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Dexamethasone Attenuates Microvascular Malformations and Vascular Leakage During Prolonged Ex Vivo Perfusion of Mouse Hearts

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Abstract

This study examined whether dexamethasone attenuates microvascular malformations and vascular leakage in ex vivo-perfused mouse hearts using the Miniature Tissue Culture System (MTCS). The MTCS is an ex vivo perfusion model that enables prolonged culture of mouse hearts. Mouse (C57BL/6) hearts were cultured ex vivo for 3–7 days in the MTCS, perfused in the presence or absence (control) of dexamethasone (100 nM). Immunostaining and quantification were performed to analyze attenuated microvascular malformations based on a decrease in CD31-positive area and an increase in capillary count. Vascular leakage was assessed using Evans blue perfusion, myocardial basement-membrane architecture was evaluated by laminin immunostaining, and the anti-inflammatory effect was analyzed with NF-κB-p65 immunostaining and quantification. Dexamethasone treatment attenuated microvascular malformations compared with controls, as evidenced by decreased CD31-positive area (mean ± SD = 8.91% ± 3.78% versus 34.51% ± 7.08%, $p < 0.0001$; $n = 7$) and increased capillary count (mean ± SD = 14.27 ± 3.70 versus 2.32 ± 1.62 capillaries per 10^4 μm^2, $p < 0.0001$; $n = 7$). Reduced Evans blue extravasation and preservation of laminin-positive basement-membrane architecture in the myocardium were observed. NF-κB-p65-positive area was decreased (mean ± SD = 3.17% ± 0.76% versus 6.13% ± 2.35%, $p = 0.0338$, $n = 5$). These findings suggest that dexamethasone may improve preservation of the cardiac microvasculature during prolonged ex vivo perfusion.

Keywords: microvasculature; capillary remodeling; endothelial cells; vascular leakage; dexamethasone; donor heart preservation; ex vivo perfusion; Miniature Tissue Culture System

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1. Introduction

End-stage heart failure is associated with increasing prevalence and poor prognosis, with one-year mortality rates ranging from 25%–75% [1]. Patients suffer from persistent symptoms, including breathlessness, swelling due to fluid retention, fatigue, and reduced exercise tolerance [1]. For selected patients, heart transplantation remains the only definitive treatment, but its availability is severely limited by the shortage of suitable donor hearts [1–3]. Expanding the donor pool is therefore a major clinical priority. A key limitation in this effort is the relatively short period for which donor hearts can currently be safely preserved with ex vivo heart perfusion (EVHP). Extending cardiac preservation time could provide a wider window for donor–recipient matching and allocation, thereby improving access to suitable donor organs [3–7]. Importantly, prolonged ex vivo preservation could also enable the development of therapeutic interventions to assess, recover, or repair donor hearts that currently do not meet transplantation criteria, potentially further expanding the pool of transplantable organs [3–7].

The myocardial microvasculature is particularly vulnerable during prolonged ex vivo preservation. Endothelial barrier dysfunction can result in vascular leakage and tissue fluid accumulation, while inflammatory and mechanical stresses during perfusion can promote endothelial activation and vascular remodeling [8–12]. Maintaining a functional and structurally organized microvascular network is therefore important for preserving myocardial integrity and function during EVHP. Experimental models that allow prolonged perfusion are needed to define these processes and to test interventions aimed at limiting perfusion-induced vascular changes.

The Miniature Tissue Culture System (MTCS) enables prolonged ex vivo perfusion and culture of whole mouse hearts [13–15]. Previous work using this model demonstrated extensive cardiac remodeling during prolonged culture and showed that these responses can be modified by altering the mechanical or molecular environment [13]. Here, we used the MTCS to characterize the vascular response to prolonged ex vivo perfusion and to test whether this response could be modulated pharmacologically. We hypothesized that the anti-inflammatory glucocorticoid dexamethasone would attenuate microvascular malformations during prolonged perfusion. We therefore assessed endothelial organization, capillary abundance, vascular leakage, myocardial basement-membrane architecture, and NF-κB-p65 staining in mouse hearts perfused in the presence or absence of dexamethasone.

2. Results

2.1. Microvascular Malformations Appear Between Day 4 and Day 7 of Ex Vivo Mouse Heart Culture

To assess changes in the (micro)vasculature during ex vivo culture, whole mouse hearts were cultured in the MTCS. The MTCS perfusion system consists of eight units. Per unit, a mouse heart is perfused with medium that flows from the medium reservoir through a pump and bubble trap to the perfusion chamber containing the mouse heart (Figure 1A). Subsequently, the medium returns to the reservoir for recirculation. Between day 4 and day 7 of ex vivo culture, microvascular malformations appeared, which we defined as morphological reorganization of the vasculature, visualized by endothelial marker CD31 (indicated by the red arrowheads in Figure 1B).

