Stem cell-mediated regeneration of the infarcted heart
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Improved wound healing and remodeling after cryoinjury compared to ligation induced myocardial infarction

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Submitted
Abstract

Objective Permanent coronary artery ligation and myocardial cryoinjury in mice are both frequently used as experimental animal models for acute myocardial infarction (AMI). Since the early pathophysiology of a specific experimental AMI model is critical for the interpretation of the eventual study outcome, we performed a detailed comparative study on the early (2-28 days) histological, morphological and functional changes associated with wound healing after ligation and cryoinjury.

Method Doppler-echocardiography was used to assess left ventricular dimensions, systolic function and cardiac output on days 4, 7 and 28 after ligation and cryoinjury. A 3-lead electrocardiogram was simultaneously recorded. Cardiomyocyte replacement, collagen deposition, inflammatory cell infiltration and neovascularization in the infarct area were determined on days 2, 4, 7, 14 and 28 by histology.

Results Ligation resulted in marked lumen dilatation, wall thinning, decreased systolic function and cardiac output, evident 4 days after AMI. In contrast, cryoinjury resulted in only modest lumen dilatation on day 28 post-AMI, and no decrease in systolic function. Both ligation and cryoinjury were associated with infarct-typical changes in ECG early after infarction, which were however more severe after ligation. Inflammatory cell infiltration and neovascularization were increased after cryoinjury compared to ligation. Scar formation was accelerated after cryoinjury compared to ligation.

Conclusion We show that wound healing and functional outcome following cryoinjury differs considerably from that following permanent coronary artery ligation. Cryoinjury results in an increased inflammatory response, accelerated wound healing, a different electrophysiological response, less pathological remodeling and a better functional outcome. These important pathophysiological differences should be considered when deciding on the use of a specific experimental AMI model.
Introduction
Acute myocardial infarction (AMI) is characterized by ischemic tissue damage followed by a wound healing response. This response involves many different aspects, such as inflammation, scar formation, tissue remodeling, changes in electrophysiology, and cardiac function, for which several murine models of AMI have been established over the past decades.

The ligation model, in which the left anterior descending (LAD) coronary artery is permanently ligated, is the most extensively used mouse model of experimental AMI [1,2]. The cryoinjury model, in which a frozen probe is applied directly to the left ventricular (LV) epicardial surface to inflict tissue damage, has also been used in many AMI studies. In particular, the cryoinjury model has been proposed and used as an adequate AMI model to test the effects of cardiac cell transplantation due the simplicity and reproducibility of the application of the injury [3-5].

The decision for the use of a specific murine AMI model should be considered and, more than technical aspects, the pathophysiology of the experimental model should be recognized for the interpretation of the study outcome. Since the ability for regeneration of myocardial tissue is limited, adequate wound healing is a critical step in the early pathophysiology after AMI. Myocardial wound healing starts by infiltration of inflammatory cells that promote wound healing by removal of necrotic cell debris as well as by production of growth factors and cytokines that modulate neovascularization [6]. Inflammatory cell infiltration is followed by scar formation by which prevents structural fragility. Furthermore, during these early wound healing responses tightly controlled architectural changes of the heart take place, which are a major determinant of the functional outcome after AMI [6,7].

Although fundamental for the use of experimental AMI models, a detailed comparative study on the early histological, morphological and functional changes associated with wound healing after ligation and cryoinjury is lacking. We hypothesize that, given the disparate type of injury induced by ligation or cryoinjury, the two insults exhibit differences in wound healing. Especially, since it has been shown that cryoinjury, in sharp contrast to ligation, does not lead to overt heart failure within 4 weeks [8,9].

Materials and Methods
Animals. Twelve-week-old male C57BL/6JOlaHsd mice (Harlan Nederland, Horst, The Netherlands) were housed individually in a room with conventional conditions. Mice received standard pelleted diet (RMH-B 10mm, Arie Blok, Woerden, The Netherlands) and water ad libitum. Drinking water was supplemented with 1mg/ml 5-bromo-2-deoxyuridin (BrdU, Sigma, St. Louis, USA) for 3 days prior to killing. All procedures performed on mice were approved by the local committee for care and use of laboratory animals and were performed according to strict governmental and international guidelines on animal experimentation.

