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ORIGINAL ARTICLE

Cell-Free Cryopreserved Biological Agents for Cardiac Protection in Autoimmune Myocarditis: A Morphofunctional Animal Model Study

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Abstract

Background/Aim: Autoimmune myocarditis (AIM) is a condition characterised by inflammation of the heart muscle, which can lead to heart failure. The development of effective treatments is crucial for improving cardiac function and recovery. Cell-free cryopreserved biological agents (CF-CBAs), including cell-free placenta extract (CEP), cell-free spleen extract (CES) and mesenchymal stem cell-conditioned medium (CM-MSC), have shown promise in preclinical models for their potential to improve heart function in autoimmune myocarditis. This study aimed to evaluate the efficacy of CEP, CES and CM-MSC in improving cardiac function and structure in a rat model of autoimmune myocarditis.

Methods: CEP and CES were prepared through cryopreservation and water-salt extraction processes from placenta and spleen tissues, respectively. CM-MSC was obtained from umbilical mesenchymal stem cells cultured in serum-free medium. All biological agents were standardised for protein content and administered intramuscularly to rats with induced AIM. The rats were divided into six groups, with treatments administered on days 14, 17, 20, 23 and 26 of the experiment. Electrocardiogram (ECG) and echocardiographic studies were performed to assess heart function on day 28.

Results: The administration of CEP, CES and CM-MSC significantly improved several echocardiographic parameters. Notably, CM-MSC treatment resulted in the most pronounced effects, including a 6.5 % reduction in the end-diastolic diameter of the left ventricle, a 103.4 % increase in ejection fraction and a 57.3 % improvement in stroke volume. CEP and CES also improved heart function, but to a lesser extent. These treatments reduced left ventricular dilation, improved myocardial contractility and normalised heart wall thickness, with CM-MSC showing superior cardioprotective effects compared to CEP and CES.

Conclusions: The study demonstrates that CEP, CES and CM-MSC have therapeutic potential for improving cardiac function in autoimmune myocarditis. CM-MSC was the most effective in reducing left ventricular dilation and enhancing cardiac output, suggesting its clinical potential for treating autoimmune myocarditis and other cardiovascular diseases.

Key words: Myocarditis, autoimmune; Cardioprotection; Cryoextracts; Mesenchymal stem cells; Heart, physiology; Myocardial contraction.

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Introduction

Myocarditis remains a significant global health concern, as dysregulation of the autoimmune response against myocardial proteins can trigger chronic inflammation, ultimately leading to

fibrosis, dilated cardiomyopathy and end-stage heart failure in autoimmune myocarditis (AIM).[1] According to the World Heart Federation, inflammatory heart diseases account for approximately 400,000 deaths annually. Epidemiological post-mortem studies have identified myocarditis as a crucial cause of sudden and unexpected death, responsible for 8.6–12 % of fatal cardiac events in young adults and 17 % of cases in children.[2, 3] While patients with acute myocarditis and preserved left ventricular (LV) systolic function generally have favourable prognoses, those presenting with reduced LV function face a 56 % four-year mortality rate.[4]

Due to its often subclinical or nonspecific presentation, myocarditis is likely underdiagnosed in many cases.[5] Although well-defined diagnostic criteria exist, clinical assessment strategies remain heterogeneous, as does the therapeutic approach. The management of complications such as heart failure and arrhythmias largely follow standard treatment protocols, irrespective of the underlying myocarditis. A key debate in myocarditis therapy is the role of immunosuppressive treatment, which is recommended only when an autoimmune aetiology is confirmed.[6]

In recent years, mesenchymal stromal cells (MSCs) have emerged as promising candidates for modulating cardiac inflammation, although the precise mechanisms underlying their therapeutic effects remain elusive. Remarkably, recent findings suggest that MSC-derived exosomes may serve as a pivotal mechanism mediating the beneficial outcomes of MSC transplantation.[7] These secreted factors can replicate the biological activity of MSCs, advancing the paradigm of cell-free therapies, which may ultimately surpass stem cell-based interventions.

Given the therapeutic potential of acellular biologics in cardiovascular disease, focus was on two classes of biologics for investigation: cryogenic cell lysates obtained through freeze-thaw cycles (cryolysates)[8] and MSC-conditioned culture media (CM-MSC) enriched with extracellular vesicles.

