].

2.7. Transmission Electron Microscopy for MSC-EV characterization

MSC-EVs were fixed with 4% PFA for 5 min, and one drop (2.00 × 109 particles) of EVs was placed on a 400-mesh holey-film grid for 10 min. After washing with PBS, the MSC-EVs were stained with 1% uranyl acetate for 2 min. The sample was then washed with PBS and finally observed with a Tecnai G2 (FEI; Thermo Fisher Scientific, Waltham, MA, USA) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System; Münster, Germany) digital camera.

2.8. Immunophenotyping of MSC-EVs

MSC-EVs were characterized by flow cytometry using the MacsPlex Exosome Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), following manufacturer's instructions. Briefly, 1.00 × 109 particles of MSC-EVs were loaded in a 1.5 mL tube and diluted to 120 µL with MACSPlex buffer. Then, a mix of 15 µL of exosome capture beads and 15 µL of antibody were added to each tube and incubated for 1 h at RT in agitation. For blank control, only MACSPlex buffer was used. Then, 500 µL of MACSPlex buffer was added to each tube and the EVs were centrifuged at 3000× g for 5 min. Finally, 500 µL of the supernatant was discarded and the samples were resuspended and transferred to a flow-cytometry tube. Samples were analyzed using a Cytoflex (Beckman Coulter, Brea, CA, USA) flow cytometer.

2.9. Histological analysis

To evaluate microscopic signs of damage, samples were taken from the ventricles and interventricular septum and then analyzed. Immediately after collection, samples were fixed in 10% buffered formalin. After 24 hours of fixation, all samples were embedded in paraffin blocks, then sectioned into 5-micron slices, and then stained with hematoxylin-eosin. Signs of hemorrhage, edema, coagulative necrosis and cellular infiltration were evaluated in the histological samples. Histological scoring was applied according to an evaluation of the percentage of altered cells over the total number of cells observed as follows: 0 (< 1%), 1 (1-25%), 2 (25-50%), 3 (50-75%), 4 (>75%).

To evaluate apoptosis in cardiac tissue, caspase-3 levels were measured by immunohistochemistry. The streptavidin-biotin-peroxidase technique (BIO SPA, Milan, Italy) was followed with Cleaved Caspase-3 antibody (dilution 1:2000, Cell Signaling). After incubating the samples with 0.3% hydrogen peroxide in methanol for 30 minutes, the sections were incubated overnight in a humidified chamber at 4°C with the primary antibody. The sections were then washed in PBS and incubated with the secondary antibody (biotin-linked anti-rabbit IgG) for 30 min at room temperature. They were subsequently incubated with streptavidin-biotin-peroxidase for 25 minutes at room temperature. Then, they were incubated for 10 minutes

in DAB chromogen solution (0.02% diaminobenzidine and 0.001% hydrogen peroxide in PBS), the sections were immediately washed with PBS and then with water. After washing, sections were counterstained (hematoxylin), dehydrated, and fixed with DPX (Fluka). The results were expressed as the total number of positive cells in 10 fields at 40x magnification.

2.10. Transmission Electron Microscopy for Mitochondrial Evaluation

Small cardiac tissue samples (about 1–2 mm3) were fixed in 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 O.N. at 4 °C, subsequently postfixed in osmium tetroxide 1% in 0.1 M sodium cacodylate buffer for 2 h at 4 °C and embedded in an Epon–Araldite resin mixture. Semithin sections were stained with toluidine blue. Ultrathin sections (60–70 nm) were obtained with a Leica Ultracut EM UC7 ultramicrotome, counterstained with uranyl acetate and lead citrate, and viewed with a Tecnai G2 (FEI) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System, Olympus Italia S.r.l., 20054 Segrate, Italy) digital camera.

2.11. Mitochondrial analysis

Changes in mitochondrial cristae morphology were quantified by counting mitochondria with reduced lamellar density and/or abnormal lamellar structure and expressing these mitochondria relative to the total number of mitochondria counted.

2.12. Preparation of tissue samples

Biopsies derived from pig hearts were homogenized with a Polytron in 4 vol/g tissue of 10 mM Tris-HCl buffer pH 7.6, containing 1 mM DTT (dithiothreitol), 0.5 M sucrose, and 0 KCl. .15 M. The homogenates were centrifuged at 20,000 rpm for 60 min at 4 ◦C (Beckman model J2/21). The supernatants were collected and stored at −80◦C for biochemical analysis. From each animal, a sample was taken from the left ventricle, one from the right ventricle and one from the interventricular septum, which were processed independently. The determination of proteins in the samples was carried out using the BCA method (BCA protein assay kit, Thermofisher).

2.13. Determination of SOD activity

To determine the enzymatic activity of SOD, the supernatant of the cardiac tissue homogenates was used. A commercial kit was used, and the manufacturer's instructions were followed (SOD determination kit, 19160, Sigma). Briefly, the kit uses the highly water-soluble tetrazolium salt, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt] which produces a water-soluble formazan dye upon reduction with a superoxide anion. The reduction rate con ●O2 is linearly related to xanthine oxidase (XO) activity and is inhibited by SOD. Therefore, the IC50 (50% SOD inhibition activity) can be determined by a colorimetric method, measuring the absorbance at 440 nm, which is proportional to the amount of superoxide anion. Thus, SOD activity as inhibition activity can be quantified by measuring the decrease in absorbance at 440 nm. To do this, a solution containing the WST is added to 20µL of sample or standard and mixed. Then, a solution containing xanthine oxidase is added and incubated at 37°C for 20 minutes. Once the incubation is completed, the absorbance is measured at 450nm.