Surgical procedures. Under general isoflurane (2.5 %) anesthesia mice (n = 14-20 per group per time point) were intubated and ventilated using a mechanical ventilator (Hugo Sachs Elektronik, March-Hugstetten, Germany). Buprenorfine (0.03 mg/kg) was given subcutaneously prior to surgery as analgetic. The heart was exposed through a left
lateral thoracotomy. Two models for myocardial infarction were used, a LAD ligation and a cryoinjury model: 1) For the LAD ligation model, the LAD coronary artery was localised using a dissecting microscope and permanently ligated with a non degradable 6-0 non-absorbable Prolene suture above branching of the LAD. Absence of blood flow was verified visually. 2) For the cryoinjury model, a round 3 mm diameter metal probe was cooled to −196°C with liquid nitrogen and applied directly to the LV free wall for 10 sec. After the frozen myocardium had thawed the procedure was repeated two times to the same area, as previously described [10]. Sham-operated control mice underwent the same procedure, except for induction of myocardial infarction. The intercostal space and skin were closed in layers. Mice received 100% oxygen until wakening after which the endotracheal tube was withdrawn. Animals were given 100% oxygen via nasal cone until recovery.

**Doppler-echocardiography.** Transthoracic Doppler-echocardiography was performed in sham-operated (n=5) and cryoinjury (n=8) and ligation (n=11) infarcted mice pre-operatively and 4, 7 and 28 days post-AMI using a digital cardiac ultrasound platform (SONOS 5500, B1 software package, Philips Medical Systems, The Netherlands) with a 15 MHz linear scanner (M- and B-Mode) and a 12 MHz short focal length-phased array transducer (Doppler). Mice were shaved and lightly anesthetized by isoflurane (mean 0.2% in oxygen) anesthesia via nasal cone and were allowed to breathe spontaneously. Mice were placed in a left decubital position on a feedback-controlled heating pad to maintain body temperature at 37°C. B-mode guided M-mode echocardiography was performed in the parasternal short-axis view at the midpapillary level. End-diastolic and end-systolic dimensions of the LV-cavity and thickness of the anterior and posterior walls were assessed, all measurements were performed according to the leading-edge method [11]. End-diastolic measurements were obtained at the peak of the R-wave of the ECG and end-systolic measurements were obtained at the time of minimal internal chamber dimensions. Fractional shortening (FS) as measure of LV systolic function, was calculated from the M-mode LV dimensions using the equation:

\[
FS(\%) = \left(\frac{LVEDD - LVESD}{LVEDD}\right) \times 100
\]

with LVEDD / LVESD as LV end-diastolic / end-systolic diameter. Ejection fraction (EF%) was calculated using the equation:

\[
EF(\%) = \left(\frac{LVEDV - LVESV}{LVEDV}\right) \times 100
\]

LV end-diastolic volume (EDV) and end-systolic (ESV) were calculated according to Teichholz et al.[12].

For determination of systolic outflow of the left ventricle, pulsed wave Doppler signals were obtained by placing the sample volume parallel to the flow in an modified apical view into the left ventricular outflow tract. Cardiac output was calculated as described before [13] and normalized for body weight. Mitral inflow was assessed by placing the sample volume into the left ventricle near the septum. After the final measurement, mice were killed by cervical dislocation and the hearts were rapidly removed. The wet weight of the heart and length of the left anterior tibia were measured.
**Electrocardiography.** A 3-lead surface ECG was recorded simultaneously to Doppler-echocardiography. P-wave duration, RR interval, PQ interval and QRS duration were measured. QRS was measured from the Q-wave onset to the return of the S-wave to the isoelectric line.

**Tissue preparation.** Two, 4, 7, 14 and 28 days post-AMI mice were killed by cervical dislocation and the hearts were excised immediately. Hearts were snap-frozen in liquid nitrogen and stored at -80 °C (n = 4 per group per time point), or fixed in 2% glutaraldehyde (GA, MERCK, Darmstadt, Germany) for at least 24 h (n = 3 per group per time point), or fixed in 2% paraformaldehyde (PF, MERCK, Darmstadt, Germany) for 3 hours (n = 2 per group per time point). Hearts fixed in GA were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) and hearts fixed in PF were embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer’s instructions.