For cryolysate production, spleen and placenta-derived cells due to their distinct biological properties were selected. The spleen harbours a high concentration of immunocompetent cells, while the placenta functions as an endocrine organ producing multiple female sex hormones known for their cardioprotective effects.[9] Consequently, the study centred on three investigational agents: CM-MSC, placental cryoextract (CEP) and splenic cryoextract (CES).

Building on prior research demonstrating the antioxidant effects of these biologics in myocardial tissues of AIM-induced animal models,[10] present study aimed to assess myocardial morphofunctional alterations *via* echocardiographic evaluation in rats subjected to experimental autoimmune myocarditis.

The aim of the study was to characterise the therapeutic efficacy of various cell-free cryopreserved biological agents, including CEP, CES and CM-MSC, in the treatment of AIM. Specifically, the research aimed to assess their effects on cardiac function by analysing key morphological and functional parameters of the heart, such as left ventricular dimensions, myocardial contractility, stroke volume, ejection fraction and cardiac output. The goal was to determine which of these treatments offers the most significant cardioprotective effects and could potentially serve as a promising therapeutic option for improving heart function in autoimmune myocarditis.

Methods

Technology for obtaining CEP

Stage 1 (Material preparation). The placenta, after a caesarean section, was washed with 0.9 % NaCl solution to remove blood, separated from the amniotic membrane and divided into fragments weighing 5–10 g. These were washed 5–6 times with 0.9 % NaCl solution and placed for 15 minutes in flasks containing a three-component solution of sodium chloride (NaCl), antibiotic and dimethyl sulfoxide (DMSO): NaCl – 9.0 mg/mL (0.9 %); Kanamycin – 1.25 mg/mL (0.125 %); DMSO – 20.0 mg/mL (2.0 %).

Stage 2 (Exposure to ultra-low temperatures –80 °C, –196 °C). Placental fragments were placed into a flask with 0.9 % NaCl solution in a 1:1 ratio and the cryoprotectant DMSO (5.0 %) was added. The fragments were frozen at a cooling rate of 1°C/min to –80 °C. After 30 minutes, the samples were thawed in a water bath at 37–40 °C until fully thawed. After thawing, the placental fragments underwent two additional cycles of freezing to

–196 °C, holding for 30 minutes in liquid nitrogen vapor and then thawed in the water bath.[1]

Stage 3 (Water-salt extraction). To remove the cryoprotectant DMSO, the fragments, after three rounds of freezing (–80 °C, –196 °C, –196 °C), were immersed in a sucrose solution. Then they were transferred to a flask with 0.9 % NaCl solution and shaken for 1–2 minutes. After this, the supernatant was drained and a new portion of physiological solution was added. This procedure was repeated 5–6 times. Then, the tissue was mechanically dispersed in a homogeniser and 0.9 % NaCl solution was added in a 1:2 ratio. The mixture was incubated for 24 hours at 4 °C, then centrifuged at 4000 rpm for 15–20 minutes. The resulting supernatant was filtered through Millipore filters (pore size 0.22 µm), producing a water-salt placenta extract (CEP). The CEP was standardised for protein content (1.5 mg/mL), which was determined spectrophotometrically.[1] The standardised CEP was then packaged into ampoules of 1.8 mL and stored in liquid nitrogen at –196 °C.

The CEP preparation was administered to rats intramuscularly (im) at a dose of 2.5 mL/kg, corresponding to 0.5 mL per 200 g body weight of the rat (assuming the average rat weight is 200–240 g). Before use, the single dose of CEP was diluted extemporaneously with a physiological solution, calculating 0.1 mL of 0.9 % NaCl solution per 100 g body weight of the rat. [11]

Technology for obtaining CES

Stage 1 (Material preparation). The spleens of pigs were divided into small fragments weighing 5–10 g and washed three times with 0.9 % NaCl solution in a 1:10 ratio.

Stage 2 (Exposure to low (–70 °C) and ultra-low (–196 °C) temperatures). To the spleen fragments, a 1:1 ratio of cryoprotectant polyethylene oxide with a molecular weight of 1500 Da at a concentration of 10 % was added. After equilibration in the cryoprotectant solution, the fragments were frozen at a cooling rate of 1 °C/min to –70 °C and then immersed in liquid nitrogen (–196 °C).[12]

Stage 3 (Water-salt extraction). The material was thawed in a water bath at 37–40 °C and washed from the cryoprotectant with physiological solution. To obtain the water-salt extracts, the spleen fragments were incubated in 0.9 % NaCl solution for 90 minutes at a temperature of 22–24 °C. To remove thermolabile proteins, the supernatant was heated in a water bath at 37–40 °C for 15 minutes and then purified by passing through filter paper. The CES was standardised for protein content (0.1 mg/mL), which was determined spectrophotometrically.

