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Three-dimensional culture of heart tissue constructs from heart progenitor cells on a polycaprolactone scaffold - optimization

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Myocardial damage resulting from ischemia leads to the irreversible loss of cardiac function. Consequently, there is a critical need to develop effective therapies capable of regenerating damaged heart muscle. Tissue engineering (TE) enables the creation of functional tissues *in vitro* using cells, biologically active molecules, and scaffolding methods. To advance research on heart tissue culture on polycaprolactone (PCL) scaffolds, it is important to optimize culture methods to ensure adequate nutrient supply. In this study, different sera and various concentrations of heart tissue extracts were evaluated for their ability to support cardiac pseudo-tissue formation on PCL scaffolds using progenitor cells at different stages of differentiation. The results confirm the biocompatibility of PCL with cardiac progenitor cells. Cardiac progenitors, collected on the 8th embryonic day (8 ED), demonstrated the greatest potential for forming heart pseudo-tissue constructs when cultured with horse serum (HS) and an extract from homogenized hearts of 14-day-old chicken embryos as an additive to the culture medium. Cardiac progenitor cells collected on 12 ED exhibited the highest potential for creating pseudo-heart tissue constructs in the presence of HS, even without the addition of homogenized heart extract. Moreover, a high concentration of the extract reduced their ability to form structured tissue constructs. Overall, HS promoted more intensive cell proliferation in both 2D, and 3D cultures compared to foetal bovine serum (FBS). The absence of a vascular-like system limits the development of large tissue constructs, leading to nutritional deficiency and subsequent tissue degradation.

KEY WORDS: tissue engineering / multipotent cells / chicken embryo

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Any deformation or damage to the heart can have serious consequences, because the cells of this muscle have a minimal regeneration capacity. Damage can result from inadequate blood supply, such as in myocarditis, which is most often caused by a viral infection or from bacteria, certain medications, toxins, or autoimmune diseases [Giacomelli *et al*. 2017, Saxton *et al*. 2022]. Damage to the heart muscle causes the loss of cardiomyocytes, which are then replaced by fibrous scar tissue, ultimately leading to heart failure. Currently, the only treatment option for heart failure is a whole-organ transplant, but many people in need cannot access this option due to a lack of donors or very high costs. Alternative therapies include coronary artery bypass [McCarthy 2008] and intracoronary stent or balloon angioplasty [Kobo *et al.* 2020, Ogawa *et al*. 2023]. Patients who fail revascularization can be managed with mechanical circulatory support devices or pumps [McCarthy 2008; Vargo et al. 2020]. However, these approaches do not regenerate damaged myocardium but only support heart function, typically for a limited time. Therefore, it is extremely important to develop effective therapies that can help regenerate damaged heart muscle and prevent heart failure [Sharma *et al*. 2019].

One strategy is to use cells with high proliferative and cardiogenic potential. These could be induced pluripotent stem cells (iPSCs) genetically matched to the recipient [Sharma *et al.* 2026]. Before reaching that stage, it is essential to define optimal culture conditions using scaffolds that mimic the cardiac extracellular matrix. Key variables include the differentiation state of cells seeded onto the scaffold and additives that stimulate proliferation and differentiation toward cardiomyocytes. Progenitor cells from model organisms provide a practical system for preliminary studies that inform these parameters.

Cardiac progenitor cells (CPCs) have high potential for restoring normal heart function in patients suffering from cardiac diseases and heart failure [Augustine *et al.* 2021, Arenal *et al*. 2022]. CPCs differ from embryonic stem cells because they have a predetermined differentiation pathway, and therefore their ability to self-renew and differentiate into other cell types is limited [Birket and Mummery 2015]. They proliferate readily and generate cells of the three main cardiac lineages: cardiomyocytes, smooth muscle cells, and endothelial cells. These cells are also responsible for maintaining heart homeostasis under physiological and pathological conditions [Xu *et al*. 2017]. According to Barreto *et al.* [2019], advances in biomaterials that mimic the native cardiac microenvironment show promise in enhancing the regenerative functions of CPCs while also integrating with host tissue.

Modern tissue engineering (TE) enables the creation of functional tissues *in vitro* using cells, biologically active molecules, and scaffolding methods. Recent technological advances in TE, including the fabrication of biocompatible 3D scaffolds and the use of pluripotent and multipotent stem cells for tissue or organ regeneration, offer new prospects. Scaffolds based on polymers such as polycaprolactone (PCL) are widely used in various TE applications due to their biocompatibility and ability to support cell proliferation [Allafchian *et al.* 2018, Hasan *et al*. 2018].