**Histology.** Sections (2 µm) of the ventricles of T-7100 embedded hearts, cut transversally in the middle third between base and apex, were stained with toluidin blue (Fluka Chemie, Buchs, Switzerland) and mounted in Permount (Fisher, New Jersey, USA). Masson’s trichrome, which stains collagen blue and myocardial cells red, was performed according to standard protocol on cryo sections of 6-8 mm, fixated in 4% formaldehyde for 1 h at room temperature, followed by Bouin fixative at 60 °C for 5 min.

**Immunohistochemistry.** Cryosections (5 µm) from snap-frozen hearts, cut transversally in the middle third between base and apex, were stained by immunoperoxidase staining for neutrophils (monoclonal rat anti-mouse neutrophils, Serotec Ltd, Oxford, UK), macrophages (monoclonal rat anti-mouse F4/80, Serotec Ltd, Oxford, UK), T-lymphocytes (polyclonal hamster anti-mouse CD3ε, BD Pharmingen, San Diego, USA), as previously described [10]. Sections of the T-8100 embedded hearts were stained by immunoperoxidase for BrdU (monoclonal mouse anti-BrdU, Sigma, St Louis, USA). Sections were pretreated with 0.1 % trypsin in 0.1 M Tris buffer (pH 7.8), containing 0.1% CaCl$_2$ at 37 °C for 15 min. DNA was denatured by incubation with 1 M HCl at 60 °C for 30 min, followed by incubation with 0.05 % pepsin (Roche Diagnostics, Mannheim, Germany) in 0.35 M HCl at 37 °C for 15 min. Colour development was performed with 3.3-diaminobenzidine-tetrahydrochloride (DAB; Sigma, St. Louis, USA) and sections were counterstained using toluidin blue.

**Quantitative Analysis.** All evaluations were performed by at least two investigators independently on blinded samples. The initial infarct size was quantified in 1.25x microscopic images of Masson’s Trichrome stained sections of hearts 2 days after infarction using computerized planimetry (Leica Qwin version 4.0, Leica Microsystems, USA). Inflammatory cells were quantified by counting the number of red stained cells (AEC) with blue stained nuclei (hematoxilin) in digital micrographs (x400) taken equally distributed over six areas in the infarct area. Small (1 endothelial cell nucleus), middle-sized (2-6 endothelial cell nuclei) and large (>6 endothelial cell nuclei) blood vessels were quantified on toluidine blue stained T-7100 sections by counting their number in photographs (x400) taken equally distributed over six areas at the epicardial site of the infarction. Average numbers were calculated and expressed as number per mm$^2$. 
Statistical analyses. Data are expressed as mean ±SEM. The data were analyzed using statistical software (GraphPad Prism, version 3.00, GraphPad Software Inc.). Inter-observer agreement was evaluated by paired samples t-testing. The statistical significance of differences in the findings was evaluated by a one-way ANOVA followed by posthoc Tuckey’s or Dunett’s test, as appropriate. A difference of P<0.05 was considered statistically significant.

Results
Survival rate, infarct area and heart weight. All mice (100%) subjected to either cryoinjury or sham-operation survived the operation and the intended study period and had normal postoperative recovery. Survival in the ligation group was lower. Sixty-nine mice were subjected to ligation, of which 52 (75%) survived. Representative cross-sections of the ventricles of ligation and cryoinjury infarcted hearts 2 and 28 days post-AMI are shown in figure 1.
Ligation and cryoinjury resulted in infarcts of similar size, resp. 34 ± 9 % and 29 ± 1 % of the LV area at day 2 post-MI. Coronary artery ligation resulted in a transmural infarct localized to the LV free wall and apex. Cryoinjury infarcts were non-transmural and located at the LV free wall. The apex was not involved.
Body weights (± 27.5 g) and tibial lengths (± 18.4 mm) remained similar in all groups during the time period investigated. On day 28 the weight of the ligation infarcted hearts was significantly increased compared to both sham-operated and cryoinjury infarcted mice (0.252 ± 0.024 lig. vs. 0.133 ± 0.012 sham and 0.126 ± 0.004 cryo.; P<0.001).

