Journal of Biomedical Optics 9(4), 779–787 (July/August 2004)

Detection of myocardial cell damage in isolated rat hearts with near-infrared spectroscopy

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Abstract. One hallmark of cell death resulting from prolonged ischemia is cell membrane disruption. We apply optical spectroscopy to gauge membrane disruption in isolated rat hearts by monitoring (1) the washout of myoglobin (Mb) and (2) the accumulation of an exogenous contrast agent in permeabilized cells. The contrast agent, a neodymium (Nd) chelate, has several absorptions in the visible and near-IR, and when present in the perfusate, it cannot penetrate cellular membranes. When membrane integrity is disrupted, however, it is expected to accumulate within the intracellular space; moreover, cellular Mb is expected to wash out. To test this hypothesis, rat hearts ($n=12$) are perfused with Krebs-Henseleit buffer (KHB), followed by perfusion with KHB in which a 5 mM Nd-DTPA solution is present. Membrane damage is then induced by infusion of digitonin into the Nd-KHB perfusate to provide a digitonin concentration of 2.5, 5, or 10 μg/mL. After 30 min of infusion, Mb levels fall to $46\pm14\%$ of baseline levels and Nd-DTPA rises to $161\pm19\%$ of predigitonin levels. No apparent dependence of total membrane disruption on digitonin concentration over the concentration range studied is found, although higher concentrations do lead to more rapid membrane disruption.
[DOI: 10.1117/1.1751125]

Keywords: optical spectroscopy; neodymium; myoglobin; isolated heart; digitonin; membrane disruption.

Paper 03090 received July 7, 2003; revised manuscript received November 14, 2003; accepted for publication December 19, 2003.

1 Introduction

Coronary artery disease (CAD) is one of the leading causes of death in industrialized countries, resulting in 26% of all deaths in Canada in 1998. It is characterized by restricted blood flow to the heart tissue (ischemia) and hence reduced oxygen supply that, left untreated, leads ultimately to cardiac cell death. One of the hallmarks of ischemia-induced cell death is disruption of the cellular membranes. Noninvasive methods to detect the membrane disruption characteristic of myocardial infarctions (heart attacks) include proton magnetic resonance imaging with gadolinium or dysprosium chelates[1,2] (e.g. Gd-DTPA) and detection of released soluble proteins[3] (e.g., lactate dehydrogenase, creatine kinase, troponin, and myoglobin). While these techniques are effective for noninvasive, *in vivo* diagnosis of myocardial injury, there is also a need for sensitive monitors of membrane disruption in exposed hearts (both in the operating theater and in research studies) that these techniques do not meet. The approach that we introduce here to fill this niche is based on optical spectroscopy. Visible/near-infrared (IR) spectroscopy has proven to be an effective measure of blood and tissue oxygenation, which serve as indicators of hypoxia and ischemia,[4,5] but oxygenation values provide no direct information regarding membrane integrity. In this paper, we propose two ways in which visible/near-IR spectroscopy can be applied to the characterization of cell membrane disruption in cardiac tissue.

The first approach capitalizes on the optical absorptions of myoglobin (Mb), a heme protein similar in structure to hemoglobin, but normally restricted to the cytoplasm within muscle cells. Although the extent of its role in oxygen transport under normal conditions is unclear,[6] it binds oxygen efficiently and acts as an oxygen buffer when the blood oxygen supply is restricted.[7] While Mb cannot normally pass through the cell membrane, when membranes are disrupted in the presence of perfusion, it drains into the vascular space and washes out of the tissue, reducing the overall tissue Mb concentration.[3] Since it has strong absorptions in the visible spectrum, Mb washout may be monitored by visible absorption spectroscopy as an indicator of reduced membrane integrity. Since the visible and near-IR absorptions of Mb are very difficult to differentiate from those of hemoglobin (Hb), the presence of Hb may impede observation of Mb washout. To monitor Mb most accurately, then, a Hb-free blood substitute (Krebs-Henseleit buffer) was used in this study to perfuse the heart.

The second approach presented here to gauge membrane disruption exploits the near-IR absorptions of neodymium. Neodymium (Nd) is a lanthanide metal in the same row of the periodic table as gadolinium and dysprosium, both of which have been used as contrast agents in magnetic resonance imaging experiments,[8,9] and it exhibits several distinct, narrow

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absorptions in the visible and near-IR spectral regions.[10,11] When bound to a chelating agent such as diethylene-triamine-pentaacetic acid (DTPA) or triethylene-tetraamine-hexaacetic acid (TTHA), it becomes nontoxic with only minimal alteration to its absorption spectrum. Since it cannot pass through the cell membrane under normal conditions, the Nd complex administered intravascularly is confined to the vascular and interstitial spaces in cardiac tissue. However, membrane disruption would be expected to lead to penetration of the complex into the intracellular space. In that event, the overall tissue concentration of the Nd complex would increase due to an increase in its distribution volume, and this concentration increase should be detectable spectroscopically.