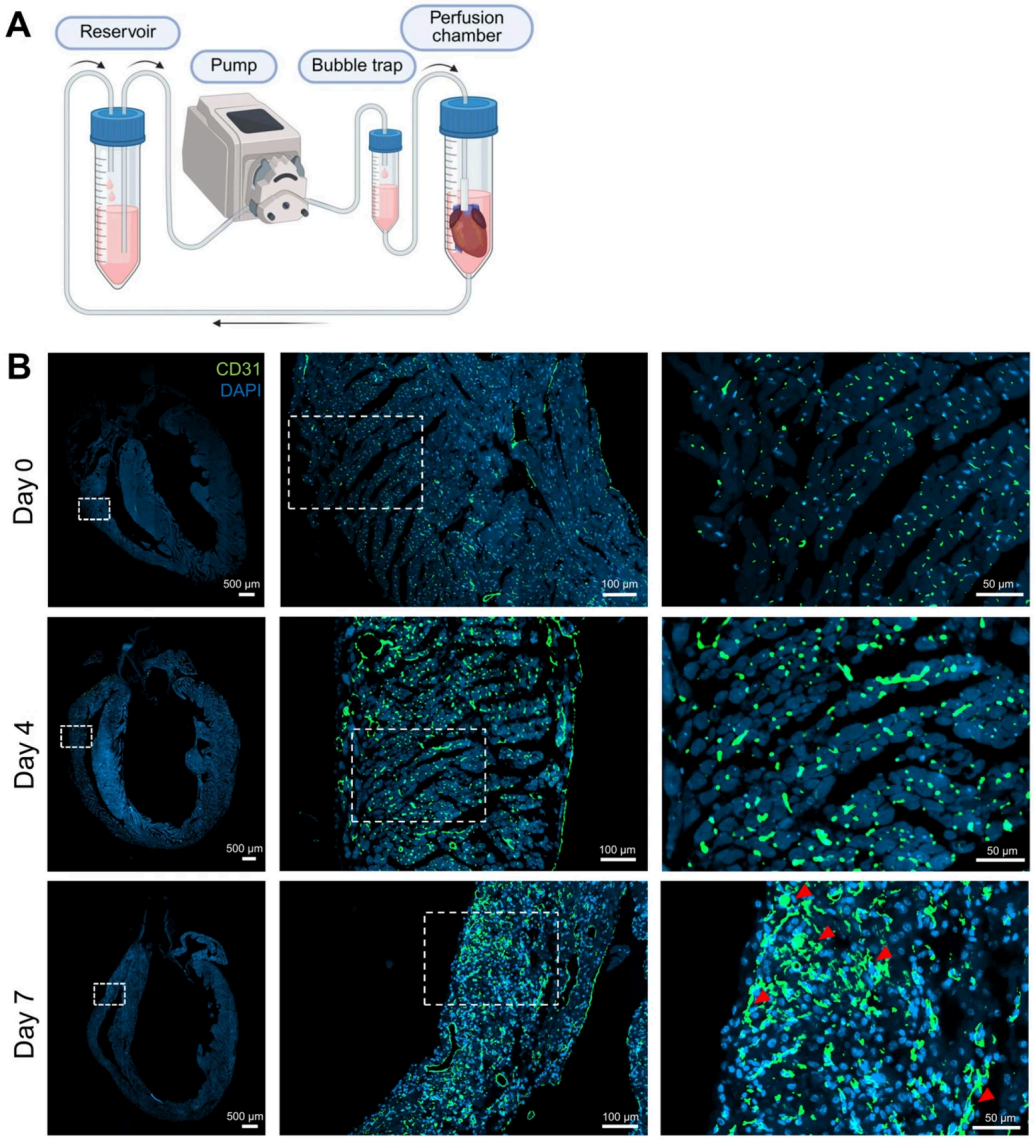


Figure 1. Microvascular malformations appeared between day 4 and day 7 of ex vivo mouse heart culture. (**A**) Schematic figure of a Miniature Tissue Culture System unit used for ex vivo mouse heart culture, consisting of a pump, bubble trap, perfusion chamber, and reservoir for (recirculating) medium (Created in BioRender. Goumans, M. (2027) https://BioRender.com/saf58xt); (**B**) Microvascular malformations in ex vivo-cultured mouse hearts appear between day 4 and day 7 of culture, as indicated by representative immunofluorescence images of CD31-positive endothelial structures in mouse hearts cultured ex vivo for 0, 4, and 7 days (red arrowheads indicate microvascular malformations). Green: CD31. Blue: DAPI. Scale bars from left to right: 500 µm, 100 µm, 50 µm.

2.2. Dexamethasone Reduces Vascular Leakage in Ex Vivo-Cultured Mouse Hearts

To assess vascular leakage, mouse hearts cultured ex vivo were perfused with Evans blue dye at the end of culture. Dark blue dots indicate leakage of vascular fluid into surrounding tissue (Figure 2A). Mouse hearts cultured ex vivo for 3 or 4 days showed multiple intense blue regions, indicating profound vascular leakage (n = 3). To test whether vascular leakage in ex vivo-cultured mouse hearts can be attenuated, dexamethasone was added to the perfusion medium. Hearts treated with dexamethasone showed reduced Evans blue dye staining (n = 3; Figure 2B), indicating decreased vascular leakage compared to untreated control hearts. Together, these observations suggest reduced vas-

cular leakage with dexamethasone administration to the perfusate.