Figure 1. Representative cross-sections of the ventricles infarcted hearts stained by Masson’s trichrome staining 2 (A) and 28 (B) days after ligation and 2 (C) and 28 (D) days after cryoinjury. The extent of the infarct is indicated by arrows.
Doppler-echocardiography. The heart rate was similar in all groups at all time points (± 580 bpm) during echocardiography. Data are shown in Figure 2. All pre-operative baseline data were similar between groups and did not change in time in the sham-operated mice. Ligation led to marked LV inner-diameter dilatation early post-AMI. The LV end-diastolic lumen area (LVDA, Fig. 2A), the end-diastolic diameter (LVEDD, Fig. 2B) and the end-systolic diameter (LVESD, Fig. 2C) of ligation infarcted mice were significantly increased post-AMI, and further lumen dilatation occurred after day 4 (LVDA, P < 0.001; LVEDD, P < 0.05; LVESD P < 0.001). After cryoinjury, no changes were found in LVDA. On day 28, the LVEDD and LVESD were significantly increased compared to the pre-operative baseline data (pre-op. cryo vs cryo. day 28 LVEDD and LVESD P < 0.01), indicating less pronounced and later lumen dilatation occurring after cryoinjury compared to ligation.

Figure 2. Doppler-echocardiography in sham-operated and cryoinjury and ligation infarcted mice pre-operative and 4, 7, and 28 days post-MI. A: Left ventricular end-diastolic lumen area (LVDA). B: LV end-diastolic diameter (LVEDD). C: LV end-systolic diameter (LVESD). D: Fractional shortening (FS). E: Ejection fraction. F: Cardiac output (CO). sham, ligation, cryoinjury. * P < 0.05; ** P < 0.01; *** P < 0.001 vs. sham. Bars represent mean ± SEM.

After ligation, the LV posterior wall thickness (LVPW) was significantly decreased in diastole, during the period investigated (P < 0.001), whereas after cryoinjury no changes were found (data not shown). No changes were found in both models in LVPW in systole, intraventricular septal thickness, LV length and E/A ratio (data not shown).

FS and EF, as indicators for systolic pump function, were significantly reduced after ligation during the entire time period investigated (FS and EF, P<0.001). After cryoinjury no changes in FS or EF were found during the time period investigated. Therefore, in contrast to ligation, cryoinjury did not cause reduction of LV pump function within 4 weeks post-AMI.

In both models, cardiac output (CO) did not significantly change in time compared to the pre-operative baseline data of the particular group. However, on day 7 CO was
significantly reduced in ligation infarcted mice compared to both sham-operated and
cryoinjury infarcted mice (P< 0.05), and on day 28 to sham-operated mice only (P<
0.05).

Electrocardiography. Electrocardiographic measurements are summarized in table 1.

Table 1

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<td>11 ± 0.4</td>
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<td>28 d</td>
<td>10 ± 0.3</td>
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*a* n, group size; *P*, P-wave duration; RR, RR interval; PQ, PQ interval; QRS, QRS duration

b* P < 0.05 vs. cryoinjury; d* P < 0.001 vs. sham and cryoinjury
Representative ECGs from ligation and cryoinjury infarcted mice pre-operative and 4, 7 and 28 days post-AMI are shown in fig. 3.

Figure 3. Representative lead I surface ECGs from ligation (left) and cryoinjury (right) infarcted mice pre-operative and 4, 7 and 28 days post-MI. The P-waves, QRS, and T-waves are indicated.

Sham-operated mice showed normal ECGs at all time points investigated, comparable to the pre-operative ECGs of ligation and cryoinjury infarcted mice, with no changes in the measured intervals. All mice subjected to ligation, exhibited a strikingly pathological and infarct-typical ECG, which persisted over the entire study period. The ECG manifestations of ligation infarcted mice included presence of a pathological Q, diminished R wave amplitude, and an inverted T-wave. The QRS interval was significantly prolonged compared to cryoinjury on day 7 and 28 compared to cryoinjury infarcted mice (P < 0.05 and 0.001 resp.) and compared to sham-operated mice on day 28 (P < 0.001). No significant differences were observed in ligation infarcted mice in P-wave duration, RR interval, PQ interval at any time point post-AMI studied.
The ECG of cryoinjury infarcted mice showed a pathological Q, and a diminished R-wave amplitude, and an inverted T-wave similar to ligation. However, the ECGs of 4 out of 8 cryoinjury infarcted mice showed a fragmented QRS, indicating a possible bundle branch block. No significant differences were observed in cryoinjury infarcted mice in P-wave duration, RR interval, PQ interval and QRS duration at any time point post-AMI studied.

