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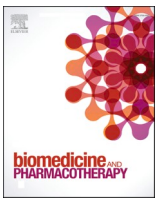

Protective effects of mesenchymal stem cells-derived extracellular vesicles against ischemia-reperfusion injury of hearts donated after circulatory death: Preliminary study in a pig model

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ABSTRACT

Introduction: Insufficient supply of cardiac grafts represents a severe obstacle in heart transplantation. Donation after Circulatory Death (DCD), in addition to conventional donation after brain death, is one promising option to overcome the organ shortage. However, DCD organs undergo an inevitable more extended period of warm unprotected ischemia between circulatory arrest and graft procurement. Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) have shown remarkable protective effects against ischemia-reperfusion injury. Thus, we aimed to enhance grafts preservation from DCD donors, through treatment with MSC-EVs.

Methods: Female pigs were euthanized by barbiturate overdose and after 20 min of a flat EKG, the chest was opened, the heart harvested and subsequently connected to an extracorporeal perfusion machine. MSC-EVs, isolated by ion exchange chromatography, were added to the perfusion solution (1×10^{11} particles) and the heart was perfused for 2 h. Then, heart tissue biopsies were taken to assess histological changes, mitochondrial morphology, antioxidant enzyme activity and inflammation mediators' expression. Biochemical parameters of myocardial viability were assessed in the perfusate.

Results: The treatment with MSC-EVs significantly prevented mitochondria swelling, mitochondrial cristae loss and oxidative stress in cardiac tissue. The protective effect of MSC-EVs was confirmed by the delayed increase of the cardiac-specific enzymes CK and TnC in the perfusate and the reduction of caspase-3+ cells in tissue sections.

Conclusion: MSC-EVs improve graft quality by preserving the mitochondrial ultrastructure protecting the myocardium against oxidative stress, reducing apoptosis of cardiac cells and preventing the increase of pro-inflammatory cytokines.

Abbreviations: **ALT:**, alanine aminotransferase; **AST:**, aspartate aminotransferase; **CAT:**, catalase; **CK:**, creatinin kinase; **DBD:**, donors after brain death; **DCD:**, Donation after Circulatory Death; **GGT:**, gamma-glutamyl transferase; **GPx:**, glutathione peroxidase; **IRI:**, ischemia-reperfusion injury; **IVS:**, Interventricular septum; **IL-x:**, interleukin-x; **LV:**, left ventricle; **MSC-EVs:**, mesenchymal stromal cell-derived extracellular vesicles; **ROS:**, reactive oxygen species; **RV:**, right ventricle; **SOD:**, superoxide dismutase; **TEM:**, transmission electron microscopy; **TnC:**, troponin C.

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1. Introduction

Heart transplantation is the treatment of choice in patients where other treatments have failed and there are no other therapeutic alternatives available to improve organ function and the patient's quality of life [1]. It is performed in patients with chronic heart failure, cardiomyopathy, coronary artery disease, and valvular disease, among others [2]. Donor hearts can come from donors after brain death (DBD) or donors after circulatory death (DCD). DCD are those donors from whom life support is withdrawn in a controlled manner and who have suffered extensive and irreversible brain damage, but who do not meet the criteria for brain death. From a clinical point of view, DBD hearts are preferred over DCD hearts since, in the former group, it is possible to maintain adequate perfusion of the organs until procurement is completed, although DCD organs generally come from younger donors with fewer comorbidities [3]. However, due to the increase in life expectancy and technological advances in the treatment of end-stage heart failure and other pathologies, the number of patients on the waiting list demanding a heart transplant currently exceeds the availability of organs [4]. Furthermore, this imbalance tends to worsen over the years given that the characteristics of donors are changing towards older donors. In this sense, as the age of the donor increases, comorbidities such as obesity, diabetes or high blood pressure also increase, which translates into poorer quality of the organs, which aggravates the need for organs and leads to having to accept marginal organs for cardiac transplantation [4].

In this scenario, hearts from DCD have been reconsidered and adopted as an alternative source of organs for transplantation [5,6]. However, the main limitation to the widespread use of DCD organs is that they inevitably suffer a certain degree of ischemic injury during the time of withdrawal of the donor life support [6] and the "no-touch" period (necessary to certify the death of the donor), which varies according to the laws of each country. During this time of unprotected warm ischemia, cellular damage sets in, which is further exacerbated once coronary circulation is restored. This damage, known as ischemia-reperfusion injury (IRI), is characterized by the loss of cellular homeostasis due to intracellular imbalances, such as calcium overload and the production of reactive oxygen species (ROS), which lead to the activation of apoptosis, pyroptosis and cell death due to necrosis [7]. ROS are commonly produced due to cellular metabolism and, at physiological concentrations, are involved in the regulation of growth factor signaling, hypoxic response, inflammation and immune response in mammalian cells [8]. However, in pathological conditions, the increase in these intermediates causes direct damage to cellular DNA, proteins, and lipids, in addition to activating stress response pathways, which initiate a cytokine-mediated cascade that ultimately recruits cells of the immune system, such as neutrophils and macrophages, amplifying tissue damage [9].

Furthermore, mitochondria are critical organelles in a high-energy-consuming organ like the heart and play a central role in its homeostasis. In IRI, mitochondria are one of the main targets of oxidative damage, leading to mitochondrial edema (mitochondrial swelling), loss of mitochondrial cristae, membrane potential and, eventually, cell death [10,11]. Therefore, preserving the integrity and functionality of mitochondria is essential to ensure the organ's viability.

In this sense, mesenchymal stem cells derived-extracellular vesicles (MSC-EVs) have been demonstrated to convey many of the pro-regenerative, anti-apoptotic and anti-inflammatory effects characteristic of their cells of origin. MSCs-EVs limit the excessive inflammatory response inhibiting M1 macrophages polarization and effector T lymphocytes and stimulating the M1 macrophages polarization and the proliferation of regulatory T cells [12–14]. Moreover, in animal models, MSC-EVs enhance the functional recovery of the kidney and heart following IRI, as well as liver regeneration [15,16].

Given all the above, we aimed to achieve an efficient and safe therapeutic based on the rational use of MSC-EVs in a preclinical model of heart transplantation to better preserve the organs and thus contribute to increasing the pool of organs available for transplantation.