PCL is a nonpolar aliphatic polyester derived from petrochemical feedstocks that has attracted great interest in scaffold production because of its cell compatibility, bioresorbability, and the ease of processing. In addition, it has been approved by the American Food and Drug Administration (FDA) for use in several medical products, such as drug delivery devices and sutures [Dikici *et al.* 2020]. Moreover, studies have shown that the arrangement of PCL nanofibers plays a crucial role in the speed and efficiency of cardiomyocyte differentiation. Cardiomyocyte differentiation increases significantly when cells are cultured on aligned nanofiber PCL [Azari *et al*. 2021].

Proper cell or tissue culture growth requires essential nutrients and active factors in the medium, which are readily supplied by sera of various origins. The most used sera for *in vitro* culture are those of bovine origin (adult, neonatal or foetal). Horse serum (HS) has also been used as an alternative for supplying growth factors and hormones [Leicht *et al.* 2003]. Primary cultures of chick myoblasts were able to differentiate in both media tested, with Dulbecco's modified Eagle medium containing HS being a more efficient medium for cell fusion [Lawson *et al*. 2000]. Therefore, HS may initiate increased cell differentiation in cultures compared to foetal bovine serum (FBS). Embryonic skeletal muscle tissue extracts, in turn, as additives to the medium at concentrations 0.5-2% are proven to stimulate cell proliferation [Bałaban *et al*. 2021].

Further work on heart tissue culture using PCL scaffolds requires the evaluation of how factor-rich supplements added to the medium influence the growth of cardiac pseudo-tissue on PCL. In our experiment we will test FBS versus HS and varying concentrations of heart tissue extract. We will also compare the efficiency of pseudo-tissue formation by cardiac progenitors at different differentiation stages.

Material and methods

Production of a polycaprolactone scaffold

Polycaprolactone pellets (Sigma-Aldrich, Darmstadt, Germany) were heated on a hotplate to 80°C until they reached a plastic state. Thin and long polymer threads of about 0.01-0.1 mm in diameter were obtained using a metal rod, and then small polycaprolactone coils were formed. The scaffolds were disinfected in 70% ethanol and left to dry completely.

Preparation of chicken embryos

Fertilized eggs of Ross chickens were supplied by a local commercial hatchery (Lasocice, Poland). They were incubated under standard conditions (37°C, 60% relative humidity, rotated twice daily) for the appropriate time points up to the 8th, 12th, and 14th days of embryonic development (8, 12, and 14 ED).

Based on the Polish Act on Animal Experiments (Journal of Laws 2015, item 266), the research on chicken embryos does not have to be submitted to the local ethics committee.

Obtaining and culturing cardiac progenitor cells on PCL scaffolds

On the appropriate day, chicken embryos were extracted from the shell cut open with sterile instruments that had previously been washed in 70% ethanol. The embryos were decapitated and cleaned of foetal membranes on sterile Petri dishes. Embryos were then transferred to a new Petri dish containing sterile phosphate-buffered saline (PBS; Gibco, Paisley, UK). Cardiac muscle was isolated and then cleaned of excess blood in PBS. Next, the tissue was transferred to a 1.5 ml Eppendorf with 300 µl trypsin and minced into smaller pieces.

After 10 minutes, the tissues were homogenized using sterile insulin syringe with needles sized 0.9 x 40 mm, 0.7 x 40 mm and 0.4 x 15 mm. Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) with 10% heat-inactivated FBS (Gibco, Paisley, UK) and 1% antibiotics-antimycotics (Gibco, Paisley, UK) was then added to the cell suspension to inactivate trypsin. The tubes were centrifuged at 1,200 rpm for 6 min at room temperature. The supernatant was discarded and the resulting pellet was gently pipetted in 4 ml of fresh medium before plating the cells into a culture flask. The scaffolds were placed in wells coated with agarose to minimize adherent growth. Appropriate mixtures of medium, sera, and extracts were pipetted into individual wells to condition the PCL fibres. After reaching 80–90% confluence, the cells were harvested and transferred onto the scaffolds as a concentrated 50 µL suspension. The plate was placed in an incubator under standard conditions (37°C, 5% CO_2, 95% relative humidity) for 7 days.

Preparation of embryonic heart extract

On 14 ED, chicken embryos were sacrificed and heart muscles were extracted as described above. Hearts were collected in the ice-cold Dulbecco's phosphate-buffered saline (DPBS; Gibco, Paisley, UK) on ice until the procedure was completed. The tissue was then homogenized for 15 minutes in the minimal volume of DPBS (Gibco, Paisley, UK) using tissue homogenizer (Polytron PT 2100, Kinematica AG, Littau-Lucerne, Switzerland) in a cool room, with the 30,000-rpm rotor speed. The homogenate was transferred to centrifuge tubes and spun at 3,000 rpm for 10 minutes at 4°C. The supernatant was collected and centrifuged again at 12,000 rpm for 15 minutes at 4°C. The final supernatant was aliquoted into cryogenic tubes and stored at -80°C.