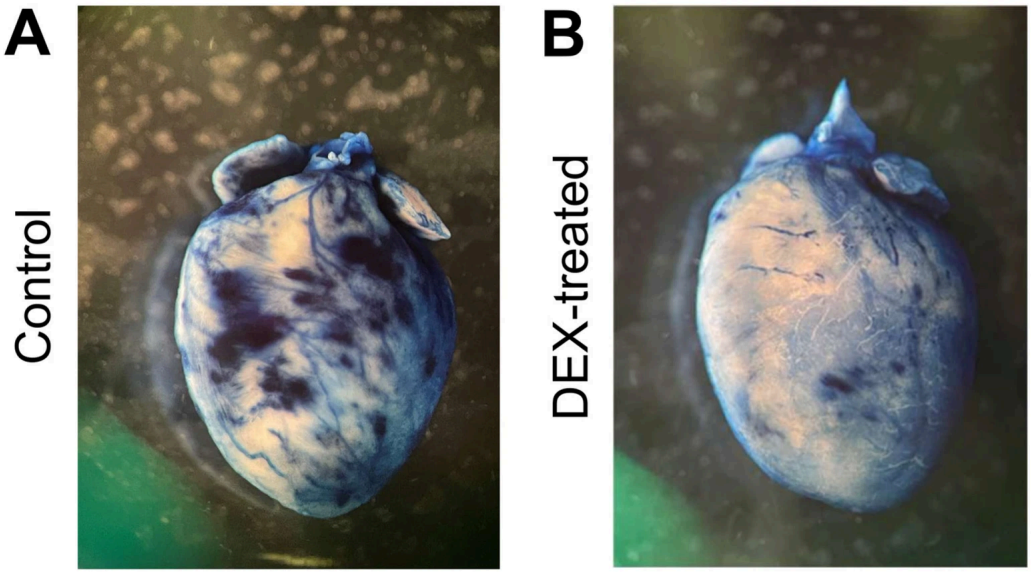


Figure 2. Dexamethasone (DEX) reduces vascular leakage in ex vivo-cultured mouse hearts. (**A**) Vascular leakage in mouse heart cultured ex vivo for 4 days, visualized by Evans blue perfusion; (**B**) Reduced vascular leakage in DEX-treated mouse hearts cultured ex vivo for 4 days, visualized by Evans blue dye extravasation.

2.3. Dexamethasone Protects the Myocardial Basement-Membrane Architecture in Ex Vivo-Cultured Mouse Hearts

Laminin immunostaining was used to assess myocardial basement-membrane architecture. Compared with non-cultured hearts, untreated control hearts cultured ex vivo for 7 days showed marked disruption of the laminin-positive basement-membrane network (Figure 3A,B). To test whether the myocardial basement membrane in ex vivo-cultured mouse hearts can be protected, dexamethasone was added to the perfusion medium in mouse hearts cultured ex vivo for 7 days. In dexamethasone-treated hearts, the laminin pattern was better preserved and more closely resembled that of non-cultured hearts (Figure 3C). The laminin-positive myocardial basement-membrane architecture results were supported by collagen-IV staining (Figure 3D–F). Similarly, collagen-IV staining showed that dexamethasone treatment prevented disruption of the myocardial basement-membrane architecture in mouse hearts cultured ex vivo for 7 days compared to untreated control mouse hearts cultured ex vivo for 7 days, resembling the non-cultured group. These representative images therefore suggest preservation of myocardial basement-membrane architecture with dexamethasone treatment.