2. Materials and methods

2.1. Establishment of the porcine model of cardiac preservation

Animals were anesthetized by intramuscular injection of tiletamine/zolazepam (5 mg/kg). Venous access was performed in the auricular vein, the animals were intubated, and general anesthesia was induced with 8 % sevoflurane. Then, animals were euthanized by administering thiopental (20 mg/kg). According to Italian law, we waited 20 min before proceeding with the procurement of the heart. No medication or intervention was administered during this "no-touch" period, in which

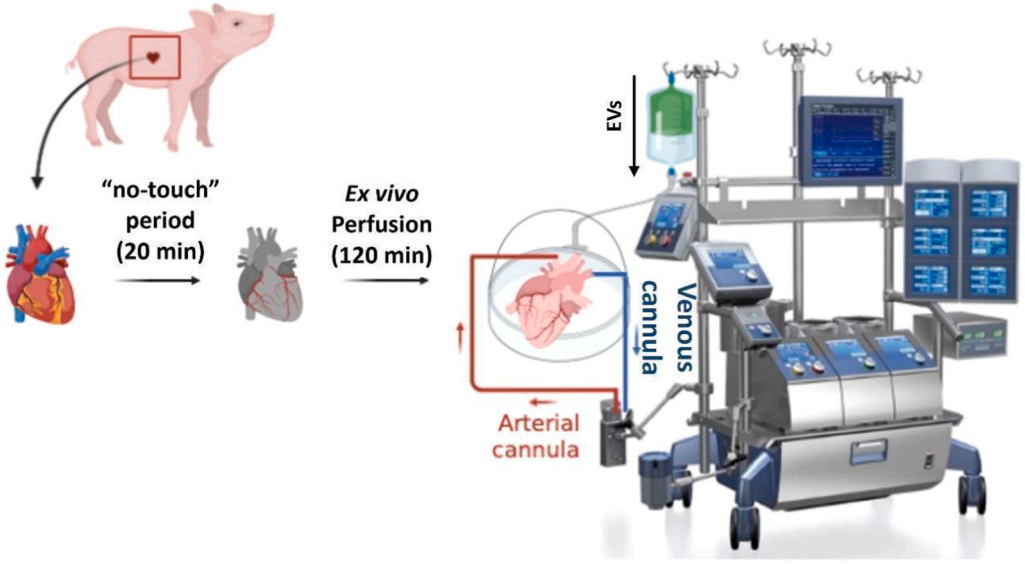

Fig. 1. Schematic representation of the perfusion protocol. After the death of the animal was declared, the heart was left in the animal's body for 20 minutes, according to Italian Law ("no-touch" period). The heart was then removed and connected to a perfusion machine, where it was perfused with a mixture of the animal's own leucodepleted blood and a preservation solution, formulated ad hoc, for 120 min. At the beginning of the perfusion, the extracellular vesicles ($1x10^{11}$ particles) or an equivalent volume of PBS+500 mM NaCl (control group) were added.

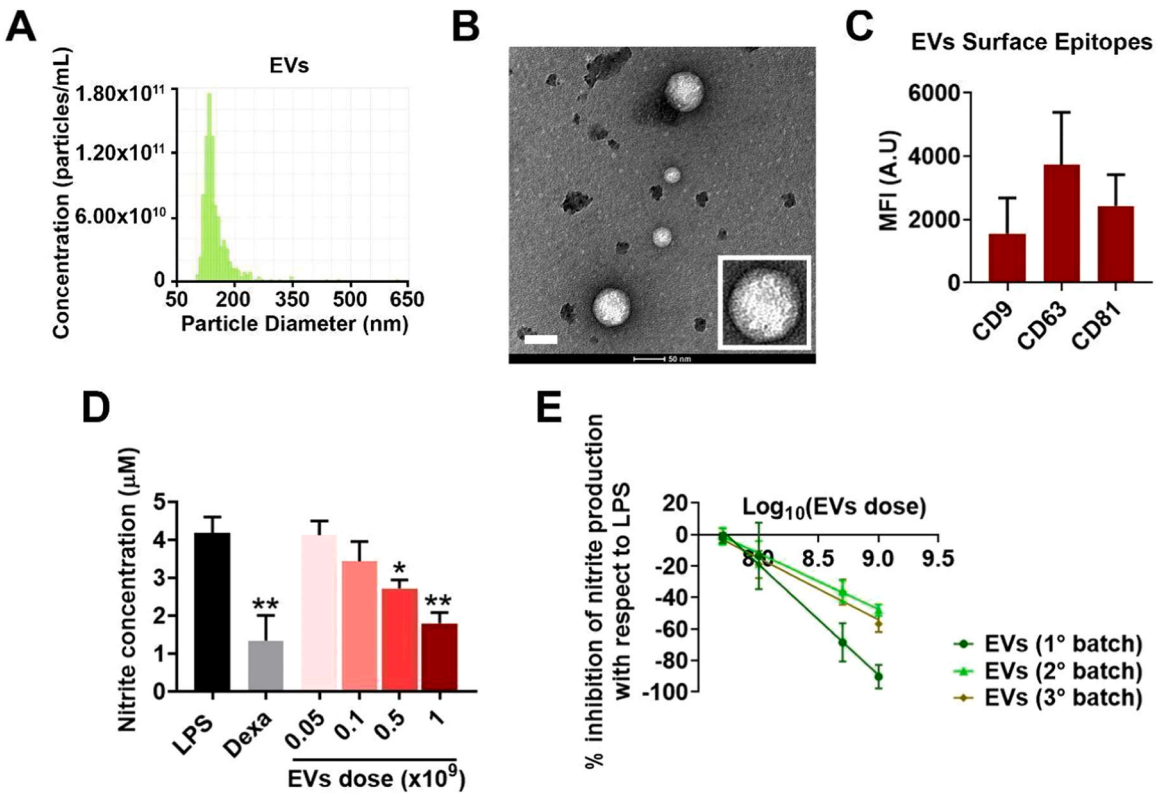

Fig. 2. EV characterization. EV particle size and distribution were assessed by tRPS (A) while TEM assessed its morphology. Scale bar: 50 nm (B). EV surface markers CD9, CD63 and CD81 were assessed by flow cytometry. The biological activity of EVs was evaluated before using them in the *ex-vivo* model. A macrophage assay evaluated EVs' anti-inflammatory activity (D, E). Results were expressed as mean ± SD and significance was determined for independent samples using one-way ANOVA with post-hoc Tukey's test (n = 3). *P<0.05; **P<0.01.

ventilation had been interrupted. After 20 min, the animal's heart was removed and connected to the perfusion machine for 120 min. The perfusion circuit consisted of an air and an oxygen inlet intended to maintain an adequate oxygen pressure, a carbon dioxide inlet to regulate the pH through pCO2, and an inlet to administer drugs and the MSC-EVs (1×10^{11} particles). After 120 min, tissue and perfusate samples were collected for biochemical, histological, inflammation mediators and oxidative stress analysis. The model scheme is outlined in Fig. 1.