Medium replacement

Culture medium exchange was performed by transferring scaffolds containing heart tissue constructs from old plates to new ones with fresh culture medium to reduce the risk of infection and provide a fresh supply of nutrients. Medium replacement was performed every 7 days.

First, 0.5 ml of low-gelling-temperature 2-hydroxyethyl agarose (Sigma) was applied to each well of a 12-well plate. After the agarose solidified, 2 ml of medium was added. The scaffolds with tissue constructs were then transferred to fresh media in new plates using sterile forceps. The cultures were then placed in an incubator under standard conditions for another 7 days.

Stimulating supplements

An extract from homogenized chicken embryo hearts isolated on day 14 of embryonic development was used as an additive to stimulate proliferation and contraction of heart cells. The extract was added at a concentration of 10 µl/ml and 20 µl/ml of medium to selected wells of the culture plate.

Visualization of polycaprolactone fibres

Image showing the morphology and structure of PCL scaffolds was taken with a scanning electron microscope JEOL JSM-6390LV (JEOL Ltd., Japan).

Morphology assessment

A Leica DMi8 inverted microscope was used to assess the morphology of heart constructs growing on PCL scaffolds. Images were taken on days 7 and 14 of *in vitro* tissue culture.

Cell viability/metabolic activity testing

The PrestoBlue Cell Viability Assay (Thermo Fisher Scientific) was used to assess cell viability. A 96-well plate was seeded with 100 µl of cardiac cell suspension and incubated for 24 hours. The medium was then replaced with DMEM medium containing the following combinations of sera and stimulating additives: 10% pure FBS (FBS); 10% FBS supplemented with 10µl/ml heart cell extract (FBS10); 10% FBS with 20µl/ml of heart cell extract (FBS20); 10% pure HS (HS); 10% HS with 10µl/ml heart cell extract (HS10); 10% HS with 20µl/ml heart cell extract (HS20). Each variant was tested in triplicate. The same number of wells were designated for a negative control where the wells were supplemented with appropriate media. Then, 10 µl of PrestoBlue reagent was added to each well and incubated for 24 hours under standard conditions. Fluorescence was measured using a microplate reader (Infinite M200, Tecan, Durham, NC, USA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Statistical analysis

Statistical analyses were performed using Statgraphics Plus 4.1 software (StatPoint Technologies, Warrenton, Virginia, USA) and GraphPad Prism v10.0.0 (GraphPad Software, Boston, MA, USA). To assess the normality of the data distribution, standardised skewness and kurtosis were calculated. Levene's test was used to assess homogeneity of variances. For non-normal data, the Kruskal–Wallis test was used to compare medians; box-and-whisker plots were used to visualise group differences. For normally distributed data, differences between experimental groups were analysed using one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$. Data are presented as means ± standard deviation.

Results and discussion

Visualization of polycaprolactone fibres

Figure 1 shows a scanning electron microscope (SEM) photograph of PCL fibres.

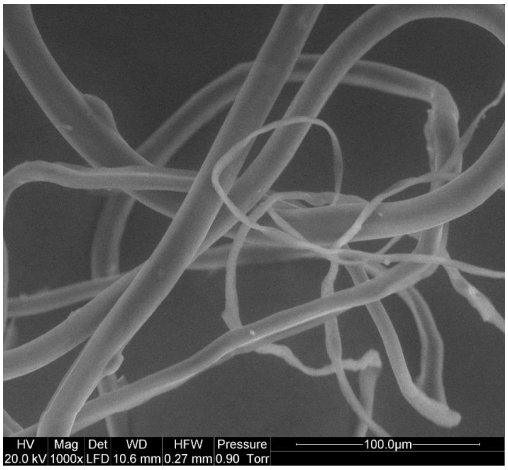

Fig. 1. Polycaprolactone (PCL) fibres prepared manually as observed in an SEM image. Scale bar 100 µm.

The polymer fibres varied in thickness (approximately 5-20 µm) and formed random tangles, which caused differences in pore size. The lack of connections between the fibres resulted in a highly elastic scaffold.

Morphology of constructs

Figures 2 and 3 show the results obtained for 8 ED, using FBS and HS supplementation in the culture medium, respectively.

Cells from 8 ED easily colonized polycaprolactone scaffolds. Cells in the control group had a more oval morphology, whereas those treated with heart extract took on an elongated shape. Cells supplemented with the extract (Fig. 2B, C, E, F) proliferated faster and probably began differentiation, judging by their elongated morphology and near-parallel arrangement. Increased proliferation was indicated by the faster growth of the construct compared to cultures without extract supplementation.