Isolated rat hearts were employed in this study as a model to explore the feasibility of using Mb and Nd as markers of membrane damage. The hearts were perfused with Krebs-Henseleit buffer to which a Nd chelate had been added at low concentration, and membrane disruption was achieved by infusion of digitonin into the perfusate (digitonin has been shown to disrupt cardiac cellular membranes under normoxic conditions effectively without significantly altering cellular morphology[12]). Changes in Nd and Mb tissue concentrations were monitored by visible/near-IR spectroscopy of the cardiac tissue. After 30 min of digitionin infusion, Mb band intensities typically fell to approximately half their original values, while Nd-chelate band intensities rose by 60% over their initial values.

2 Materials and Methods

2.1 *Surgery and Perfusion*

Sprague-Dawley rats ($n=12$) weighing 300 to 400 g were anesthetized with pentobarbital [120 mg/kg, intraperitoneal (ip)], leading to a rapid onset of anesthesia. When the animal was unresponsive to the toe pinch test, the chest cavity was opened and the heart was removed. The heart was then attached to a Langendorff perfusion setup and perfused through the aorta with phosphate-free Krebs-Henseleit buffer (KHB), which contained (in mM): NaCl (118), $NaHCO_3$ (25), KCl (4.7), $CaCl_2$ (1.75), $MgSO_4$ (1.2), EDTA-2Na (0.5), and glucose (11). The perfusate was bubbled with 95% O_2/5% CO_2 to maintain pO_2 at 500 to 600 mm Hg and pH at 7.35 to 7.45.

Once the heart had been removed and suspended, a latex balloon was placed in the left ventricular (LV) cavity and connected to a pressure transducer, to assess mechanical function. After cannulation and initiation of cardiac perfusion by peristaltic pump (constant flow), the LV balloon was filled gradually with water to reach optimal mechanical parameters. Left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressure, heart rate (HR) and perfusion pressure (PP) were monitored as indicators of cardiac function. Perfusion pressure in the Langendorff model is the aortic pressure, and when divided by coronary flow, serves as an indicator of coronary resistance. The pressure-rate product (PRP), defined as (LVSP-LVEDP)$\times$HR, provided a useful measure of cardiac performance.

In addition to the Krebs solution already described (KHB), a solution of KHB with neodymium (Nd-KHB) was also prepared. Low-sodium KHB was mixed with 100-mM stock solutions of Nd-DTPA ($n=5$) or Nd-TTHA ($n=4$) to bring the overall Nd concentration to 5 mM. These stock solutions were prepared by mixing equimolar amounts of $NdCl_3$ and DTPA or TTHA (free acids) in water, and titrating the solution with NaOH to bring the pH to 7.4. The presence of NaCl in the stock solutions made it necessary to use low-sodium KHB to maintain normal Na^+ levels in the mixed perfusate. TTHA was initially used as a chelate. Since TTHA also binds some Ca^{2+}, $CaCl_2$ was added to maintain the free $[Ca^{2+}]$ at 1 mM, the level normally present in KHB. DTPA, however, did not bind sufficient calcium in the low concentrations used here to warrant addition of $CaCl_2$, so DTPA was used in later experiments.

2.2 *Experimental Protocol*

Hearts ($n=12$) were initially perfused with normal KHB to ensure stable cardiac function. After this equilibration period, perfusion was switched to the Nd-KHB solution ($n=9$). Once tissue Nd concentrations stabilized (10 min), digitonin was infused into the Nd-KHB perfusate at a steady flow rate to provide a perfusate digitonin concentration of 2.5 ($n=2$), 5 ($n=3$), or 10 ($n=4$) μg/mL. Infusion of digitonin was maintained for a minimum of 30 min, inducing significant membrane damage at all three digitonin concentrations. After halting digitonin infusion, perfusion with Nd-KHB was continued for an additional 5 min. Finally, the hearts were again perfused with Nd-free KHB for approximately 10 min to observe washout of Nd from the tissue.

The three other hearts were perfused with the Nd-free Krebs throughout the entire experiment to assess the influence of the Nd chelate (if any) on digitonin-induced Mb washout. In these experiments, digitonin was infused into the KHB after the equilibration period, at a concentration of 10 μg/mL. After 30 min, digitonin infusion was stopped and perfusion with normal KHB continued for 10 min.

2.3 *Spectroscopy*

Broadband visible/near-IR light from a fiber optic illuminator (Oriel Model 77501, Stratford, Connecticut) was transmitted to the heart through one arm of a bifurcated fiber optic bundle. The common illumination/collection probe tip was placed in gentle contact with the left ventricle of the heart, where scattered light was gathered and transmitted to a visible/near-IR spectrometer (Control Development, Inc., South Bend, Indiana). The emitting and collecting fiber optic filaments were arranged in two concentric rings at the probe tip (collecting filaments formed the inner ring and the radial spacing between the rings was 2 mm). Spectra were acquired continuously (0.75 s acquisition time) throughout the experimental protocol (400 to 1100 nm, 0.5-nm spacing). Blocks of 20 spectra were averaged to yield one spectrum every 15 s. Cardiac functional data were recorded every minute throughout the protocol.