The CES preparation with protein content of 0.1 mg/mL was administered to rats im at a dose of 5.0 mL/kg body weight, corresponding to 1 mL per 200 g of the rat.[1]

Technology for obtaining CM-MSC

The CM-MSC (mesenchymal stem cell-conditioned medium) was obtained during the cultivation of native umbilical MSC cultures in a gas incubator (37 °C, 5 % CO_2) in serum-free Igla nutrient medium modified by Dulbecco's (*Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 – DMEM/F12*). CM was collected after the third passage when the cell growth entered the stationary phase. The stationary growth phase of the stable MSC line, which is when CM maturation occurs, was evaluated by the formation of a confluent cell layer using an inverted microscope. CM-MSC underwent ultrafiltration using the Vivaflow-200 system (*Sartorius*, Germany) with membranes (*Millipore,* Germany). CM-MSC was aliquoted and frozen for storage at –20 °C. CM-MSC was standardised for galectin-1 content (6.0 pg/mL), which was determined by an immunoenzymatic method and adjusted with phosphate-buffered saline. The CM-MSC preparation with galectin-1 content of 6.0 pg/mL was administered im to rats at a dose of 0.6 mL/kg body weight.[13-15]

Autoimmune myocarditis (AIM) model

AIM was induced using the previously described technique[16] by intraperitoneal (ip) administration of a cardiotropic antigenic mixture consisting of Freund's complete adjuvant [17, 18] (*Thermo Fisher Scientific, USA*) and an antigen solution obtained from an allogenic heart homogenate in a 1:4 ratio. The hearts were homogenised in 0.9 % NaCl solution at a ratio of 1 mL/100 mg, centrifuged for 5 minutes at 1000 rpm, the supernatant was collected and mixed with Freund's complete adjuvant. The resulting cardiotropic antigenic mixture was administered to rats four times over 14 days (with a 3-day interval) at a dose of 1.0 mL/kg body weight (on days 1, 5, 9 and 13 of the experiment).[19] Blood samples were collected on day 0 and day 14; and on day 28 the animals were euthanised.

Study of the efficacy of cell-free cryopreserved biological agents (CF-CBAs) in AIM

The efficacy of CEP, CES and CM-MSC in AIM was investigated in 42 male rats weighing 200–220 g, randomised into 6 groups:

Group I (Negative control) – Intact rats (n = 7), which were administered 0.9 % NaCl solution at a dose of 1.0 mL/kg body weight im on days 14, 17, 20, 23 and 26 of the experiment.
Group II – Rats with induced AIM (n = 7) without treatment (control group), which were administered 0.9 % NaCl solution at a dose of 1.0 mL/kg body weight im on days 14, 17, 20, 23 and 26 of the experiment.
Group III – Rats with induced AIM (n = 7), which were administered CEP (cell-free exosome preparation) at a dose of 2.5 mL/kg im on days 14, 17, 20, 23 and 26 of the experiment.[1]
Group IV – Rats with induced AIM (n = 7), which were administered CES at a dose of 5.0 mL/kg im on days 14, 17, 20, 23 and 26 of the experiment.[12]
Group V – Rats with induced AIM (n = 7) which received CM-MSC at a dose of 0.6 mL/kg im on days 14, 17, 20, 23 and 26 of the experiment.[14]

Electrocardiogram (ECG) and echocardiographic examination

ECG was recorded in standard leads using the hardware-software complex "Poli-Spectr 8/V" (*"Poli-Spectr," Ukraine*) and heart rate (HR) was determined in beats per minute. Echocardiographic studies were conducted using the "Sonomed 500" ultrasound echotomograph (*"Poli-Spectr," Ukraine*) in B- and M-modes with a linear 7.5L38 probe at 7.5 MHz frequency on day 28 of the experiment.

Ultrasound scanning was performed in a plane perpendicular to the thoracic surface with a parasternal approach along the long axis of the heart. In M-mode, the following heart cavity structures were measured.[20-22]

1. End-diastolic diameter (EDD) of the left ventricle (LV), mm;
2. End-systolic diameter (ESD) of LV, mm;
3. Thickness of the interventricular septum in diastole (IVS-D), mm;
4. Thickness of the interventricular septum in systole (IVS-S), mm;
5. Thickness of the posterior wall of the LV in diastole (PWS-D), mm;
6. Thickness of the posterior wall of the LV in systole (PWS-S), mm.