Cells from 8ED cultured with HS showed a higher tendency to colonize polycaprolactone scaffolds. They acquired an elongated shape, suggesting the integration of individual cells and the formation of a shared niche on the scaffold. These cells differentiated more rapidly even not supplemented with the extract (Fig. 3A). The numbers of cells supplemented with the extract increased visibly compared to the cells without extract supplementation.

Figures 4 and 5 show the results obtained using inverted microscope for 12 ED, using FBS and HS supplementation of culture medium respectively.

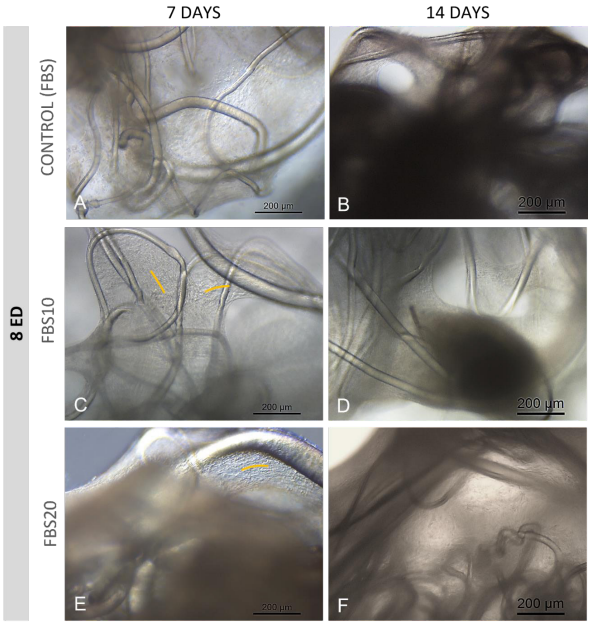

Fig. 2. Constructs of heart tissue created from progenitors taken from chicken embryo at day 8 of embryonic development in culture with FBS supplementation A) control - cell culture without additives, day 7; B) control - cell culture without additives, day 14; C) culture with 10 µl/ml extract addition, day 7; D) culture with 10 µl/ml extract addition, day 14; E) culture with 20 µl/ml extract addition, day 7; F) culture with 20 µl/ml extract addition, day 14. Yellow lines indicate direction of elongated cells in the pseudo-tissue strands. Scale bar 200µm. Magnification 100x.

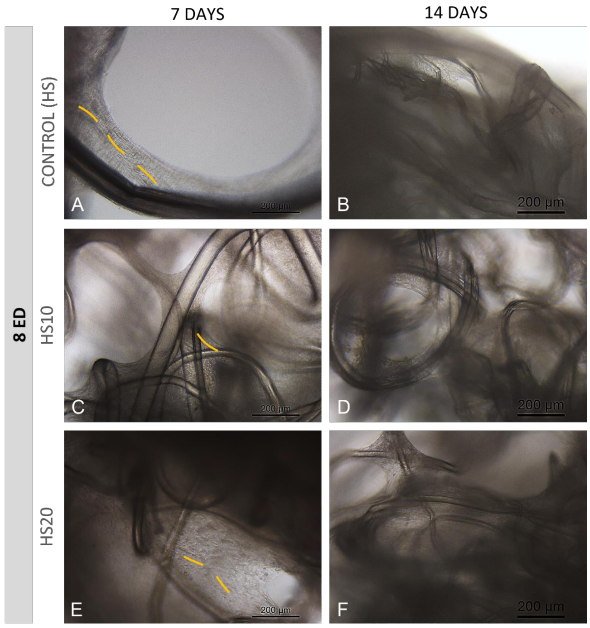

Fig. 3. Constructs of heart tissue generated from progenitor cells isolated from chicken embryos at ED8, cultured in HS-supplemented medium. A) control culture without additives, day 7; B) control culture without additives, day 14; C) culture with 10 µl/ml extract, day 7; D) culture with 10 µl/ml extract, day 14; E) culture with 20 µl/ml extract, day 7; F) culture with 20 µl/ml extract, day 14. Yellow lines indicate the orientation of elongated cells within the pseudo-tissue strands. Scale bar: 200 µm. Magnification: 100×.