2.14. Determination of CAT activity

To determine the enzymatic activity of CAT, the supernatant of the cardiac tissue homogenates was used. A commercial kit was used, and the manufacturer's instructions were followed (Catalase activity assay kit, ab83464, Abcam). Briefly, the catalase present in the sample reacts with hydrogen peroxide (H2O2) to produce water and oxygen. Unmetabolized H2O2 reacts with a probe to produce a product that can be measured colorimetrically at 570 nm or. Therefore, the catalase activity present in the sample is inversely proportional to the signal obtained. To do this, the samples and standards are added to the wells in a 96-well plate. Stop solution is added to the sample control wells and incubated at 25ºC for 5 min. Then, H2O2

solution is added to the wells and incubated for 30 min at 25ºC, when stop solution is added. Finally, the developer mixture is added, incubated for 10 min at 25ºC and the absorbance is read at 570 nm.

2.15. Determination of GPx activity

To determine the enzymatic activity of GPx, the supernatant of the cardiac tissue homogenates was used. A commercial kit was used, and the manufacturer's instructions were followed (Glutathione peroxidase activity assay kit, ab102530, Abcam). Briefly, glutathione peroxidase (GPx) oxidizes GSH to produce GSSG as part of the reaction in which it reduces cumene hydroperoxide. Glutathione reductase (GR) then reduces GSSG to produce GSH, and in the same reaction consumes NADPH. The decrease in NADPH (measured at 340 nm) is proportional to GPx activity. To do this, 20µL of the sample is loaded into a 96-well plate, then the reaction mixture is added and incubated for 15 min at room temperature to exhaust all the GSSG in the sample. Cumene hydroperoxide is then added and analyzed with a microplate reader after at least 5 minutes.

2.16. Determination of carbonylated proteins

For the determination of carbonylated proteins, the supernatant of the cardiac tissue homogenates was used. A commercial kit was used, and the manufacturer's instructions were followed (Protein Carbonyl Content Assay Kit, ab126287, Abcam). Briefly, the protein carbonylation determination assay is based on the reaction of DNPH with protein carbonyls. The DNP hydrazones formed in this reaction can be quantified at an absorbance of 375 nm. To do this, DNPH is added to the samples and incubated for 10 min. TCA (trichloroacetic acid) is then added to the samples and incubated for 5 min. Then centrifuged for 2 min, the supernatant is discarded, and the pellet is washed with acetone. Finally, a guanidine solution is added to resolubilize the pellet and it is transferred to the 96-well plate to measure the absorbance at 375 nm.

2.17. Tissue Repair Mediators Analysis

Tissue Samples Preparation. The biopsies derived from the pigs' hearts were homogenized with Polytron in 4 vol/g of tissue of 10 mM Tris–HCl Buffer pH 7.6, containing 1 mM DTT (dithiothreitol), 0.5 M sucrose and 0.15 M KCl. The homogenates were centrifuged at 20,000 rpm for 60 min at 4 °C (Beckman model J2/21). The supernatants were collected and stored at −80 °C for biochemical analysis, as described below

Estimation of the Total Amount of Proteins. The total amount of proteins in the cellular extracts was assessed by the Folin phenol reagent method [7], using growing concentrations of bovine serum albumin as standard.

Total protein extracts were analyzed to quantify pro-inflammatory cytokines and chemokines, which were simultaneously measured in each protein extract with Luminex xMAP®® technology (Luminex Corporation, Austin, TX, USA). Each measurement was performed in duplicate. Quantitative analyses were performed with Luminex xPONENT 3.1 Software using a five-parameter logistic curve fitting. Data were expressed as pg per mg tissue.

2.18 Statistical analysis

One-way ANOVA with Kruskal–Wallis test were used for multiple comparisons. Median ± range was used. Student's unpaired t-test was used for independent samples. Data were analyzed using GraphPad software. P-values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0001$.

References

1. Lucchinetti E, Lou PH, Hatami S, Qi X, Clanachan AS, Freed DH, et al. Enhanced myocardial protection in cardiac donation after circulatory death using Intralipid® postconditioning in a porcine model. Can J Anaesth. 2019;66: 672–685. doi:10.1007/S12630-019-01322-X
2. White CW, Ambrose E, Müller A, Li Y, Le H, Thliveris J, et al. Avoidance of Profound

Hypothermia During Initial Reperfusion Improves the Functional Recovery of Hearts Donated After Circulatory Death. Am J Transplant. 2016;16: 773–782. doi:10.1111/AJT.13574

3. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. J Vis Exp. 2019;2019. doi:10.3791/58430

4. Malvicini R, De Lazzari G, Tolomeo AM, Santa-Cruz D, Ullah M, Cirillo C, et al. Influence of the isolation method on characteristics and functional activity of mesenchymal stromal cell-derived extracellular vesicles. Cytotherapy. 2024;26: 157–170. doi:10.1016/J.JCYT.2023.11.001

5. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8: 315–317. doi:10.1080/14653240600855905

6. Malvicini R, Santa-Cruz D, Tolomeo AM, Muraca M, Yannarelli G, Pacienza N. Ion exchange chromatography as a simple and scalable method to isolate biologically active small extracellular vesicles from conditioned media. PLoS One. 2023;18: e0291589. doi:10.1371/JOURNAL.PONE.0291589

7. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193: 265–275. doi:10.1016/s0021-9258(19)52451-6