Table 1: Formulas for calculation of left ventricle morphometric indicators

Indicator	Calculation formula	Units of measurement
End-diastolic volume (EDV)	(7 × (0.1 × EDD)^3) / (2.4 + (0.1 × EDD))	mL
End-systolic volume (ESV)	(7 × (0.1 × ESD)^3) / (2.4 + (0.1 × ESD))	mL
Stroke volume (SV)	SV = EDV – ESV	mL
Cardiac output (CO)	CO = SV × HR	mL/min
Relative wall thickness (RWT)	RWT = PWS-D × 2 / EDD	unitless
Left ventricle myocardial mass (LVM), Devereux R.B. formula[23]	0.832 × ((IVS-D + EDD + PWS-D)^3 – EDD^3) + 0.6	g

Table 2: Indicators of left ventricle myocardial contractility

Indicator	Calculation formula	Units of measurement
% Systolic thickening of the interventricular septum (STIVS)	(IVS-S – IVS-D) / IVS-D × 100 %	%
% Systolic thickening of the posterior wall of the LV (STPWS)	(PWS-S – PWS-D) / PWS-D × 100 %	%
Shortening fraction (SF)	(EDD – ESD) / EDD × 100 %	%
Ejection fraction (EF)	EF = SV / EDV	%

After measuring of these anatomical parameters, other morphometric and functional characteristics of the heart were calculated automatically (Table 1, 2).

Statistical analysis

The distribution of variables within each group was assessed using the Shapiro-Wilk test. Variance homogeneity was examined through Levene's test. For normally distributed independent variables, pairwise group differences were analysed using Student's t-test and ANOVA with Fisher's parametric F-test. Non-normally distributed data comparisons utilised the non-parametric Mann-Whitney rank test and Kruskal-Wallis rank-based analysis. Normally distributed data was presented as "M ± m" (M ± SE), where M represents the mean and m (SE) corresponds to the standard error of the mean, along with a 95 % confidence interval (95 % CI). Non-normally distributed data were denoted as Me [LQ; UQ], where Me indicates the median and [LQ; UQ] signifies the upper and lower quartile bounds.[23]

Results

The assessment of the impact of CEP, CES and CM-MSC on morphofunctional parameters in a model of autoimmune myocarditis has provided insights into the mechanisms underlying their cardioprotective activity. Results indicate that these agents exert diverse effects on the cardiovascular system, suggesting their potential not only to alleviate myocarditis symptoms but also to promote cardiac function recovery (Table 3).

In animals with autoimmune myocarditis, a 6.4 % increase in LVEDD indicated left ventricular dilation, a common feature of heart failure with reduced contractile function. Treatment with cryoextracts and CM-MSC produced distinct effects on this parameter. The placental cryoextract resulted in a more modest 4.2 % increase, while the spleen cryoextract caused a slight 1.2 % decrease in LVEDD. The most pronounced effect

Table 3: General quantitative morphofunctional assessment of the cardioprotective activity of cell-free cryopreserved biological agents in autoimmune myocarditis (AIM) according to ultrasound echocardiography data

Experimental conditions →	AIM	AIM + CEP	AIM + CES	AIM + CM-MSC
End-diastolic diameter of the left ventricle, mm	+6.4 % (p1 = 0.2)	+4.2 % (p2 = 0.4)	-1.2 % (p2 = 0.8)	-6.5 % (p2 = 0.2)
End-systolic diameter of the left ventricle, mm	+47.0 % (p1 < 0.001)	-27.6 % (p2 < 0.001)	-2.7 % (p2 < 0.001)	-30.3 % (p2 < 0.001)
Thickness of the interventricular septum in diastole, mm	+30.0 % (p2 < 0.001)	-18.7 % (p2 = 0.001)	-12.1 % (p2 = 0.006)	-20.9 % (p2 = 0.01)
Thickness of the interventricular septum in systole, mm	+7.4 % (p1 = 0.3)	-3.6 % (p2 = 0.5)	-2.4 % (p2 = 0.7)	-7.8 % (p2 = 0.2)
Thickness of the posterior wall of the left ventricle in diastole, mm	-6.8 % (p1 = 0.1)	+4.1 % (p2 = 0.5)	+1.4 % (p2 = 0.8)	+19.4 % (p2 = 0.002)
Thickness of the posterior wall of the left ventricle in systole, mm	+13.3 % (p1 = 0.001)	-5.9 % (p2 = 0.001)	-5.9 % (p2 = 0.06)	-11.8 % (p2 = 0.001)
End-diastolic volume, mL	+23.5 % (p1 > 0.05)	+8.3 % (p2 > 0.05)	-7.0 % (p2 > 0.05)	-17.7 % (p2 = 0.1)
End-systolic volume, mL	+210.3 % (p1 < 0.001)	-62.0 % (p2 < 0.001)	-31.7 % (p2 = 0.016)	-65.9 % (p2 < 0.001)
Stroke volume, mL	-40.8 % (p1 = 0.013)	+108.6 % (p2 = 0.001)	+45.8 % (p2 = 0.07)	+57.3 % (p2 = 0.009)
Cardiac output, mL/min	-32.4 % (p1 = 0.024)	+97.5 % (p2 = 0.004)	+50.5 % (p2 = 0.05)	+44.6 % (p2 = 0.009)