Experimental activities with animals were carried out per the current Italian legislation on using animals for experimental purposes (D. Lgs 26/2014). A local ethics committee and the Italian Ministry of Health approved this study. Please see the online supplement for detailed methods.

3. Results

3.1. MSC-EV characterization

TRPS analysis of the MSC-EVs demonstrated a homogeneous population with particle sizes mostly below 100 nm (Fig. 2A). Transmission electron microscopy (TEM) identified the EVs as a group of heterogeneous spheroids with sizes ranging from 30 to 250 nm (Fig. 2B). The RPS and TEM analysis confirmed that almost all the nanoparticles isolated in the present work could be called "small EVs", according to the MISEV2018 criteria [11]. No apoptotic bodies were detected. The EV surface markers were analyzed and the classical tetraspanin (CD63, CD9 and CD81) were identified in our samples (Fig. 2C). To corroborate the biological activity of the EVs preparations before using them in the *ex-vivo* model, and to compare their relative potency, we performed an LPS-stimulated macrophage assay [17]. As can be seen in Fig. 2D and E, EVs preparations used to treat the hearts (one preparation per heart), presented a significant anti-inflammatory activity, although the EV1 preparation exhibited greater relative potency than the other two preparations (Y=-70.3X + 543; IC50=2.75x108 vs Y=-35.5X + 272; IC50=1.17x109 and Y=-40.1X + 253; IC50=1.02x109, respectively).

3.2. Extracellular vesicles ameliorate cardiac damage after ischemia-reperfusion

Biochemical parameters were monitored and analyzed in the perfusion fluid every 30 minutes, during the 120 min of perfusion. A continuous increase in glucose concentration was observed throughout the perfusion, with no observable differences between the two groups (Fig. 3A). Regarding the pH, a slight acidosis was maintained during the first hour of perfusion and was then adjusted to physiological values for both groups (Fig. 3B). An attempt was made to hold a slight hyponatremia to avoid calcium overload, which was more marked in the control group (Fig. 3C). Regarding potassium, normokalaemia levels were maintained throughout the perfusion, to avoid hyperpolarization of cardiac cells (Fig. 3D). Regarding chloride, slight hypochloremia was maintained throughout the perfusion (Fig. 3E). Regarding Magnesium, hypermagnesemia was maintained, since it has been shown that it has anti-arrhythmogenic properties and can prevent calcium overload (Fig. 3F). Regarding ionic calcium, hypocalcemic levels were maintained during perfusion, to avoid calcium overload of cardiomyocytes, which can lead to cell death (Fig. 3G). These results indicate that treatment with extracellular vesicles does not influence the biochemical parameters analyzed, which can be regulated and adjusted thanks to the perfusion system.

Then, the enzymatic activity of cardio-specific enzymes, such as creatinine kinase (CK) and troponin C (TnC), and non-cardio-specific enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) was assessed. As can be seen in Fig. 4, treatment with MSC-EVs delays the increase in