Cardiomyocyte replacement and collagen deposition. In both models there was a rim of non-injured cardiomyocytes in the subendocardium, which remained present at all time points investigated, likely due to the close proximity of the oxygenated blood in the ventricle (Fig. 4C, D, E, G and H). The infarct areas in both models demonstrated the presence cardiomyocyte remnants on day 2 post-AMI (fig. 4A and E). The cardiomyocyte remnants in the ligation infarcts had an aligned appearance, whereas they were fragmented in the cryoinjury infarcts.

**Figure 4.** Progression of the wound healing process in cross-sections of ligation (A-D) and cryoinjury infarcts (E-H) stained by Masson’s trichrome (blue, collagen; red, myocardial cells) on day 2 (A, E), 4 (B,F ), 7 (C, G) and 28 (D, H) post-MI. In the ligation infarcts, cardiomyocyte remnants have an aligned appearance on day 2 post-MI (A) and are still present on day 4 post-MI (B). In the cryoinjury infarcts, the cardiomyocyte remnants are fragmented on day 2 (E). On day 4, the cryoinjury infarcts demonstrate that the cardiomyocytes are replaced by high numbers of inflammatory cells and collagen (F). On day 7, both ligation and cryoinjury infarcts show collagen deposition, but the amount of collagen per area is increased in cryoinjury infarcts (G) compared to ligation infarcts (C). On day 7 and 28, progressive thinning of the left ventricular wall is visible in ligation infarcts (C, D), but not in cryoinjury infarcts (G, H). Furthermore large vascular structures (asteriks) and collagen are observed in the infarcts in both models. Original magnification: A-H x200; insets in B and F x1000.
In ligation infarcts aligned cardiomyocyte remnants were still present on day 4 (fig. 4B). In contrast, 4-days-old cryoinjury infarcts demonstrated a complete clearance of cardiomyocyte remnants and higher inflammatory cell density compared to ligation infarcts (fig. 4F), demonstrating a faster progression of cardiomyocyte replacement by granulation tissue, as compared to ligation infarcts. Furthermore, collagen deposition was hardly observed in the ligation infarcts on day 4, whereas deposited collagen fibrils, were present throughout the cryoinjury infarcts. On day 7, in both ligation and cryoinjury infarcts parallel-organized collagen fibrils were present, however, collagen deposition was higher in cryoinjury compared to ligation infarcts (fig. 4C and G). On day 28, both infarcts appeared as scar tissue, containing parallel-organized collagen fibers and large blood vessels (fig. 4D and H).

**Inflammatory cell infiltration.**

![Inflammatory cell infiltration graphs](image)

**Figure 5.** Inflammatory cell density in ligation and cryoinjury infarcts. A and B: Neutrophil density. C and D: Macrophage density. E and F: T-lymphocyte density. The number of neutrophils is higher in the cryoinjury infarcts compared to ligation infarcts on day 2 and 4. The number of macrophages is higher in cryoinjury infarcts 2 and 7 days post-MI as compared to ligation infarcts. The cryoinjury infarcts show an increase in numbers of T-lymphocytes as compared to ligation infarcts until 14 days post-MI. *P < 0.05; **P < 0.01; ***P < 0.001. Bars represent mean ± SEM.
Infiltrating cells were hardly observed in hearts of sham-operated mice. Infarcts in both models demonstrated rapid infiltration with inflammatory cells, albeit in different quantities. In both models, neutrophils were only present during the first week post-AMI, with peak numbers at days 2 and 4 (fig. 5A and B). The number of neutrophils was significantly higher in the cryoinjury infarcts at these time points (cryo. vs. lig. day 2 P < 0.05, day 4 P < 0.01), but decreased rapidly in cryoinjury infarcts after day 4. Ligation infarcts demonstrated a more gradual decline, with higher neutrophil numbers on day 7 compared to cryoinjury (P < 0.001).

Macrophage numbers gradually increased in both infarcts, with peak numbers on days 4 and 7 (fig. 5C and D). The number of macrophages was significantly higher in cryoinjury infarcts 2 and 7 days post-AMI as compared to ligation infarcts (cryo. vs. lig. day 2 P < 0.01, day 7 P < 0.001). After the first week, macrophages remained present in similar numbers in both types of infarct.

T-lymphocytes were only sporadically present in ligation infarcts in numbers comparable to controls (fig. 4E). The cryoinjury infarcts showed significantly increased numbers of T-lymphocytes (fig. 4F), which were observed as cell clusters, compared to ligation infarcts until 14 days post-AMI (cryo. vs. lig. day 2, 4, 14 P < 0.01, day 7 P < 0.001). The number of T-lymphocytes present were low compared to the numbers of neutrophils and macrophages on the same time points, i.e. 10 fold lower than macrophages on day 7. Mast cells were only sporadically observed in both types of infarcts and control myocardium (not shown).