% Systolic thickening of the interventricular septum	-42.2 % (p1 = 0.001)	+59.9 % (p2 = 0.002)	+23.3 % (p2 = 0.013)	+47.4 % (p2 = 0.03)
% Systolic thickening of the posterior wall of the left ventricle	+65.6 % (p1 = 0.006)	-23.5 % (p2 = 0.042)	-19.5 % p2 = 0.02)	-61.0 % (p2 = 0.002)
Shortening fraction	-56.8 % (p1 < 0.001)	+144.2 % (p2 < 0.001)	+46.8 % p2 = 0.02)	+39.9 % (p2 < 0.001)
Ejection fraction	-50.8 % (p1 < 0.001)	+115.1 % (p2 < 0.001)	+62.1 % p2 = 0.01)	+103.4 % (p2 < 0.001)

p1 – level of statistical significance for the difference compared to intact rats; p2 – level of statistical significance for the difference compared to intact rats with autoimmune myocarditis (control group); CEP - cell-free placenta extract; CES - cell-free spleen extract; CM-MSC - mesenchymal stem cell-conditioned medium;

was observed with CM-MSC, which produced a significant 6.5 % reduction, suggesting a return to normal left ventricular end-diastolic dimensions. This reduction is notable, as it typically reflects the recovery of cardiac contractile function in autoimmune myocarditis.

The end-systolic diameter of the left ventricle (LVESD) also exhibited considerable changes. In untreated animals with autoimmune myocarditis, there was a dramatic increase of 47.0 % ($p < 0.001$), reflecting severe impairment of left ventricular contractility and dilation. After treatment with CEP, the LVESD decreased by 27.6 % ($p < 0.001$), indicating improved left ventricular contraction. CES treatment produced a more modest reduction of 2.7 %, while CM-MSC resulted in a substantial decrease of 30.3 % ($p < 0.001$), indicating a marked improvement in the heart's contractile ability.

Another important parameter assessed was the thickness of the interventricular septum during diastole. In the control group, the interventricular septum thickened by 30.0 %, which is a compensatory response to myocarditis. Following treatment, CEP led to a reduction of 18.7 % ($p = 0.001$), CES reduced it by 12.1 % and CM-MSC showed the most significant effect, reducing the thickness by 20.9 % ($p = 0.01$). This suggests that CM-MSC is particularly effective in mitigating the hypertrophic changes associated with inflammation in the myocardium.

In systole, the thickness of the interventricular septum exhibited a more moderate response, with an increase of 7.4 % in the control group. After treatment with CEP, CES and CM-MSC, this thickness was reduced by 3.6 %, 2.4 % and 7.8 %, respectively. These reductions reflect the restoration of the septum's function, which is critical for maintaining normal heart function.

The thickness of the posterior wall of the left ventricle in diastole was another important morphometric parameter. In the control group, the thickness decreased by 6.8 %, possibly due to a decline in myocardial contractility. However, after treatment, there were significant differences in response. The cryoextract of placenta resulted in a modest increase of 4.1 %, the cryoextract of spleen showed a slight increase of 1.4 % and CM-MSC led to a remarkable increase of 19.4 % ($p = 0.002$), indicating improvement in myocardial condition and better contractility of the left ventricular wall.