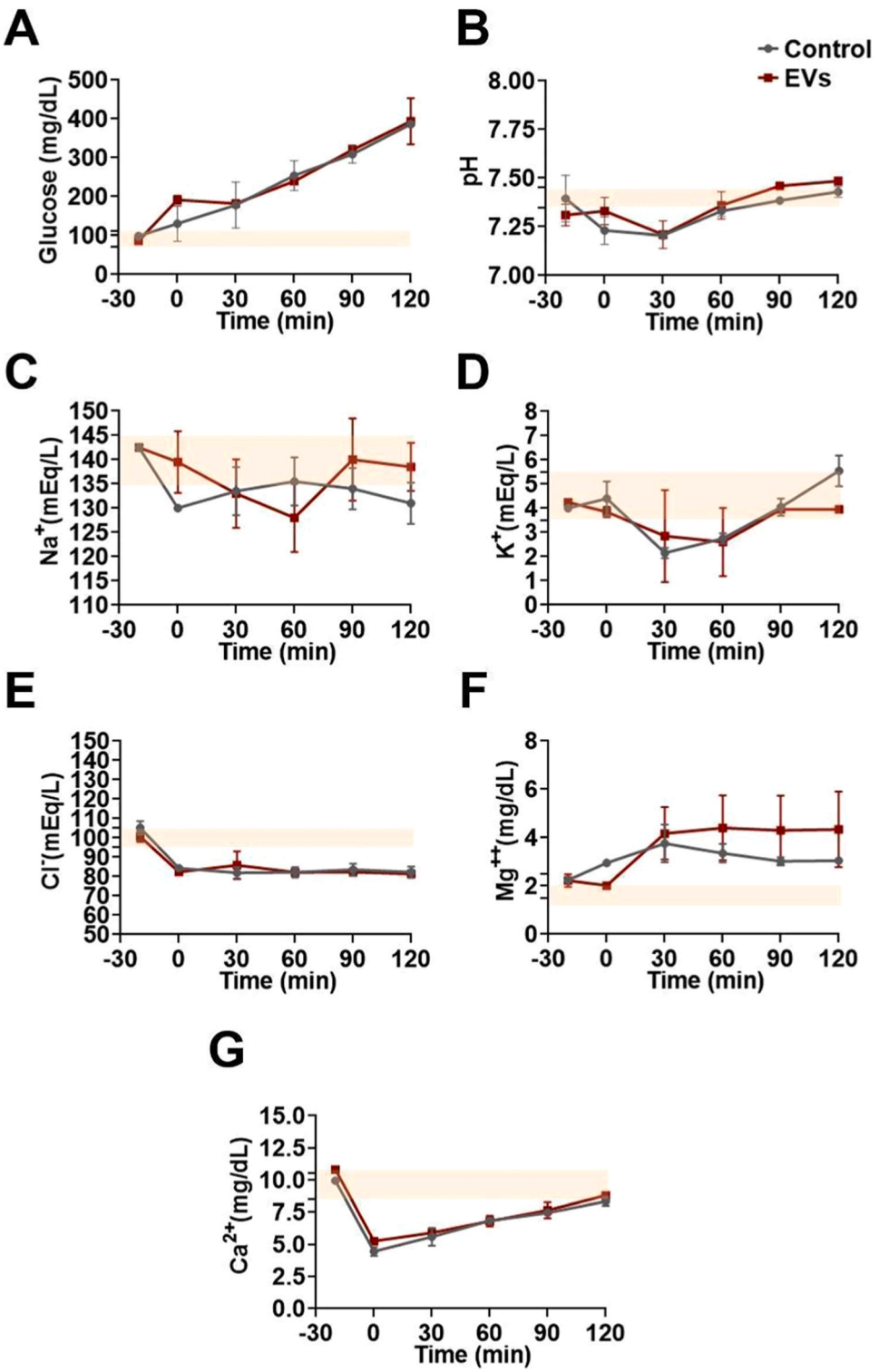

Fig. 3. Biochemical parameters of perfusate. Biochemical analyses of the perfusate were performed every 30 min, throughout the *ex-vivo* perfusion protocol, for the control group and the group treated with EVs. Glucose (A), pH (B), Na+ (C), K+ (D), Cl- (E), Mg++ (F) and Ca++ (G) were analyzed. The usual range of each analyte is indicated in pink. N=3 for each group. Results were expressed as mean ± SD and significance was determined for independent samples using Student's unpaired t-test (n = 3).

the enzyme's activity in the perfusate, compared to the control group, this effect being more noticeable in the first hour of perfusion for CK, TnC and AST (Fig. 4A, B and C, respectively). Likewise, ALT also shows a delay in the increase in perfusion fluid for the EV-treated group, but finally reaches the same levels as the control group (Fig. 4D). Finally, GGT maintains considerably lower and constant levels in the group treated with EVs, compared to the control group (Fig. 4E). These results indicate that treatment with extracellular vesicles would protect cardiac cells from ischemia-reperfusion damage, delaying cellular damage by approximately 30–60 min, compared to the control.

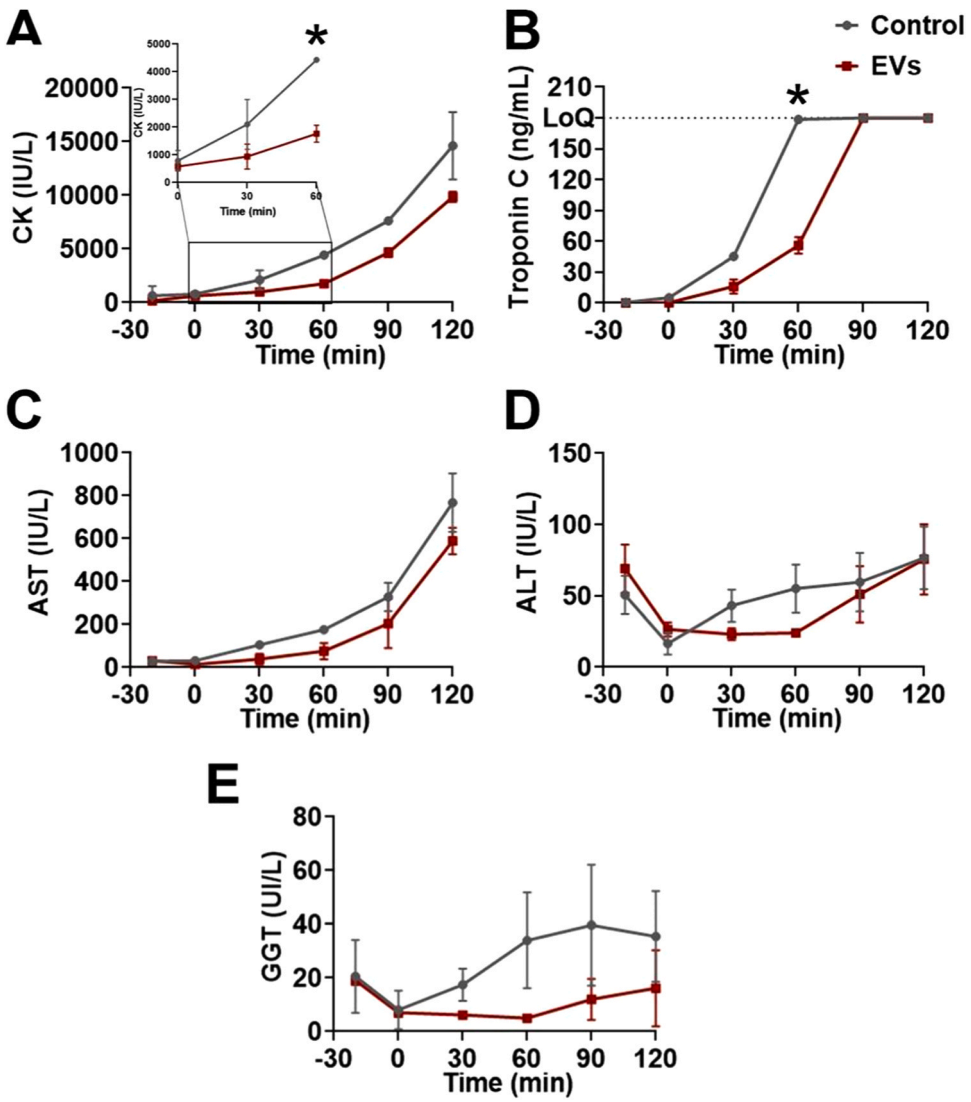

Fig. 4. Analysis of the enzymatic activity in the perfusate. The enzymatic activity of the cardiac-specific enzymes CK and TnC was analyzed in the perfusate (A and B, respectively). Note that for TnC the upper limit of quantification (LoQ) is reached (180 ng/mL). In addition, the activity of AST, ALT and GGT (C, D and E, respectively) was also analyzed. Results were expressed as mean ± SEM and significance was determined for independent samples using Student's unpaired t-test (n = 3) for each group. *P<0.05; **P<0.01.