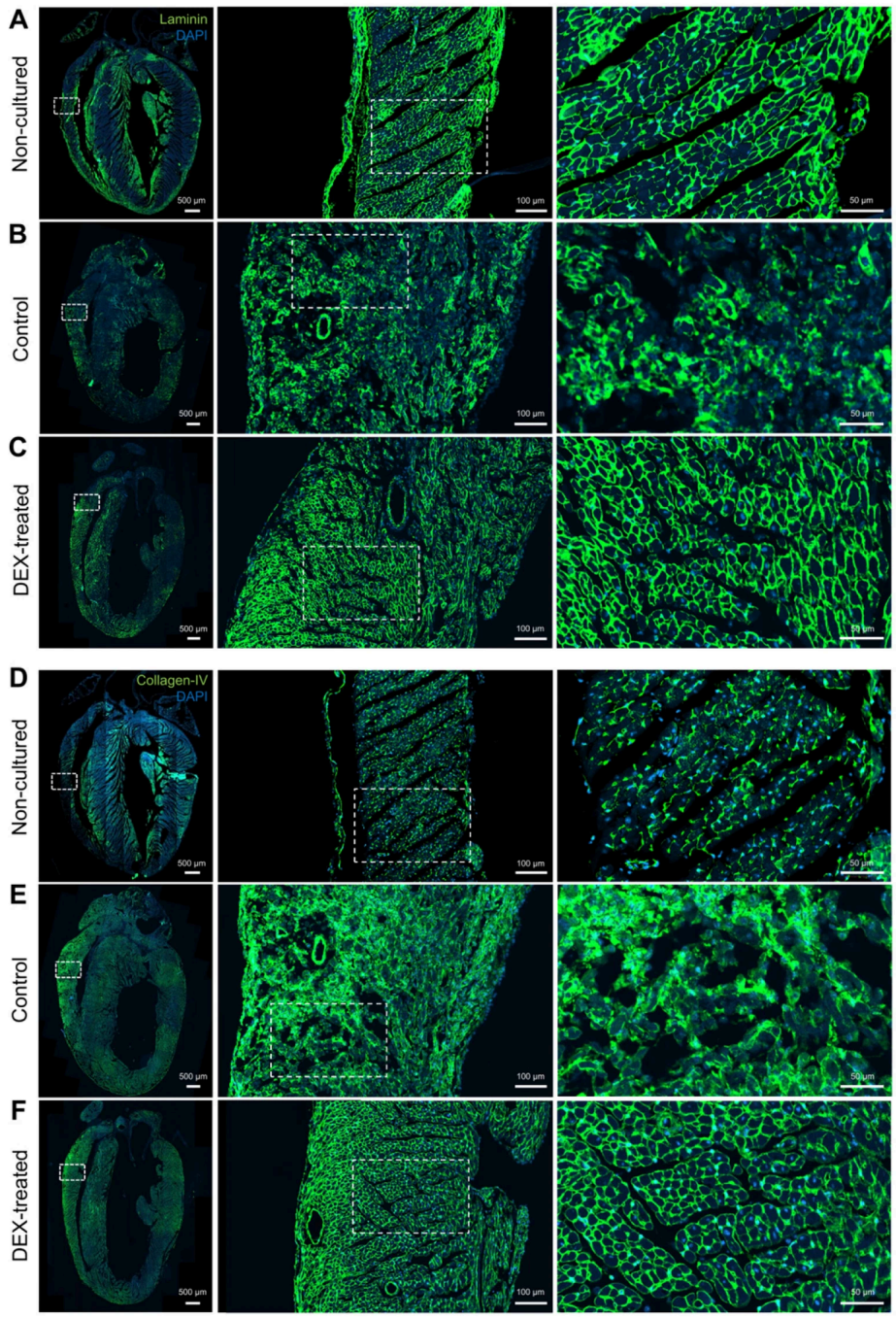


Figure 3. Dexamethasone preserves the myocardial basement-membrane architecture in ex vivo-cultured mouse hearts. (**A**,**B**) Disruption of basement membrane indicated by laminin staining in representative immunofluorescence images of mouse hearts cultured ex vivo for 7 days (**B**) compared to non-cultured hearts (**A**); (**C**) Basement-membrane structural integrity in DEX-treated mouse hearts cultured ex vivo for 7 days (**C**), resembling non-cultured hearts (**A**); (**D**,**E**) Disruption of basement membrane indicated by collagen-IV staining in representative immunofluorescence images of mouse hearts cultured ex vivo for 7 days (**E**) compared to non-cultured hearts (**D**); (**F**) Basement-membrane structural integrity in DEX-treated mouse hearts cultured ex vivo for 7 days, resembling non-cultured hearts (**D**). Green: laminin (**A**–**C**) or collagen-IV (**D**–**F**); blue: DAPI; scale bars from left to right: 500 µm, 100 µm, 50 µm.

2.4. Dexamethasone Attenuates Microvascular Malformations and Preserves Capillary Abundance in Ex Vivo-Cultured Mouse Hearts

To determine whether dexamethasone attenuates microvascular malformations and preserves the microvasculature, mouse hearts cultured ex vivo for 7 days were stained for endothelial cell marker CD31. Microvascular malformations were observed in untreated control hearts, in which CD31-positive endothelial structures were reorganized into an extensively altered vascular network with loss of capillaries, in contrast to the abundant capillary structures in non-cultured hearts (Figure 4A,B). Treatment with dexamethasone attenuated microvascular malformations and preserved capillary abundance (Figure 4C), comparable to the non-cultured hearts (Figure 4A). The preserved capillary abundance is apparent from the pattern of small, punctate CD31-positive structures (indicated by red arrowheads, Figure 4A,C).