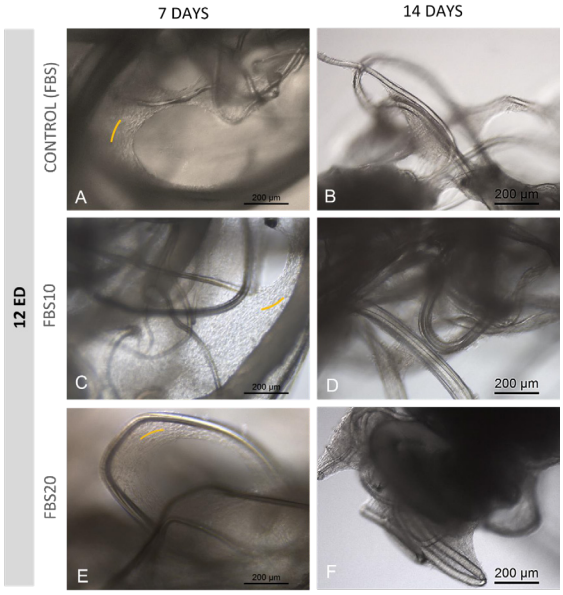

Fig. 4. Constructs of heart tissue generated from progenitor cells isolated from chicken embryos on 12 ED, cultured in FBS-supplemented medium. A) control culture without additives, day 7; B) control culture without additives, day 14; C) culture with 10 µl/ml extract, day 7; D) culture with 10 µl/ml extract, day 14; E) culture with 20 µl/ml extract, day 7; F) culture with 20 µl/ml extract, day 14. Yellow lines indicate the orientation of elongated cells within the pseudo-tissue strands. Scale bar: 200 µm. Magnification: 100×.

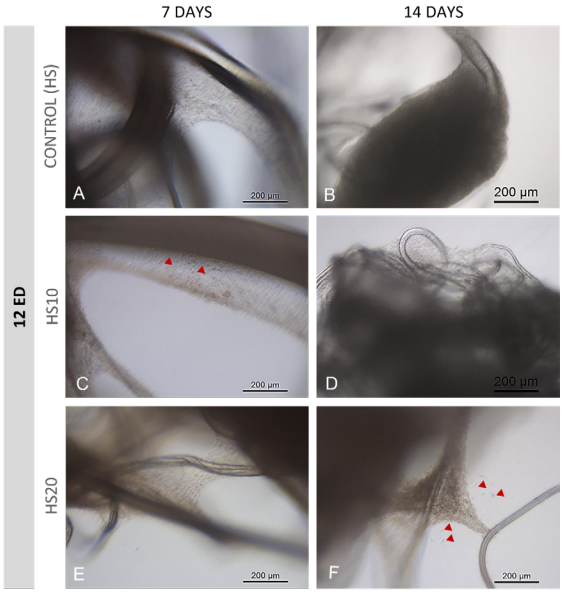

Fig. 5. Constructs of heart tissue generated from progenitor cells isolated from chicken embryos at embryonic day 12 (12 ED), cultured in HS-supplemented medium. A) control culture without additives, day 7; B) control culture without additives, day 14; C) culture with 10 µl/ml extract, day 7; D) culture with 10 µl/ml extract, day 14; E) culture with 20 µl/ml extract, day 7; F) culture with 20 µl/ml extract, day 14. Red arrowheads indicate cells that have lost their elongated morphology and intercellular connections. Scale bar: 200 µm. Magnification: 100×.

Cells from 12 ED colonized the scaffolds in significantly greater numbers than cells from 8 ED with the same rerum addition. Their proliferation rate and differentiation level were notably higher, as evidenced by the parallel arrangement

of cells in the construct after 7 days, even without extract supplementation. Dense networks of branched pseudo-tissue with complex architecture were formed. After 14 days of culture in medium with the extract, excessive proliferation led to construct overgrowth, which eventually resulted in cell death due to limited nutrient availability and inefficient removal of metabolic by-products. In the last image (Fig. 4F), agglomerates of dead cells are visible, indicating loss of integrity and tissue-like structure due to cell accumulation.

Following HS supplementation, ED-derived cells colonized the scaffolds easier than in other experimental conditions, both in terms of embryonic development stage and serum type. However, differentiation was evident only in the control culture after 7 days. High proliferation levels led to thicker pseudo-tissue sheets, visible as reduced transparency of scaffold strands. After extract supplementation, even after 7 days cells in pseudo-tissue started to show symptoms of losing direct connections between them. They seemed to be connected mainly to the extracellular matrix they had produced (Fig. 5C, F). Cell proliferation was so intense that rapid hypertrophy and excessive accumulation of cell mass occurred (Fig. 5D, E, F). As a result, malnutrition and metabolic waste accumulation were likely to have caused cell death.

Cell viability/metabolic activity assay

Cell viability was expressed as fluorescence intensity after subtracting the blank readings. For CPCs from 8 ED, standardized skewness and kurtosis for all groups were within -2 to +2, indicating approximate normality. Levene's test showed homogeneity of variances ($\alpha = 0.05$), supporting the use of one-way ANOVA to evaluate group differences. Means ± standard deviations are shown in Figure 6. To corroborate these findings, the Kruskal-Wallis test was also performed and indicated significant differences in medians at the 95% confidence level; a box-and-whisker plot is shown in Figure 7.