3.3. MSC-EV administration reduces histological injury in cardiac grafts

Hematoxylin-eosin staining was performed on cardiac tissue sections to evaluate the effect of MSC-EV treatment on the cardiac organ. In this sense, treatment with EVs reduced both edema and hemorrhage areas in the tissue, and the necrotic regions (Fig. 5A). Furthermore, the analysis of caspase-3 by immunohistochemistry revealed that the treatment reduced the number of apoptotic cells, evidenced by a lower number of caspase-3+ cells, than the control group (Fig. 5A). The quantification of the histological score (for hemorrhage, edema, and necrosis) and the number of caspase-3^{+} cells per field at 40X is shown in Fig. 5B.

3.4. MSC-EV reduces mitochondrial damage and oxidative stress

The mitochondrial cristae density in the different cardiac regions was analyzed to evaluate mitochondrial damage. Representative microphotographs of mitochondrial ultrastructure are shown in Fig. 6A. The analysis revealed a loss of the mitochondrial cristae density in the control group. At the same time, the treatment with MSC-EVs effectively maintained the density of the mitochondrial cristae (Fig. 6B). Also, the number of abnormal mitochondria significantly increased in the control group, while treatment with EVs was effective in maintaining the mitochondrial structure (Fig. 6C). These results indicate that treatment with MSC-EVs would help prevent and reduce tissue damage (hemorrhage, edema, and necrosis) and ultrastructural damage to mitochondria.

Moreover, ischemia-reperfusion injury is mainly mediated by an increase in reactive oxygen and nitrogen species and an inadequate or insufficient response of the antioxidant system. This leads to damage to DNA, proteins, lipid peroxidation and mitochondrial dysfunction. In this sense, the enzymatic activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were analyzed. A significantly lower activity of the antioxidant enzymes, SOD, and catalase, was found in the groups treated with MSC-EVs compared to the control group. Also, a significantly lower content of carbonylated proteins was observed in the MSC-EV treated group (Fig. 6D).

3.5. Tissue Cytokines, Chemokines and Tissue Repair Mediators Have a Lower Expression after MSC-EVs Treatment

Pro-inflammatory cytokines (such as IL-1ra, IL-2 and IL-6) promote

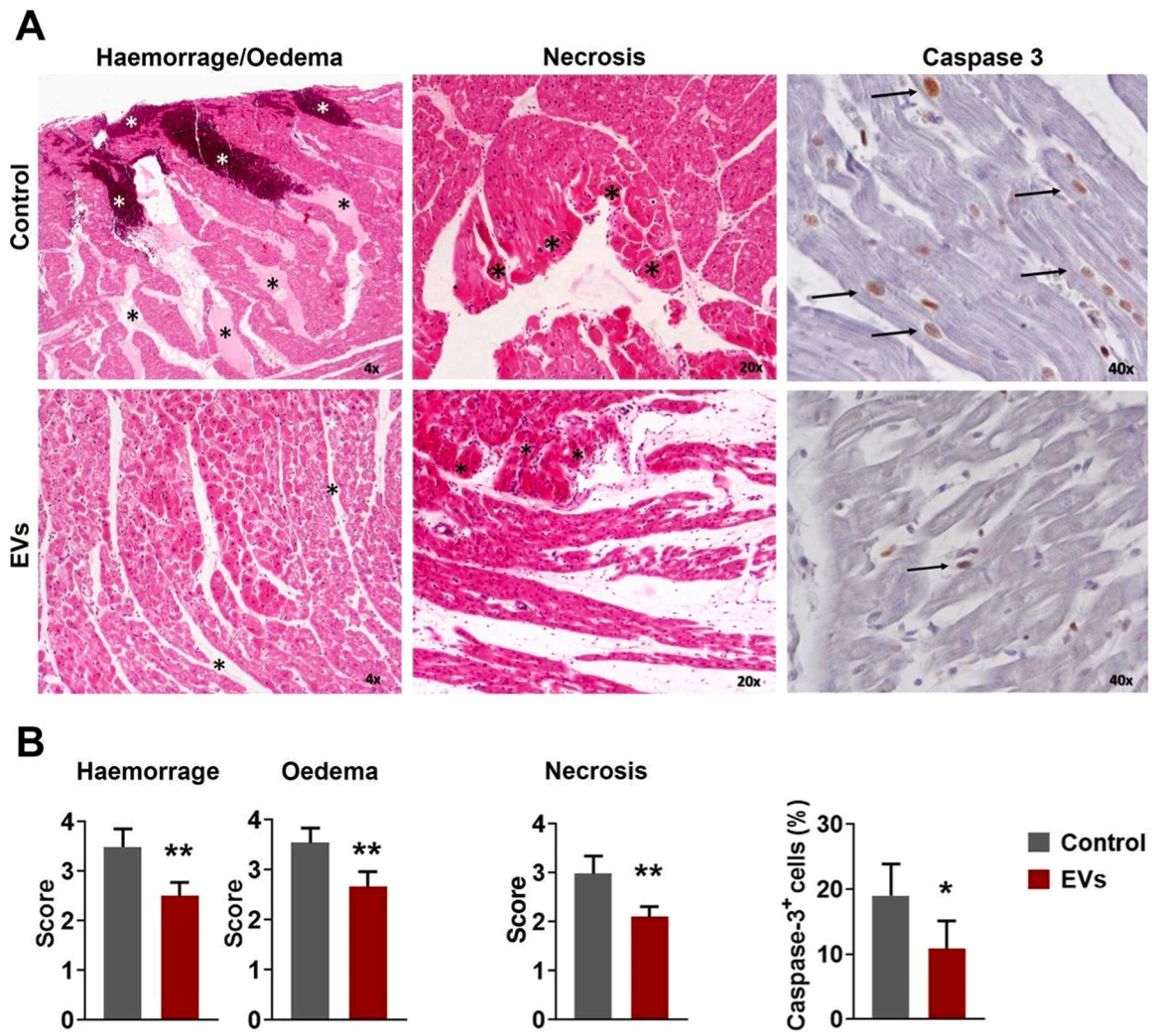

Fig. 5. Histological analysis of the heart**.** Histological section samples of pig hearts were stained with hematoxylin-eosin and analyzed for hemorrhage and edema (*black asterisks and white asterisks, respectively, 4x magnification) and tissue necrosis (black asterisks, 20x magnification). Additionally, immunohistochemical staining for caspase-3 was performed to analyze apoptosis (A). Quantification of the histological score and the total number of caspase-3^{+} cells (10 fields at 40x) (B). Results were expressed as mean ± SD and significance was determined for independent samples using Student's unpaired t-test (n = 3) for each group. *P<0.05; **P<0.01.