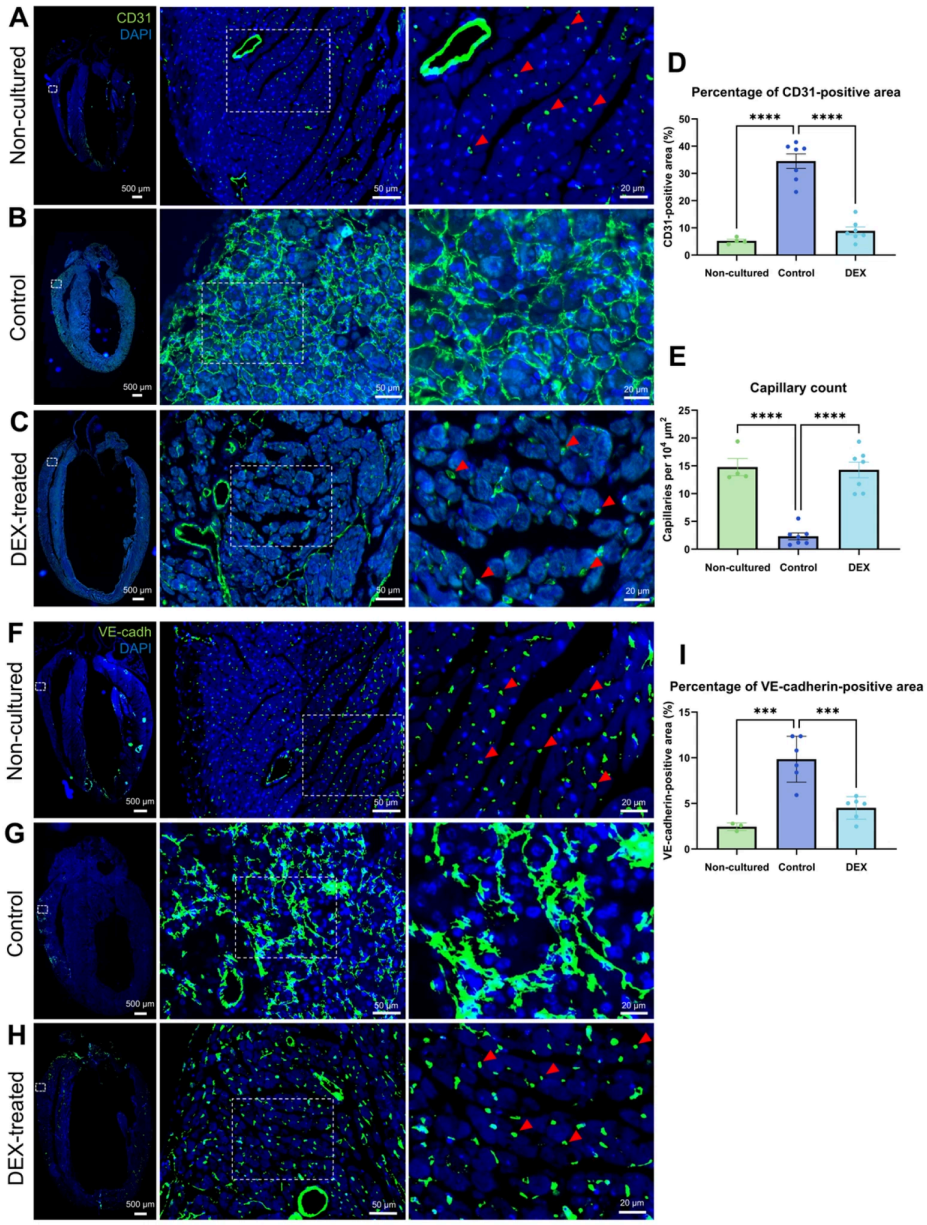


Figure 4. Dexamethasone attenuates microvascular malformations and preserves capillary abundance in ex vivo-cultured mouse hearts. (**A**,**B**) Microvascular malformations as demonstrated by CD31-positive stained endothelial cells in these representative pictures of mouse hearts cultured ex vivo for 7 days (**B**) compared to non-cultured mouse hearts (**A**); (**C**) Attenuated microvascular malformations and preserved capillary abundance (red arrowheads indicate capillaries) by DEX (**C**), comparable to non-cultured mouse hearts (**A**); (**D**) Significantly decreased percentage of

CD31-positive area in DEX-treated mouse hearts cultured ex vivo for 7 days compared to untreated control mouse hearts cultured ex vivo for 7 days, approximating the percentage of CD31-positive area in non-cultured mouse heart (non-cultured versus control: $p < 0.0001$; control versus DEX: $p < 0.0001$, DEX versus non-cultured: $p = 0.9583$). Quantification based on CD31-positive immunofluorescence staining; (**E**) Significantly increased capillary count in DEX-treated mouse hearts cultured ex vivo for 7 days compared to controls, resembling the capillary count in the non-cultured group (non-cultured versus control: $p < 0.0001$; control versus DEX: $p < 0.0001$; DEX versus non-cultured: $p = 0.4965$). Quantification based on manual counting of CD31-positive capillaries with diameter of 2–10 µm; (**F**–**H**) Confirmation of dexamethasone's protection against microvascular malformations and preservation of capillary abundance in mouse hearts cultured ex vivo for 7 days with immunostaining of VE-cadherin-positive endothelial cells; (**I**) significantly decreased percentage of VE-cadherin-positive area in DEX-treated mouse hearts cultured ex vivo for 7 days compared to untreated control mouse hearts cultured ex vivo for 7 days, approximating the percentage of VE-cadherin-positive area in non-cultured mouse heart (non-cultured versus control: $p = 0.0002$; control versus DEX: $p = 0.0007$, DEX versus non-cultured: $p = 0.2802$). Quantification based on VE-cadherin immunofluorescent staining. Green: CD31 (**A**,**B**,**C**) or VE-cadherin (**F**,**G**,**H**), blue: DAPI. Scale bars from left to right: 500 µm, 50 µm, 20 µm. Every dot represents one mouse (**D**,**E**,**I**). $n = 7$ (**D**,**E**) or $n = 6$ (**I**) for each DEX/control group and $n = 4$ (**D**,**E**) or $n = 3$ (**I**) for each non-cultured group. Data are presented as mean ± SD. One-way ANOVA followed by Tukey's multiple comparisons test was performed to determine significant differences between the non-cultured, untreated control mouse hearts cultured ex vivo for 7 days and dexamethasone-treated mouse hearts cultured ex vivo for 7 days. *** $p < 0.001$, **** $p < 0.0001$.

Microvascular malformations were quantified based on the percentage of CD31-positive area (Figure 4D). Untreated control mouse hearts cultured ex vivo for 7 days demonstrated a significantly increased percentage of CD31-positive area compared to non-cultured mouse hearts (non-cultured: mean ± SD = 5.22% ± 1.15%, $n = 4$; control: mean ± SD = 34.51% ± 7.08%, $n = 7$; non-cultured versus control: $p < 0.0001$). Conversely, the percentage of CD31-positive area was significantly decreased in mouse hearts cultured ex vivo for 7 days when treated with dexamethasone compared to untreated control mouse hearts cultured ex vivo for 7 days (DEX: mean ± SD: 8.91% ± 3.78%, $n = 7$; DEX versus control: $p < 0.0001$). Dexamethasone treatment reduced the percentage of CD31-positive area to a level comparable to that in non-cultured mouse hearts, with no significant difference from the non-cultured baseline (DEX versus non-cultured: $p = 0.9583$).

In addition, CD31-positive capillaries were quantified by capillary counting (Figure 4E). Untreated control mouse hearts cultured ex vivo for 7 days had a significantly lower capillary count than non-cultured mouse hearts (non-cultured: mean ± SD = 14.78 ± 3.10 capillaries per 10^4 µm^2, $n = 4$; control: mean ± SD = 2.32 ± 1.62 capillaries per 10^4 µm^2, $n = 7$; non-cultured versus control: $p < 0.0001$). In contrast, treatment with dexamethasone resulted in a significantly higher capillary count in mouse hearts cultured ex vivo for 7 days compared to controls (DEX: mean ± SD: 14.27 ± 3.70 capillaries per 10^4 µm^2, $n = 7$; DEX versus control: $p < 0.0001$). Capillary count in the dexamethasone-treated group resembled the baseline capillary count in the non-cultured group with no significant difference (DEX versus non-cultured: $p = 0.4965$).