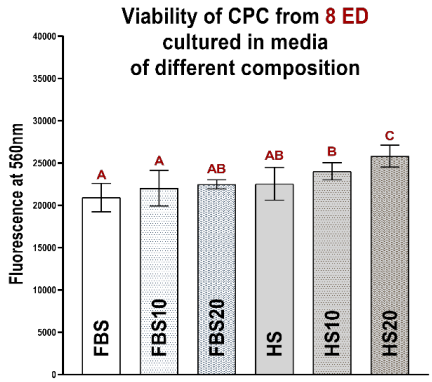

Fig. 6. Viability of cardiac progenitor cells (8 ED) depending on serum type and extract concentration (one-way ANOVA). Different letters (A-C) above the columns indicate statistically significant differences ($p \leq 0.05$).

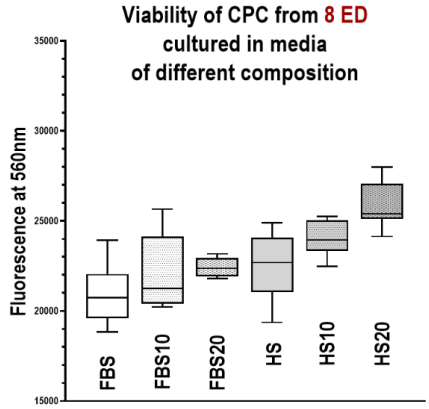

Fig. 7. Viability of cardiac progenitor cells (8 ED) by serum type and extract concentration. Results of the Kruskal-Wallis test are shown as a box-and-whisker plot, displaying the minimum, first quartile, median, third quartile, and maximum.

For CPCs on 12 ED, standardized skewness and kurtosis fell outside -2 to +2 for one group (FBS), indicating non-normality. Accordingly, medians were compared using the Kruskal-Wallis test, which showed significant differences at the 95% confidence level; see Figure 8 for the Box-and-Whisker plot.

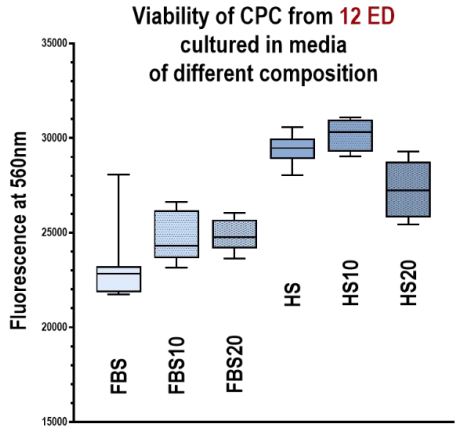

Fig. 8. Viability of cardiac progenitor cells (12 ED) depending on serum type and extract concentration. Results of the Kruskal-Wallis test are shown as a box-and-whisker plot, displaying the minimum, first quartile, median, third quartile, and maximum.

However, because for CPCs on 12 ED Levene's test showed no significant differences in standard deviations at the 95,0% confidence level and nonnormality was observed for only one group, we also performed one-way ANOVA for this dataset to identify which groups differed significantly.

Overall, the parametric and nonparametric analyses yielded consistent conclusions for CPCs on 8 ED and CPCs on 12 ED across the tested culture conditions. Each graph illustrates the effect of two different sera - FBS and HS. Comparing cell viability in cultures without extract supplementation (FBS, HS), a viability increase was observed only in progenitors from 12 ED. In Figures 6 and 7, extract supplementation positively influenced the viability of 8 ED progenitor cells, but only at the higher dose (20 µl/ml) in combination with HS. In Figures 8 and 9, viability of 12 ED progenitors was significantly stimulated by FBS in combination with the extract (FBS10, FBS20). However, when the extract was combined with HS (HS10, HS20), the effect was neutral or even unfavourable, particularly at the higher concentration (HS20).

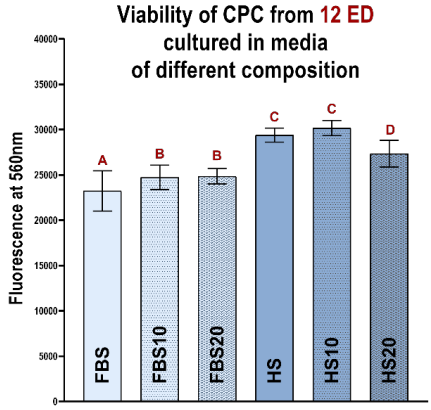

Fig. 9. Viability of cardiac progenitor cells (12 ED) depending on serum type and extract concentration (one-way ANOVA). Different letters (A-D) above the columns indicate statistically significant differences ($p \leq 0.05$).