the cell proliferation pathway involved in sterile inflammation (during ischemia damage) and immune processes. We found a significant decrease in the concentration of these cytokines in the group treated with EVs with respect to the control group and a non-significative trend for IL-1α, IL-1β and IL-18 (Fig. 7A-B). Both IL-8, a chemokine responsible for the recruitment of neutrophils and macrophages to the injury site and IL-12, a cytokine involved in the macrophage's activation, were influenced by the MSC-EV administration. We found that treatment significantly decreased the levels of these cytokines in the cardiac tissue, with respect to the untreated groups. The anti-inflammatory cytokines, IL-4, and IL-10, typically produced to counteract an ischemic insult, were significantly lower in the treated group with respect to the untreated group (Fig. 7A-B).

4. Discussion

Solid organ transplantation represents the gold standard treatment for patients with terminal organ failure. Heart failure is a perfect example, as heart transplantation remains the treatment of choice for patients with end-stage heart failure and approximately 9 % of patients on the waiting list for heart transplantation ultimately die [18]. Although the World Donation and Transplant Observatory reported 129, 681 solid organs transplanted worldwide in 2020, it is estimated that this covered less than 10 % of the global need [http://www.transplant-observatory.org/]. These data demonstrate a gap between organ transplantation's needs and their availability. Therefore, new strategies for obtaining donor organs must be explored to reduce this gap and increase the number of organs available for transplant. These strategies include DCD donors, representing around 23 % of the organs transplanted in 2020 [http://www.transplant-observatory.org/]. Currently, perfusion machines are being used to improve the function and viability of organs from DCD donors, thus increasing the number of transplants. In this sense, organ perfusion seems a valuable strategy to evaluate pre-transplant graft function and recover organs that would otherwise be discarded [19,20].

An essential advantage of *ex-vivo* organ perfusion is that it offers the potential to explore the effects of various therapeutic strategies on a fully functioning organ. MSC-EVs involve numerous physiological and pathological processes, such as tissue regeneration, immunomodulation, apoptosis, inflammation and viral infections [21–23]. In this scenario, MSC-EVs administration was associated with an elevation in the alveolar fluid clearance rate, in an *ex-vivo* perfusion model of lungs from deceased donors who did not match the criteria for transplantation [24]. Moreover, EVs derived from mesenchymal stem cells have been shown