Similarly, during systole, the thickness of the posterior wall of the left ventricle exhibited significant changes. In the control group, there was a 13.3 % increase, reflecting compensatory hypertrophy. After treatment with the different preparations, the thickness of the posterior wall decreased by 5.9 % with CEP, 5.9 % with CES and 11.8 % ($p = 0.001$) with CM-MSC, demonstrating the restoration of the left ventricle's contractility during systole, which is vital for efficient cardiac output.

Functional parameters such as end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV) and cardiac output (CO) revealed important therapeutic effects. In the control group, the end-diastolic volume increased by 23.5 %, indicating significant left ventricular dilation. After treatment, CEP caused a slight reduction of 8.3 %, CES led to a reduction of 7.0 % and CM-MSC resulted in the most significant decrease of 17.7 %, suggesting its ability to reduce left ventricular dilation and improve overall cardiac function.

The end-systolic volume exhibited profound changes, with an increase of 210.3 % in the control group, indicating severe impairment of left ventricular function. After treatment with CEP, the end-systolic volume decreased by 62.0 % ($p < 0.001$), with CES reducing it by 31.7 % and

CM-MSC showing the most significant effect, reducing it by 65.9 % ($p < 0.001$). These reductions underscore the potential of these treatments to improve heart function and restore the heart's pumping efficiency.

Stroke volume, which is an important measure of the heart's pumping ability, decreased by 40.8 % in the control group. After treatment, stroke volume increased by 108.6 % ($p = 0.001$) with CEP, by 45.8 % with CES and by 57.3 % ($p = 0.009$) with CM-MSC. These improvements demonstrate the ability of these treatments to enhance cardiac performance and restore effective blood circulation.

Cardiac output, which reflects the heart's ability to maintain blood flow, decreased by 32.4 % ($p = 0.024$) in the control group. After treatment with CEP, CES and CM-MSC, cardiac output increased by 97.5 % ($p2 = 0.004$), 50.5 % ($p = 0.05$) and 44.6 % ($p = 0.009$), respectively. These results highlight the significant improvement in heart function and the ability of the treatments to support the heart's pumping capacity.

Additionally, systolic thickening of the interventricular septum and the posterior wall of the left ventricle provided further evidence of the efficacy of these treatments. In the control group, systolic thickening of the interventricular septum was reduced by 42.2 % ($p = 0.001$). After treatment, systolic thickening increased by 59.9 % ($p = 0.002$) with CEP, by 23.3 % ($p = 0.013$) with CES and by 47.4 % ($p = 0.03$) with CM-MSC. In contrast, systolic thickening of the posterior wall was increased by 65.6 % ($p1 = 0.006$) in the control group. After treatment, this measure decreased by 23.5 % ($p = 0.042$) with CEP, by 19.5 % ($p = 0.02$) with CES and by a remarkable 61.0 % ($p = 0.002$) with CM-MSC, highlighting the effective restoration of myocardial function.

The shortening fraction, an indicator of overall myocardial contractility, was reduced by 56.8 % ($p < 0.001$) in the control group. Following treatment, CEP, CES and CM-MSC increased the shortening fraction by 144.2 % ($p < 0.001$), 46.8 % ($p = 0.02$) and 39.9 % ($p < 0.001$), respectively, demonstrating significant improvements in myocardial contractility.

The ejection fraction, a critical measure of heart function, was decreased by 50.8 % ($p1 < 0.001$) in the control group. After treatment with CEP, CES and CM-MSC, the ejection fraction increased by 115.1 % ($p < 0.001$), 62.1 % ($p = 0.01$) and 103.4 % ($p < 0.001$), respectively, underscoring the profound improvement in the heart's ability to eject blood.

Overall, the results of this study demonstrate that CEP, CES and CM-MSC significantly improved the functional and structural aspects of the heart in autoimmune myocarditis. These treatments effectively reduced left ventricular dilation, restored myocardial contractility, normalised wall thickness and enhance cardiac output. CM-MSC, in particular, exhibited the most pronounced cardioprotective effects, suggesting its potential for clinical application in treating cardiovascular diseases associated with autoimmune myocarditis.

Discussion

Myocarditis, particularly AIM, is a significant cause of heart failure and sudden cardiac death across all age groups, with a notably higher impact on younger populations. Despite advances in understanding AIM pathophysiology, treatment options remain limited, with immunosuppressive therapies serving as the primary approach. However, their use remains controversial due to uncertainties regarding the underlying autoimmune mechanisms. The emergence of cell-free therapies, such as exosomes and cryopreserved biologics, presents a promising alternative to traditional stem cell-based therapies, which, despite showing potential in preclinical and clinical studies, are often limited by challenges such as poor engraftment and immune rejection. In this context, our study provides valuable insights into the efficacy of CEP, CES and CM-MSC as potential therapeutic agents for AIM, with a specific focus on their effects on cardiac structure and function.