The results indicate that HS primarily enhances the viability of developmentally older progenitor cells (12 ED). In contrast, extract supplementation in combination with HS had a more beneficial effect on younger cardiac progenitor cells (8 ED).

Cardiac TE offers promising solutions to diseases that cause damage to the heart muscle [Tenreiro *et al*. 2021, Rayat Pisheh *et al.* 2024]. This study investigated seeding cells onto a structured, supportive platform that can be supplemented with cytokines, growth factors, or extracellular matrix peptides. The scaffold provides a biomimetic environment that resembles the physiological conditions of a tissue, in this case the heart, promoting cell adhesion and differentiation. It aligns with the literature which assumes that an optimal scaffold for cardiac repair should: reproduce the myocardial microenvironment, structure and three-dimensional organization; facilitate vascularization to supply oxygen and nutrients; meet electrical and mechanical requirements for proper tissue coupling; be easily replaceable; enhance cell survival and engraftment [Tenreiro *et al*. 2021, Rayat Pisheh *et al*. 2024].

Furthermore, an appropriate animal model, e.g. chicken, is essential for the development of basic and clinical research. In 2004, the chicken genome was sequenced by Sanger using the shotgun method and mapped using extensive physical mapping [International Chicken Polymorphism Map Consortium 2004]. Despite the differences, the 70 million base pairs of sequence highly overlap human ones [International Chicken Genome Sequencing Consortium 2004]. Many characteristics of the chicken make it an ideal model organism for embryology, medical applications, and cardiovascular research [Vilches-Moure 2019, Wittig and Münsterberg 2020]. Moreover, it has a relatively large size and is therefore particularly suitable for the analysis of heart cell differentiation and behaviour [Davey *et al*. 2007, Kain *et al.* 2014].

Cardiac progenitor cells are multipotent, and their application in cell therapy has shown great promise for repairing damaged heart tissue [Tian et al. 2015]. Bai *et al.* [2013] demonstrated that cardiac progenitors exhibit high clonality and express cardiac-specific markers (Isl1, Nkx2.5, GATA4, CD34 and Flk1). These cells can self-renew and differentiate into three types of heart cells - cardiomyocytes, smooth muscle cells, and endothelial cells - which form the main tissue structures of the heart.

Committed progenitors of these lineages appear early in the chick embryo. On 4.5-5 ED the coronary vasculature begins to develop, achieving a regular myocardial pattern by 7 ED; coronary arteries form by 8 ED and coronary veins by 12 ED. By 5 ED, progressive thickening of the compact myocardium produces a multilayered organization of myocardial fibres. From day 6 to day 8, cell density in the ventricular compact zone increases by ~80%. Myocyte proliferation declines at hatching, then rises again post-hatching [Martinsen 2005].

Regarding CPC differences between 8 ED and 12 ED, chicken studies on how embryonic stage affects CPC proliferation and differentiation are lacking. Mouse data are informative: Feridooni *et al*. [2019] showed that CPCs from E11.5 vs E17.5 embryos differ in mitochondrial content, with E17.5 CPCs containing more mitochondria; cardiomyogenic factors increased differentiation in younger CPCs. Our results partially align with these observations. Although mouse gestation lasts 18-21 days, similar to chicken embryonic development, the chicken 12 ED embryo is developmentally older than a mouse E11.5 embryo due to earlier pre-hatching development in the oviduct; 12 ED aligns more closely with mouse E17.5, albeit imperfectly [HDBR 2025]. The greater differentiation potential of mouse E11.5 CPCs is consistent with the elevated viability of chicken 8 ED CPCs treated with HS and heart extract.

This experiment corroborates Barreto *et al.* [2019] demonstrating that cardiac progenitor cells from chicken embryos successfully colonize PCL scaffolds. PCL is emerging as a promising material for fabricating scaffolds that mimic the strength, structure, and mechanical properties of the native heart [Schmitt *et al*. 2022]. Among different scaffold types, fibrous scaffolds offer superior cell adhesion and orientation along the fibres [Gizaw *et al*. 2019, Khan *et al*. 2021]. PCL was chosen as a supporting

material due to its biocompatibility, biodegradability, and adaptability to various shapes and structures [Constantinides *et al*. 2018, Jana *et al.* 2019]. The PCL scaffold enabled cardiac progenitor cells to organize into a three-dimensional structure. Studies by Azari *et al*. [2021] and Miranda-Buendia *et al*. [2022] demonstrated that PCL scaffolds effectively support cardiac progenitor cell growth, differentiation, and maturation into cardiomyocytes - an essential step in functional heart tissue formation. Despite these advantages, the ongoing research aims to further optimize scaffold properties. Factors such as pore size and fibre alignment significantly influence cell colonization efficiency [Perez-Puyana *et al.* 2021].