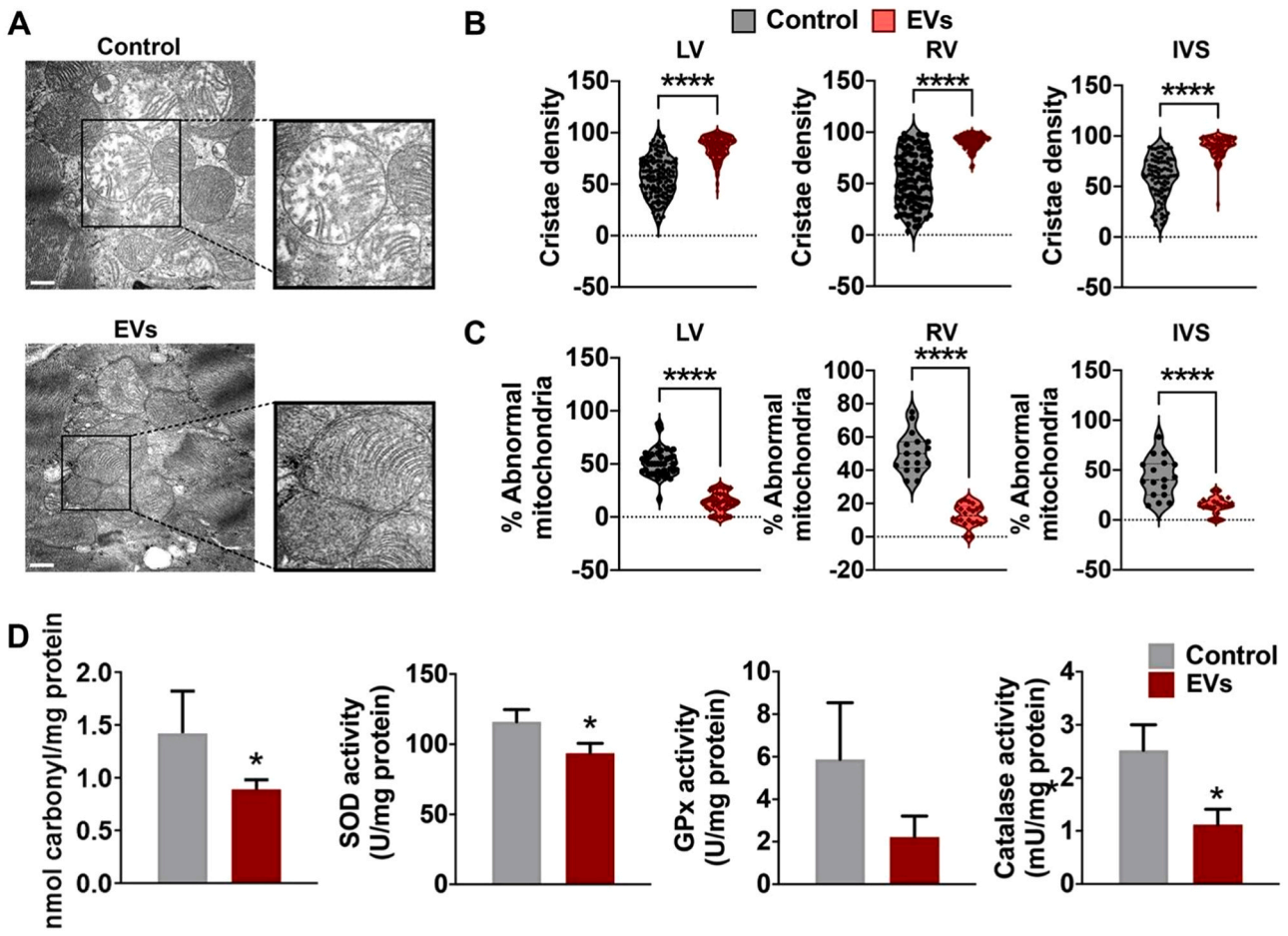

Fig. 6. Analysis of mitochondrial ultrastructure and the oxidative stress. Representative microphotographs of cardiac tissue mitochondria obtained by transmission electron microscopy (A). Analysis of mitochondria was carried out by quantifying the density of mitochondrial cristae (B) and the percentage of abnormal mitochondria (C). 30 photos per group were analyzed. Bar scale = 500 nm. The activity of the antioxidant enzymes SOD, CAT and GPx was evaluated in cardiac tissue homogenates from the regions of the left ventricle, right ventricle, and interventricular septum. In addition, oxidative damage was assessed by quantifying the content of carbonylated proteins (D). Results were expressed as median ± range and significance was determined for independent samples using one-way ANOVA with post-hoc Tukey's test (n = 3). ****P<0.0001 (B and C). Results were expressed as mean ± SD and significance was determined for independent samples using Student's unpaired t-test (n = 3) for each group. *P<0.05 (D).

to have great potential as therapeutic agents for various diseases, including myocardial ischemia, ischemic stroke, and chronic kidney disease [25–27]. In addition, more than 30 clinical trials that use MSC-EVs as therapeutic intervention are registered and are being conducted (https://clinicaltrials.gov/).

Furthermore, EVs have been used to treat myocardial I-R injury in various animal models. In a recent study, adipose tissue derived MSC-EVs (ADMSC-EVs) also showed cardioprotective effects in myocardial I-R injury. Intravenous administration of ADMSC-EVs into rats reduced the levels of creatinine kinase (CK-MB) and troponin C in a myocardial I-R injury model. Similarly, ADMSC-EVs inhibited apoptosis of cardiomyocytes in a hypoxia–reoxygenation model [28]. Moreover, rat bone marrow derived MSC-EVs abrogated ROS production, improved cardiac function, and decreased apoptosis *in-vitro* and in a model of myocardial I-R injury by prompting autophagy via activating adenosine monophosphate–activated protein kinase (AMPK) and AKT pathways [29].

Importantly, although this is a xenogenic model, in which human EVs are used on a pig heart, many studies support the idea that human MSC-EVs can be used across different species, as they have shown immunosuppressive properties, such as the modulation of T-cell responses and the promotion of regulatory T cells in murine models [30, 31]. Moreover, biodistribution studies confirm that human EVs are delivered to different organs, in xenogenic models [32–34].

In this model, we used a dose of 1×10^{11} particles per heart (weight≈120 g). On the other hand, in an experimental model of hypothermic perfusion of discarded human kidneys, an EV dose of approximately $3x10^{10}$ particles per kidney (weight≈130 g) was used [35]. However, there is a lack of studies that perform comprehensive assessments of the *in-vivo* dose-response kinetics and the administration of single *vs* multiple doses, so establishing an EV dose is often an empirical process and may depend on the species. In this regard, the reported effective dose of similar MSC-EVs was 100 μg for rats (~200 g) [36], 200 μg for rabbits (~3 kg) [37], and 1 mg for mini-pigs (~18 kg) [38]. Moreover, even though the manufacturing of GMP-compliant EVs results in a high batch-to-batch consistency, the biological activity may differ among batches for the same dose [39]. In this sense, *in-vitro* functional/potency assays should be performed, to establish the activity of each batch (for example, the IC50) for a given dose of EVs [17].

In our hands, the biochemical analysis of the plasma ions in the perfusate revealed the same behavior and trend in both treated and untreated hearts, as these parameters depend on the composition of the perfusion solution as well as the interventions that can be performed throughout the perfusion system (e.g. administration of drugs, salts, etc.). Regarding the metabolic profile, we found a constant increase in glucose levels in both groups, suggesting that the heart used pyruvate administered in the perfusion solution rather than glucose as metabolic substrate.