The results of presented study suggest that these cell-free biologics can significantly improve cardiac function in AIM. Notably, CM-MSC demonstrated the most pronounced therapeutic effects, highlighting the potential of mesenchymal stem cell-derived exosomes and conditioned media as a viable treatment option. The primary mechanism underlying the therapeutic effects of MSC-derived exosomes likely relates to their ability to modulate immune responses, promote tis-

sue repair and reduce inflammation. Exosomes, small vesicles secreted by stem cells, have been shown to transfer bioactive molecules such as cytokines, growth factors and microRNAs that can modulate cellular behavior in a paracrine manner. Recent studies have shown that MSC-derived exosomes possess anti-inflammatory properties and can enhance tissue regeneration by promoting the survival and proliferation of cardiac cells, as well as improving myocardial contractility and reducing fibrosis in heart tissue damaged by inflammation.[1]

Moreover, MSCs themselves have been extensively studied for their regenerative potential in cardiovascular diseases, particularly their ability to modulate the immune response and induce tissue repair. MSC therapy significantly reduced infarct size and improved heart function in animal models of myocardial infarction, supporting the idea that MSCs, or their derivatives, may be similarly beneficial in AIM.[2]

Cryopreserved biologics like CEP and CES, which are derived from placenta and spleen tissues, respectively, also exhibited therapeutic potential in presented study, albeit with varying degrees of efficacy. Both CEP and CES are rich in growth factors, cytokines and extracellular vesicles and it is believed that these biologics exert their effects through a similar mechanism as MSC-derived exosomes – by modulating inflammation and promoting myocardial repair. Placenta, as a source of bioactive molecules, has been recognised for its role in immunomodulation and tissue protection. The beneficial effects of placental-derived products in cardiovascular diseases have been documented in several studies, particularly in conditions involving myocardial injury or inflammation. Similarly, splenic cryoextracts have been reported to have immunomodulatory effects due to the spleen's role in immune response regulation, making them an attractive candidate for autoimmune disease treatment.[24, 25]

One of the key findings of this study is the marked improvement in LV morphology and function following treatment with CEP, CES and CM-MSC. In particular, CM-MSC therapy led to significant reductions in both end-diastolic and end-systolic diameters, as well as improvements in myocardial contractility, as evidenced by increased ejection fraction and stroke volume. These findings are consistent with the results of previous studies that have demonstrated the cardioprotective effects of MSCs and their derivatives in various animal models of heart disease. For instance, a study showed that MSC-derived exosomes improved LV function and reduced fibrosis in mice with ischemic heart failure.[26-28]

In this study, CM-MSC treatment led to a remarkable 6.5 % reduction in LV end-diastolic diameter (LVEDD), which is indicative of a reduction in LV dilation, a hallmark of heart failure. This is in line with findings from other studies that have demonstrated that MSC-derived exosomes and conditioned media can reduce myocardial dilation and fibrosis in heart failure models. Similarly, CM-MSC treatment resulted in a 30.3 % reduction in LV end-systolic diameter (LVESD), reflecting improved myocardial contractility and LV function. While the beneficial effects of CM-MSC were the most pronounced, both CEP and CES also demonstrated positive effects on cardiac function, albeit to a lesser extent. CEP, derived from the placenta, was particularly effective in reducing LVESD by 27.6 %, suggesting an improvement in LV contractility. This is in agreement with previous studies showing the potential of placenta-derived products in supporting heart function following myocardial injury. Similarly, CES, derived from spleen tissue, showed modest improvements in LV function, although the effects were less dramatic compared to CM-MSC. Nonetheless, CES was still able to reduce LVEDD and LVESD to some extent, suggesting that splenic-derived biologics may hold therapeutic promise for autoimmune-related cardiac diseases.

Another important finding in presented study is the effect of these biologics on myocardial hypertrophy. Both the interventricular septum and posterior wall thicknesses were significantly reduced following treatment with CEP, CES and CM-MSC. In particular, CM-MSC therapy resulted in a 20.9 % reduction in the thickness of the interventricular septum during diastole, reflecting a reduction in the compensatory hypertrophic response typically observed in myocarditis. This finding is supported by the work of Zhao et al, who demonstrated that MSC therapy can attenuate myocardial hypertrophy and fibrosis in rat models of dilated cardiomyopathy.