The CD31-positive area quantification results were confirmed by VE-cadherin staining and quantification (Figure 4F–I). Indeed, VE-cadherin quantification showed that dexamethasone treatment significantly decreased the percentage of VE-cadherin-positive area in mouse hearts cultured ex vivo for 7 days compared to untreated control mouse hearts cultured ex vivo for 7 days, and did not differ significantly from the percentage of VE-cadherin-positive area in non-cultured group (non-cultured: mean ± SD =

2.44% ± 0.42%, n = 3; control: mean ± SD = 9.84% ± 2.51%, n = 6; DEX: mean ± SD: 4.51% ± 1.24%, n = 6; non-cultured versus control: p = 0.0002; DEX versus control: p = 0.0007, DEX versus non-cultured: p = 0.2802). Together, these results show that dexamethasone attenuates microvascular malformations and preserves myocardial capillary abundance during ex vivo mouse heart perfusion.

2.5. Dexamethasone-Induced Effect on NF-κB-p65 Area May Indicate a Partial Anti-Inflammatory Effect During Ex Vivo Heart Perfusion

Dexamethasone is known for its anti-inflammatory effects and its interaction with NF-κB [16,17]. To investigate whether dexamethasone altered inflammatory signaling during ex vivo mouse heart perfusion, the hearts, either treated or not treated with dexamethasone, were stained for NF-κB-p65 together with VE-cadherin or CD31 to assess endothelial localization. Untreated mouse hearts cultured ex vivo for 7 days showed a larger NF-kB-p65-positive area compared to non-cultured hearts (Figure 5A,B). Dexamethasone-treated ex vivo-cultured hearts showed a lower NF-κB-p65-positive area than untreated controls (Figure 5B,C). In untreated controls, NF-κB-p65 staining was predominantly localized to the microvascular malformations, visualized by VE-cadherin (co-localization of vascular NF-κB-p65-positive nuclei indicated by the dashed white circles, Figure 5B), whereas this co-localization appeared less prominent in the dexamethasone-treated and non-cultured hearts (Figure 5A,C).

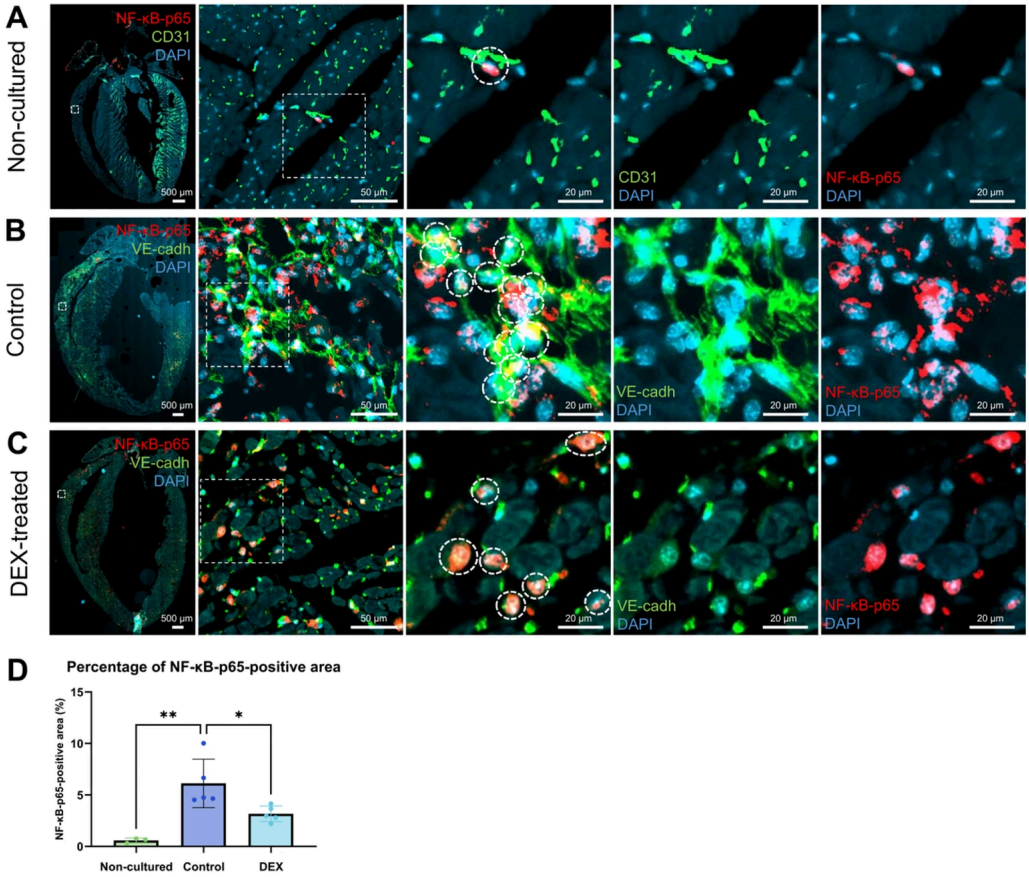


Figure 5. Dexamethasone-induced reduction in NF-κB-p65 area may indicate a partial anti-inflammatory effect during ex vivo heart perfusion. (**A**,**B**) Inflammatory response as demonstrated by NF-κB-p65-positive area in these representative pictures of untreated control mouse hearts cultured ex vivo for 7 days (**B**) compared to non-cultured mouse hearts (**A**); (**C**) Attenuated inflammatory response in DEX-treated mouse hearts cultured ex vivo for 7 days, suggested by decreased

NF-κB-p65-positive area (**C**). Reduced co-localization of NF-κB-p65 with VE-cadherin-positive capillaries (dashed white circles indicate co-localization); (**D**) Significant reduction in the percentage of NF-κB-p65-positive area in DEX-treated mouse hearts cultured ex vivo for 7 days compared to untreated control mouse hearts cultured ex vivo for 7 days, showing intermediate values in respect with the percentage of NF-κB-p65-positive area in non-cultured mouse hearts (non-cultured versus control: $p = 0.0018$; control versus DEX: $p = 0.0338$, DEX versus non-cultured: $p = 0.1086$). (**D**). n = 5 for each DEX/control group and n = 3 for each non-cultured group (**D**). Green: CD31 (**A**) or VE-cadherin (**B**,**C**), blue: DAPI. Scale bars from left to right: 500 µm, 50 µm, 20 µm. Every dot represents one mouse. Data are presented as mean ± SD. One-way ANOVA followed by Tukey's multiple comparisons test was performed to determine significant differences between the non-cultured, untreated control mouse hearts cultured ex vivo for 7 days and dexamethasone-treated mouse hearts cultured ex vivo for 7 days. * $p < 0.05$, ** $p < 0.01$.