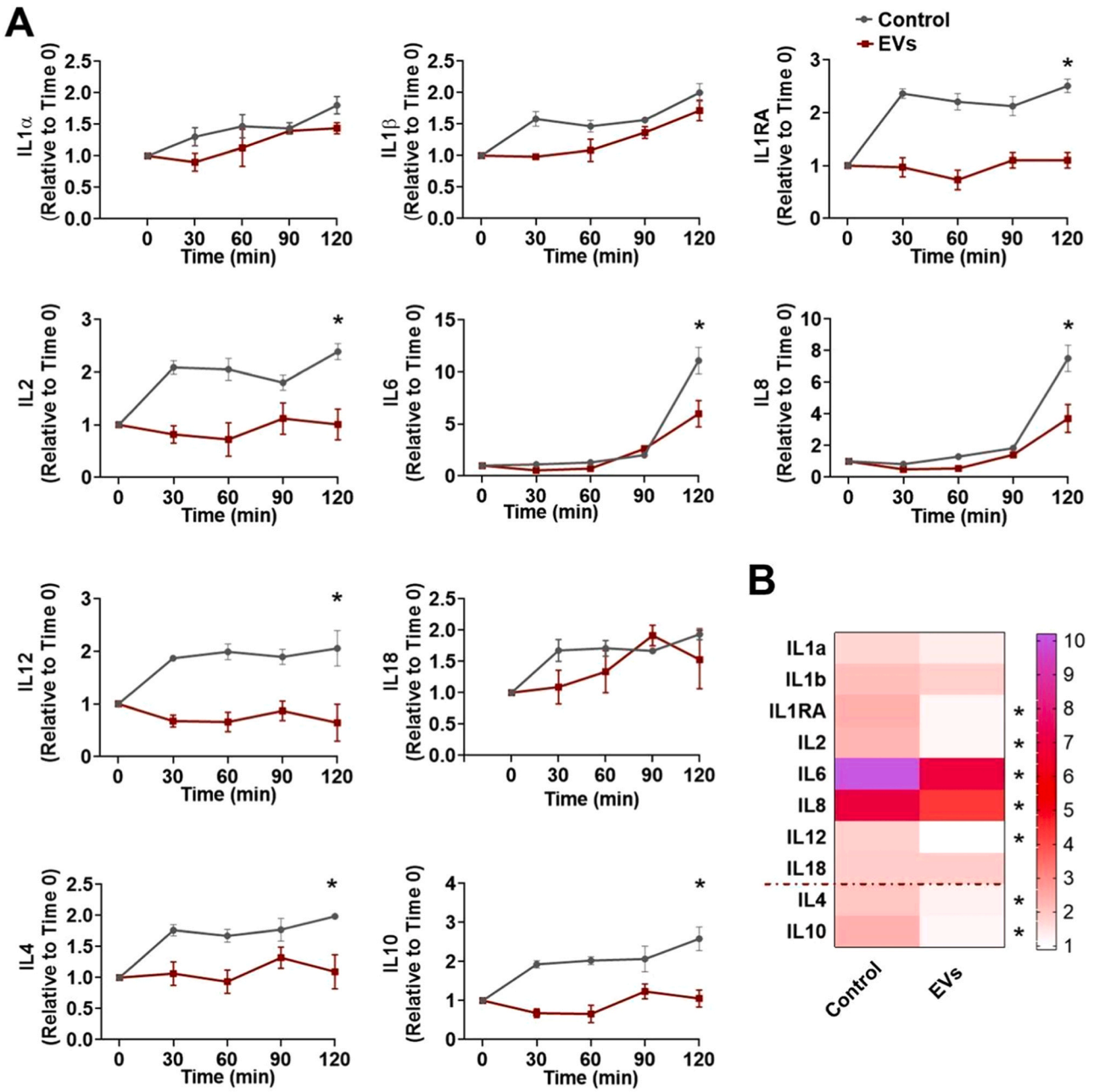

Fig. 7. Analysis of cytokines, chemokines, and inflammatory mediators. Cytokines relative values are expressed relative to time zero and normalized to each group. (B) Heatmap showing the cytokines profile relative expression (to time zero) for both groups. Results were expressed as mean ± SEM and significance was determined for independent samples using Student's unpaired t-test (n = 3). *P < 0.05.

A gradual increase in the activity of the cardiac specific enzymes CK, TnC, AST, ALT, and GGT was also observed, which is indicative of myocardial damage and cell death [40]. However, hearts treated with EVs showed a delay in the increase in troponin, CK, ALT and AST activity of approximately 30 min, compared to the control group, suggesting that EVs exert some type of protection against cellular death in the early phases of perfusion. This finding suggests that MSC-EVs can exert an immediate but transient graft protection (approximately 30 min), pointing to the possible need for repeated MSC-EV administrations to maintain the effect over time. At the end of the perfusion, there were no differences in the levels of ALT and TnC between the groups, but lower levels of activity for CK, AST and GGT were observed in the group treated with EV, compared to the control group.

The histological analysis showed that the treatment with MSC-EVs effectively prevents tissue damage since it reduces oedema, necrosis, and apoptosis, given by a lower number of caspase-3 positive cells. In this respect, Rampino and collaborators reported that discarded human kidneys perfused and treated with EVs showed a lower global score of ischemic kidney damage and renal ultrastructure was preserved, in addition, a lower renal expression of caspase-3 was found [35].

Mitochondria play a critical role in ischemia-reperfusion injury. Given that this organelle plays a fundamental role in calcium overload, ROS production and cell death, preserving the mitochondrial structure and function is crucial to the organ's viability, especially the heart, given its high metabolic demand. In this sense, an essential indicator of mitochondrial integrity is the preservation of the cristae of the inner mitochondrial membrane as it has been shown that the disorganization of mitochondrial cristae leads to mitochondrial stress, poor functioning of the respiratory chain and precedes the release of cytochrome C, with the consequent induction of the intrinsic pathway of apoptosis [41]. In

our model, treatment with MSC-EV proved effective in preserving the integrity of the mitochondrial cristae in the hearts and their ultrastructure, given that a lower number of abnormal mitochondria was observed, than the control group. These data suggest a protective role of EVs on mitochondria, which could explain the lower expression of caspase-3, observed in the treated group.

The prevention of mitochondrial damage by MSC-EVs probably also contributed to the reduction in the rate of ROS production. Indeed, analysis of oxidative stress showed that treatment with MSC-EVs led to a decrease in the expression of the antioxidant enzymes CAT and SOD in cardiac tissue, which is considered a physiological response to a lower exposure to oxidant agents, not only in mammals, but also in other animals [42,43].

Finally, by assessing the inflammatory response as a part of the evaluation of the heart, in our model, we recorded a significantly lower amount of the pro-inflammatory mediators such as IL-1ra, IL-2, IL-6 and IL-8 following the treatment with MSC-EVs. Also, the anti-inflammatory cytokines such as IL-4 and IL-10 showed a lower expression in the group treated with MSC-EVs, further pointing to their protective effect on the cardiac tissue from the ischemic insult.

5. Conclusions

Normothermic re-perfusion supplemented with MSC-EVs may be preferable when dealing with hearts harvested after prolonged warm "unprotected" ischemia. The protective effect of MSC-EVs was confirmed by the delayed increase of the cardiac enzymes CK and TnC in the perfusate, as well as for the prevention of mitochondria swelling, mitochondrial cristae loss and oxidative stress in the donor heart. In conclusion, by creating an adequate perfusion protocol after prolonged warm ischemia in DCD hearts, we showed that MSC-EVs can improve graft quality by protecting the myocardium against ischemic damage.

6. Limitations

In terms of the limitations of this study, it was performed using these three animals/groups, for ethical reasons. Moreover, due to the preliminary nature of the study and the relatively low sample size, the authors opted not to include animals of different sexes to try and obtain reliable data, yet again preliminary, by limiting confounding factors. Nonetheless, it is important to state that enrolled animals were prepubescent, therefore effects imputable to sexual hormones can be ruled out. We realize that this may still represent a limitation to the study, but when working with large animal models, and especially pigs, several husbandry technicalities have to be evaluated. Sourcing of intact males of standard commercial breeds can indeed be challenging since early castration (within the first week after birth) is the standard. In addition, intact males have to be housed singularly and can be extremely aggressive. It is therefore quite common to only include intact males when the given research protocol is either dealing with a sex-influenced pathology or aims at assessing efficacy in a larger population. Based on the results of this preliminary trial, we are planning on moving forward with further studies enrolling a more varied population, therefore also males.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117256.

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