**Vascularization.** Vascularization of the infarct area was determined by quantitative analysis of small, medium-sized and large blood vessels, defined respectively as containing 1, 2 to 6, or more than 6 endothelial cell nuclei. Both models demonstrated a significant decrease in small vessel numbers compared to sham-operated mice 2 days post-AMI (lig. P < 0.001 en cryo. P < 0.01 vs. sham; fig. 6A, B and 7A-C). However, despite the decrease, the number of small vessels was significantly higher in cryoinjury infarcts compared to ligation infarcts 2 and 7 days post-AMI (cryo. vs. lig. day 2 P < 0.01, day 7 P < 0.01). After the first week, small vessels were only sporadically observed in both types of infarcts. The number of medium-sized vessels was increased in both models compared to the number in sham-operated mice on day 4 (P < 0.01) (fig. 6C and D). After day 4, the number remained constant in ligation infarcts. The number further increased in cryoinjury infarcts (day 7 vs. day 4 P < 0.001), and was significantly increased compared to ligation after day 7 until day 28 (cryo vs. lig day 7 and 14 P < 0.001, day 28 P < 0.05).

The number of large vessels in ligation infarcts remained comparable to the number in sham-operated mice, whereas the number was significantly increased in cryoinjury infarcts as of day 7 (cryo day 7 vs. control P < 0.05) (fig. 6E and F).

Beside the increase in vessels containing multiple endothelial cell nuclei, the presence of BrdU-positive endothelial cells in vessels confirmed that the increase in middle and, in the cryoinjury, large vessels was due to vessel growth. Presence of erythrocytes in the vessels indicated that the vessels were functional (fig. 7D).
Figure 6. Blood vessel density in ligation and cryoinjury infarcts. A and B: Number of small vessels (1 endothelial cell nucleus). C and D: Number of medium-sized vessels (2-6 endothelial cell nuclei). E and F: Number of large vessels (>6 endothelial cell nuclei). Both models demonstrate a marked decrease in small vessel numbers post-MI. The number of small vessels is higher in cryoinjury infarcts compared to ligation infarcts on day 2 and 7 post-MI. The number of medium-sized vessels increased in both types of infarct. On day 7 the number of medium-sized vessels further increases in cryoinjury infarcts together with the number of large vessels compared to ligation. *P < 0.05; ** P < 0.01. Bars represent mean ± SEM.
Figure 7. Degree of vascularization in healthy and infarcted myocardium. A: Healthy myocardium showing presence of numerous small vessels (arrowheads). B and C: Ligation infarct (B) and cryoinjury infarct (C) showing decrease in small vessels on day 2 post-MI compared to small vessels in sham-operated hearts. The number of small vessels is higher in cryoinjury infarcts as compared to ligation infarcts. D: BrdU staining (brown) in a myocardial cryoinjury infarct showing presence of BrdU-positive endothelial cells (arrows) 7 days post-MI. Brown colored cells in the vessel lumen are erythrocytes. Magnification x1000.

Discussion

In order to make a well-considered decision for the use of a specific experimental AMI model, a thorough understanding of the pathophysiology of different experimental models for AMI is critical. In this comparative study between ligation and cryoinjury induced AMI we show, 1) that the inflammatory response is increased after cryoinjury, 2) that wound healing is accelerated after cryoinjury, 3) that the electrophysiological response differs between both models, and 4) that cryoinjury infarcted mice have only modest LV remodeling and a noticeable better functional outcome than ligation infarcted mice. Furthermore, the differences between both models are evident early after MI.

The inflammatory response is essential for adequate wound healing after AMI. Even though the infarcts in both models were initially of similar size, inflammatory cells, mostly neutrophils and macrophages which have strong phagocytic capacity, were increased in cryoinjury compared to ligation infarcts. Therefore, rapid clearance of cardiomyocyte remnants in the cryoinjury infarcts is probably related to the observed increased infiltration of inflammatory cells.