Quantification of NF-κB-p65 area revealed that untreated control mouse hearts cultured ex vivo for 7 days demonstrated a significant increase in the percentage of NF-κB-p65-positive compared to non-cultured hearts (non-cultured: mean ± SD = 0.58% ± 0.23%, n = 3; control: mean ± SD = 6.13% ± 2.35%, n = 5; non-cultured versus control: $p = 0.0018$) (Figure 5D). The percentage of NF-κB-p65-positive area in the dexamethasone-treated group showed intermediate values relative to the percentage of NF-κB-p65-positive area in untreated control mouse hearts cultured ex vivo for 7 days and non-cultured mouse hearts (DEX: mean ± SD: 3.17% ± 0.76%, n = 5; DEX versus control: $p = 0.0338$, DEX versus non-cultured: $p = 0.1086$). The reduction in NF-κB-p65-postive area by dexamethasone may indicate a partial anti-inflammatory effect contributing to dexamethasone's protective effect.

3. Discussion

In this study, we found that prolonged ex vivo perfusion was associated with marked microvascular malformations and loss of morphologically identifiable capillaries. Dexamethasone markedly attenuated these microvascular malformations and preserved capillary abundance. Representative imaging further suggested that dexamethasone preserved basement-membrane architecture and prevented vascular leakage. Moreover, the dexamethasone-induced reduction in NF-κB-p65 area indicates an anti-inflammatory effect during ex vivo mouse heart perfusion.

Microvascular malformations were observed in mouse hearts after 7 days of ex vivo perfusion. Vascular dysfunction has also been reported during ex vivo perfusion of larger mammalian and human hearts. Prolonged perfusion has been associated with tissue edema and progressive deterioration of cardiac function in porcine hearts [18] and in a reported human case [19]. Inflammatory and endothelial responses have been observed in porcine hearts and human donor hearts undergoing normothermic EVHP [8,20,21]. Interestingly, endothelial activation without overt evidence of endothelial injury has been reported during relatively short periods of clinical EVHP [21]. Our observations after several days of perfusion therefore suggest that prolonged exposure to the ex vivo environment may induce vascular changes that are not readily detectable during conventional shorter preservation periods.

Vascular leakage was demonstrated in the perfused ex vivo-cultured mouse hearts in our study. An interesting observation is the apparent temporal difference between vascular leakage and structural (micro)vascular remodeling. Evans blue leakage was observed after 3–4 days of ex vivo culture, whereas the pronounced abnormal endothelial structures seen by CD31 staining were predominantly apparent after 7 days. This raises the possibility that impairment of vascular barrier function precedes more extensive structural remodeling of the (micro)vasculature.

The glucocorticoid dexamethasone markedly attenuated the vascular phenotype induced by prolonged ex vivo perfusion. In addition to reducing vascular leakage of Evans blue, dexamethasone prevented the increase in CD31- and VE-cadherin-positive area observed after 7 days of ex vivo culture and preserved the number of morphologically identifiable capillaries. The laminin-positive basement-membrane architecture was also better maintained in dexamethasone-treated hearts. Together, these findings suggest that dexamethasone limits structural remodeling of the microvascular network at both cellular and extracellular levels.

Glucocorticoids are already used in clinical EVHP. Methylprednisolone is included in the perfusate used in clinical normothermic heart-perfusion protocols [6,22], and experimental studies have reported reduced myocardial edema after glucocorticoid treatment during ex vivo perfusion [22]. Dexamethasone has been reported to reduce cardiac vascular leakage in other inflammatory settings [23]. Our findings extend these observations by showing a substantial effect of dexamethasone on microvascular architecture during prolonged heart perfusion. Whether this reflects preservation of pre-existing capillaries, inhibition of maladaptive endothelial remodeling, altered angiogenic responses, or a combination of these processes remains to be determined.

Dexamethasone seems to have an anti-inflammatory effect in our ex vivo mouse heart perfusion, as suggested by the dexamethasone-induced significant reduction in NF-κB-p65 area in a small experimental group. NF-κB is a central regulator of inflammatory response and tissue remodeling, and persistent NF-κB activation can contribute to cardiac dysfunction despite context-dependent cardioprotective effects during acute injury [24,25]. Glucocorticoids can interfere with NF-κB signaling [16,17], and dexamethasone has been reported to suppress NF-κB activation in cardiovascular models [26–28] and decrease inflammatory markers in the heart [27–29]. Human donor hearts undergoing EVHP likewise show time-dependent inflammatory activation [8]. Together, these observations provide a rationale to test whether suppression of inflammatory signaling contributes to the vascular effects of dexamethasone during EVHP. Future studies should directly address this mechanism using quantitative inflammatory, endothelial barrier, and functional readouts.