In this study, the scaffold was manufactured manually, resulting in limited pore size variability and restrictions on scaffold shape and size. While sufficient for assessing culture medium additives, this may have caused uneven cell colonization and hindered cell migration due to excessively large scaffold spaces. A more effective approach to scaffold fabrication is electrospinning, which allows for precise control over scaffold architecture and customization for cardiac tissue applications [Zamani *et al*. 2021].

Electrospinning has emerged as a therapeutic platform for cardiovascular applications. Electrospun scaffolds made from biocompatible polymers, including PCL, can mimic the native extracellular matrix and support drug delivery. Contemporary cardiac TE strategies often combine nanofibers and polymers with electrical stimulation, stem-cell therapy, or controlled drug release to enhance heart function and regeneration [Broadwin *et al*. 2024]. Fibre diameter in electrospinning critically affects conductivity and other properties relevant to cardiac repair [Roacho-Pérez *et al.* 2022]. Reducing average fibre diameter increases electrical conductivity by roughly 100-fold [Abedi *et al*. 2021], which is valuable given PCL's low ionic conductivity ($\sim 10^{-11}$ S/cm) at room temperature [Zhou *et al.* 2022].

Comparing FBS and HS, our findings indicate that HS provides superior performance. FBS and HS are widely used as nutritional supplements in cell culture. FBS is preferred due to its availability and high concentrations of growth factors, hormones, amino acids, vitamins, and minerals essential for cell development [Chelladurai *et al.* 2021, Lee *et al*. 2022]. HS, on the other hand, is more similar to human serum than FBS, which may offer advantages in heart TE. Our results demonstrated a higher potential of HS in cardiac progenitor cell cultures, contradicting the findings of Franke et al. (2014). However, it should be noted that Franke et al. used a different cell type, which significantly impacts experimental outcomes.

A direct comparison of HS and FBS effects on embryonic cells in culture had not yet been performed for chicken or any other vertebrate embryos before this study. However, studies on adult human and pig heart tissues have shown that medium optimisation is essential for successful *in vitro* culture [Ou *et al.* 2019, 2020]. Similarly to muscle tissue extract [Bałaban *et al*. 2021], the extract from homogenized chicken embryo hearts supported cell proliferation. However, in 12 ED cells, proliferation occurred too rapidly, leading to an excessive pseudo-tissue growth and the accumulation of large

cell masses which probably limited oxygen and nutrient availability to cells within the agglomerates and caused metabolic waste accumulation, resulting in construct disorganization and poor cohesion. These observations highlight the need for precise control and optimization of extract dosage based on cell type and scaffold distribution.

Importantly, ensuring nutrient and oxygen supply while removing metabolic by-products is critical for the proper function of tissue constructs. Large tissue constructs lack a natural vascular system, meaning that essential substances cannot be efficiently transported to deeper tissue layers. Without a proper nutrient supply, cells can degenerate and ultimately undergo cell death. Developing strategies for vascularized TE remains a significant challenge [Sarker *et al.* 2015].

Heart TE remains an actively evolving field, and achieving fully functional cardiac tissue requires further optimization of techniques. Several critical factors, including access to growth factors and physical stimuli, affect the differentiation and organization of cardiac progenitor cells in three-dimensional structures. Despite promising experimental results, it is important to recognize that this study was conducted *in vitro* and represents a small step in the broader research landscape. Further studies are necessary to evaluate the functional capabilities of engineered heart tissue, assess the biocompatibility of PCL scaffolds, and determine long-term effects. The long-term biodegradation of PCL and its replacement by natural extracellular matrix components should be closely monitored. Despite these challenges, our study demonstrates the potential of three-dimensional heart tissue constructs on PCL scaffolds using cardiac progenitor cells from chicken embryos. This research contributes to preclinical efforts focused on heart tissue regeneration and congenital heart defect treatment.

Conclusions

Earlier-stage cardiac progenitors demonstrated an elevated potential for forming heart tissue constructs when cultured with HS and an extract from homogenized hearts of 14-day-old chicken embryos. Older CPCs collected on 12 ED exhibited a higher potential than 8 ED progenitors for creating pseudo-heart tissue constructs in the presence of HS, even without the addition of homogenized heart extract. Too high a concentration of this extract reduced their ability to form structured tissue constructs. Further research is necessary to elucidate and optimize CPC growth and differentiation on electrospun PCL scaffolds with respect to culture medium composition.

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Disclosures

The authors declare no conflicts of interest.

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