Furthermore, the increased number of macrophages will have contributed to the increased neovascularization and accelerated scar formation after cryoinjury by increased cytokine and growth factor secretion [14].
Unlike in ligation infarcts, T-lymphocytes infiltrated into the cryoinjury infarcts. However, as T-lymphocyte numbers were low compared to the numbers of neutrophils and macrophages, their contribution to the accelerated wound healing might have been insignificant. Although the vascularization remained increased in the cryoinjury infarcts, the wound healing of ligation infarcts eventually caught up with the healing of cryoinjury infarcts, in that they both were converted to scars on day 14 post-AMI. Nevertheless, major differences were found between both models in cardiac remodeling and function, which were evident early after AMI and remained present during the entire time period investigated. Ligation resulted in marked LV remodeling, as indicated by lumen dilatation and wall thinning, and decreased systolic function and cardiac output early after AMI. In contrast, cryoinjury resulted in only modest LV remodeling, indicated by lumen dilatation on day 28 post-AMI, and no decrease in systolic function. The accelerated wound healing response of cryoinjury compared to ligation infarcts described in this study, could shorten the period of susceptibility to pathological myocardial remodeling. The more rapid collagen production may have prevented structural fragility, and thereby preserved cardiac function. The difference in neovascularization after cryoinjury and ligation could be another cause for the difference in LV lumen dilatation and wall thinning, as neovascularization has been shown to reduce left ventricular remodeling and deterioration to heart failure [15].

It has also been postulated by others, studying reperfusion of ischemic myocardium or G-CSF treatment after infarction [16-18], that acceleration of the healing process limits pathological remodeling. Recently, we have shown that reperfusion after ligation leads to an increased inflammatory response and neovascularization and accelerated wound healing together with attenuated pathological remodeling compared to permanent ligation, which is similar to the present study [19].

Besides the accelerated wound healing response, also the non-transmural feature of cryoinjury, as cryoinjury infarcts are mostly epicardial leaving a border of contractile myocardium on the endocardial site, may partly account for the modest remodeling and better functional outcome. In order to create transmural injury we attempted longer application of the cryoprobe. However, this created a lesion extending to the right ventricle and left atrium and not to the endocardial site. Probably the warm blood in the ventricle unfreezes the probe before transmural tissue damage can be created. Nevertheless, also in a study were transmural cryoinjury infarcts were induced, using a cryoprobe with continuous freezing capability, no overt heart failure was observed within 4 weeks [8].

To the best of our knowledge, this study is the first to describe ECG changes after cryoinjury. We show that cryoinjury is associated with changes in ECG early after infarction, which are, at least to some extent, comparable to conduction disturbances observed after ligation. However, the ECGs of cryoinjury infarcted mice often showed signs of a bundle branch block. This might be caused by direct cryoinjury of the left bundle during the application of cryoinjury to the myocardium. Nevertheless, no differences in ECG parameters were found, indicating no severe ventricular conduction disturbances. In contrast, the QRS duration in ligation infarcted mice was significantly prolonged compared to cryoinjury infarcted mice, indicating more pronounced ventricular conduction disturbances.

Many studies have suggested the cryoinjury model as an adequate model to study cardiac cell transplantation, as the reproducibility of the injury facilitates the association of the transplanted cells with the infarcted versus the non-infarcted areas [3-5]. However, the specific pathophysiological response following cryoinjury, as presented in this study, should be considered when interpreting the study outcome. The high number of inflammatory cells present may influence the effect of cell therapy by e.g. increased phagocytotic...
capability or growth factor and cytokine production. Furthermore, timing of an experimental intervention should be considered due to the accelerated wound healing after cryoinjury. The decision for a specific murine AMI model to study potential therapeutic interventions should be based on similarities between the pathophysiology of the experimental model and the human pathophysiology following AMI. Although Dewald et al. showed that there are species-specific differences between mice and larger animals as such [20], it is to be expected that the pathohistological events after ligation share more characteristics that are common with humans, as ischemic myocardial cell death has more clinical relevance. We therefore suggest that the ligation model may be preferable over cryoinjury as an experimental AMI model for interventional studies. Nevertheless, the cryoinjury model might be a useful model to provide therapeutic clues for interventions aimed at improving wound healing after infarction, like the identification of cytokines and growth factors involved in the accelerated wound healing.

In conclusion, we show that the pathohistological and pathophysiological response following cryoinjury differ considerably from that following permanent coronary artery ligation. These important pathophysiological differences should be considered when deciding on the use of a specific experimental AMI model.

References

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