This study has several limitations. First, the MTCS is a mouse heart model and differs substantially from clinical donor heart perfusion in size, hemodynamics, oxygen delivery, and perfusate composition. In particular, the hearts were perfused with culture medium rather than blood or an oxygen-carrier-containing perfusate. Translation of the present findings to clinical EVHP therefore requires validation in larger-animal and human donor-heart models. Second, the present study primarily assessed vascular structure by histology. Although Evans blue perfusion indicates vascular leakage, the permeability phenotype was not quantitatively linked to the subsequent structural changes. Similarly, CD31-positive area, VE-cadherin-positive area, and capillary counts provide information on vascular architecture and abundance but do not directly measure endothelial barrier integrity. Finally, the reduction in NF-κB-p65 staining is associative and is based on NF-κB-p65-positive area and therefore only gives an indication of NF-kB activation and does not demonstrate that suppression of NF-κB signaling mediates the vascular effects of dexamethasone. Despite these limitations, the MTCS provides an experimental platform in which vascular changes can be studied over considerably longer periods than conventional clinical heart preservation. Candidate perfusate interventions can therefore be tested in parallel across multiple experimental groups before validation in more clinically representative models.

4. Materials and Methods

4.1. Animals

All animal experiments were performed in 2.5–5-month-old male and female C57BL/6 mice. The protocols used were approved by the animal welfare committee of the Leiden Medical Centre and complied with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Mice were anesthetized with 4% isoflurane or intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg midazolam after subcutaneous injection of 0.15 mg/kg fentanyl. Next, heart procurement was performed as described in Kruithof et al., 2024 [13].

4.2. Ex Vivo Culture of Whole Mouse Hearts

Following heart procurement, whole mouse hearts were cultured for 3–7 days in MTCS as previously described in Kruithof et al., 2024 [13]. The MTCS is an ex vivo perfusion model that enables prolonged culture of whole mouse hearts. The flow rate ranged from 1000 to 2000 µL/min. For the dexamethasone-treated group, 100 nM dexamethasone was added to the perfusion medium, as performed in Kruithof et al., 2021 [14]. The medium was refreshed on day 2. To assess vascular leakage, some hearts were perfused with 1% Evans blue (Sigma, E2129–10G, St. Louis, MO, USA) at the end of culture. Finally, all hearts were fixed overnight with 4% PFA/PBS.

4.3. Histology

To prepare the hearts for immunofluorescence, fixed mouse hearts were dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned at 6 µm. The sections were deparaffinized with xylene, hydrated in a graded alcohol series, then boiled in Antigen Retrieval Buffer (10 mmol/L Tris [pH9]/1 mmol/L EDTA/0.05% Tween-20). After antigen retrieval, sections were blocked in 1% BSA in 0.1% Tween-PBS and then incubated overnight with primary antibodies directed against CD31 (R&D Systems, AF3628, Minneapolis, MN, USA), Laminin (Sigma-Aldrich, L9393, St. Louis, MO, USA), Collagen-IV (SouthernBiotech, 1340–01, Birmingham, AL, USA), VE-cadherin (Abcam, ab205335, Cambridge, United Kingdom), NF-κB-p65 (SAB signalway Antibody, 11260, Greenbelt, MD, USA). For the antibody directed against NF-κB-p65, the Tyramide Signal Amplification biotin detection kit (NEL700A001KT, Perkin Elmer, Shelton, CT, USA) was used according to the manufacturer's instructions, followed by incubation with Alexa Fluor 555-conjugated streptavidin (Invitrogen, S32355, Carlsbad, CA, USA). For the other antibodies, sections were incubated with Alexa-conjugated secondary antibodies. Finally, sections were incubated with DAPI (Thermo Fisher, 62248, Waltham, MA, USA) and mounted using ProlongGold Antifade reagent (Thermo Fisher, P36930). The slides were scanned with Zeiss Axio Scan 7 (Carl Zeiss Microscopy, Jena, Germany) or Pannoramic 250 slide scanner (version 1.23, 3DHISTECH Ltd., Budapest, Hungary) and analyzed using Zeiss ZEN 3.8 (Carl Zeiss Microscopy) or SlideViewer (version 2.9, 3DHISTECH Ltd.).

4.4. Quantifications

All quantifications were performed with QuPath [30] (version 0.6.0). Analyses were applied to the right ventricle. To quantify the fluorescent signals of CD31, VE-cadherin, and NF-κB-p65, the 'Classify > Pixel Classification > Create Thresholder' tool in QuPath was used. The positively stained CD31-, VE-cadherin-, or NF-κB-p65-positive area was normalized to the total myocardial area of the right ventricle, visualized using DAPI staining, and multiplied by 100, yielding a percentage of positive area. For analyzing capillary count, CD31-positive capillaries with a diameter of 2–10 µm were counted manually using the 'Point' tool in QuPath. Based on the total myocardial area of the right

ventricle, visualized using DAPI staining, the number of capillaries per 10^4 μm^2 was calculated for better interpretability.

4.5. Statistics

One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad (version 10.6.1) to determine significant differences between the non-cultured, untreated control mouse hearts cultured ex vivo for 7 days and dexamethasone-treated mouse hearts cultured ex vivo for 7 days. Data are reported as the mean ± SD, with $p < 0.05$ as the significance threshold.

5. Conclusions

Taken together, these findings show that dexamethasone attenuates perfusion-associated microvascular malformations and vascular leakage during prolonged ex vivo mouse-heart perfusion and support further investigation of pharmacological strategies to preserve the microvasculature during extended EVHP.

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Abbreviations

The following abbreviations are used in this manuscript:

DEX	Dexamethasone
EVHP	Ex vivo heart perfusion
MTCS	Miniature Tissue Culture System

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