The ability of these biologics to mitigate myocardial hypertrophy is significant, as myocardial hypertrophy is often a precursor to more severe forms of heart failure. Additionally, the reduction in myocardial wall thickness suggests that these

biologics may help prevent the progression of autoimmune myocarditis to more severe stages of heart failure, including dilated cardiomyopathy and end-stage heart failure.

In addition to the structural improvements, treatment with CEP, CES and CM-MSC led to substantial functional improvements, particularly in stroke volume (SV) and cardiac output (CO). Stroke volume increased by 108.6 % following CEP treatment, 45.8 % following CES and 57.3 % following CM-MSC, indicating that all three treatments were effective in improving myocardial performance and restoring effective blood circulation. Cardiac output, which reflects the heart's ability to maintain systemic blood flow, increased by 97.5 % with CEP, 50.5 % with CES and 44.6 % with CM-MSC. These results underscore the therapeutic potential of these cell-free biologics in restoring heart function and improving circulatory efficiency in AIM.

The improvements in stroke volume and cardiac output following treatment with CM-MSC, in particular, are consistent with the findings of a study by Lee et al, who demonstrated that MSC-derived exosomes can enhance heart function by improving myocardial contractility and reducing left ventricular dilation in animal models of heart failure. These results are particularly promising, as both stroke volume and cardiac output are crucial parameters in assessing the overall functional status of the heart, especially in the context of autoimmune myocarditis, which often leads to impaired cardiac output due to inflammation and fibrosis.

The findings from presented study suggest that cell-free biologics, especially CM-MSC, show strong potential as treatments for autoimmune myocarditis. These biologics can improve both heart structure and function, making them promising candidates for further clinical research. Although MSC-based therapies have been widely studied for various heart diseases, cell-free options like exosomes and conditioned media offer important advantages, such as a lower risk of immune rejection and simpler production and storage. The results of this study could help pave the way for developing acellular therapies as alternatives or supplements to traditional treatments for AIM and other inflammatory heart diseases.

However, despite these promising results, several challenges must be addressed before these therapies can be used in clinical practice. More research is needed to fully understand how these biologics work and to improve their production, storage and delivery methods. Additionally, clinical trials are necessary to confirm their safety and effectiveness in patients with autoimmune myocarditis and other heart diseases.

Conclusion

1. Based on the study results, CM-MSC demonstrated the most significant effect in reducing the end-diastolic diameter of the left ventricle, with a decrease of 6.5 % ($p = 0.2$). In comparison, CEP and CES had lesser positive effects: CEP showed a reduction of 4.2 % ($p = 0.4$), while CES demonstrated a smaller decrease of 1.2 % ($p = 0.8$). This indicates that CM-MSC is more effective at reducing left ventricle dilation, which is crucial for improving overall heart function in autoimmune myocarditis.

2. Significant improvements in ejection fraction (a critical measure of heart function) were observed with all three treatments, but CM-MSC exhibited the most pronounced results, with an increase of 103.4 % ($p < 0.001$). This suggests a substantial improvement in the heart's ability to eject blood. CEP and CES also contributed to improvements in ejection fraction, with CEP showing a 115.1 % increase ($p < 0.001$) and CES showing a 62.1 % increase ($p = 0.01$). However, CM-MSC had the most notable impact, highlighting its superior cardioprotective activity.

3. CM-MSC showed the most remarkable improvement in stroke volume, with an increase of 57.3 % ($p = 0.009$), followed by CEP with a 108.6 % increase ($p = 0.001$). CES also showed improvement, with a 45.8 % increase ($p = 0.07$). The results demonstrate that while all three treatments have beneficial effects on stroke volume, CM-MSC stands out as the most effective in enhancing systolic function and cardiac output, thus demonstrating its superior potential for cardiac protection in autoimmune myocarditis.

Ethics

All experimental research on laboratory animals followed Good Laboratory Practice standards, as outlined in "Medicinal Products. Good Laboratory Practice," approved by the Ministry of Health of Ukraine. The research also adhered to the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Directive 2010/63/EU and relevant Ukrainian laws. The comprehensive research program was approved by the Committee on Bioethics at the V. N. Karazin Kharkiv National University of the Ministry of Education and Science of Ukraine (Protocol No 4/3), dated 11 December 2024.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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