2.4 *Spectral Analysis*

Changes in Nd and Mb concentrations and water content were monitored spectroscopically as measures of cell membrane disruption. The concentrations were determined according to the modified Beer's law relationship, $A(\lambda)=\Sigma_i \varepsilon_i(\lambda) c_i L_{\text{eff}}$, where $A(\lambda)$ is the pseudoabsorbance $(-\log I/I_0)$ at wavelength λ, $\varepsilon_i(\lambda)$ is the molar absorptivity of component i at wavelength λ, and L_{eff} is the effective photon pathlength

Table 1 Effect of digitonin on contractility and coronary resistance.

	Baseline Perfusion	After Addition of Nd Chelate	After 30 min Digitonin Infusion at Concentration Indicated (μg/mL)		
			2.5	5	10
	(n=9)	(n=9)	(n=2)	(n=3)	(n=4)
PRP (%)	100	107±25	14.5±2.1	18±1.5	7±10*
LVEDP (mm Hg)	7.5±2	8.8±5.3	77±1	56±8	77±29
PP (mm Hg)	68±5.5	78±4.5	117±2	127±20	123±10
PP/CF	4.3±0.63	5.0±0.58	10.0±0.5	12.3±3.9	13.7±1.0
		Controls for Which No Nd was Added to Perfusate (n=3)			
PRP (%)	100	—	—	—	12±17
LVEDP	6.6±0.5	—	—	—	76±2
PP	66±4.4	—	—	—	114±14
PP/CF	4.1±0.3	—	—	—	9.5±1.9

* For the 10-μg/mL group, two of the four hearts had stopped beating at the end of the digitonin treatment.

LVSP=left ventricular systolic pressure, PP=perfusion (aortic) pressure, LVEDP=left ventricular end-diastolic pressure, HR=heart rate, PRP=pressure-rate product =(LVSP-LVEDP)×HR, CF=coronary flow, PP/CF serves as an indicator of coronary resistance.

Additional baseline values (n=12): CF=15.4±1.5 mL/min, HR=230±32 bpm, LVSP=102±19 mm Hg.

through tissue, an unknown quantity governed by the physical separation of emitting and detecting optical fibres and by the tissue scattering properties.[13] To estimate the relative concentrations of the various chromophores, the tissue spectra were fit with pure component spectra measured in our lab (Nd chelates) or taken from the literature[14–16] (Mb, cytochromes, water) using a least-squares spectral fitting algorithm described previously.[17]

Although the effective path length is somewhat wavelength dependent, we have assumed it to be constant across the wavelength range of interest and absorbed it into the fitting coefficients. To minimize the impact of the wavelength dependence of the effective pathlength, each spectral fitting was performed over a limited spectral region. To derive the total Mb levels, the acquired spectra were fit over the region between 510 and 620 nm with absorptivity spectra of oxygenated Mb (MbO_2), deoxygenated Mb (dMb), and the oxidized and reduced forms of the cytochromes. Since Nd and water do not contribute substantially to the observed spectra in this region, they were not included in this analysis. To derive the Nd levels, spectra were fit over the region between 720 and 820 nm (encompassing both of the two strongest Nd absorbance bands) with the absorptivity spectra of Nd, dMb, MbO_2, and water. Cytochromes were excluded because they have no substantial absorptions in the near IR.

2.5 *Lactate Dehydrogenase Release*

In addition to Mb washout from the cells and Nd accumulation within them, we anticipated that the increased cell permeability would lead to efflux of the enzyme lactate dehydrogenase (LDH) from the cellular space into the perfusate. Release of the intracellular enzymes such as LDH and creatine kinase is widely used in cardiovascular research as an index of cellular damage.[18] Perfusate samples were taken every 10 min and LDH release into the perfusate was quantified for each sample, since LDH catalyzes oxidation of NADH. The decrease in absorption at 340 nm ($\varepsilon = 6.22\ \mathrm{mM}^{-1}\,\mathrm{cm}^{-1}$) was measured using Sigma assay kit DG1340-K at 30 °C and pH 7.5 in Beckman DU650 spectrophotometer. The total activity of LDH released (in the international units, IU=1 μmol NADH/min) was calculated as a product of LDH activity (international units per liter of perfusate), coronary flow (liters per minute), and time intervals between sampling (10 min).

2.6 *Statistical Analysis*

All statistical tests for significance were performed using Student's t test. For tests between groups of trials with different digitonin concentrations, the unpaired, one-tailed test was applied. For tests of significance between different time points within the same group of trials, the paired, one-tailed t test was applied. Differences were deemed to be significant if the resulting p value was below 0.05.

3 Results

3.1 *Cardiac Functional Data*

Addition of Nd to the perfusate did not significantly affect measures of cardiac function. This is consistent with the low toxicity demonstrated for another lanthanide complex, dysprosium-TTHA, which was used as a shift reagent in ^{23}Na-NMR studies (5 to 10-mM concentrations) of isolated rat hearts.[19,20] The transition from baseline perfusion to perfusion with Nd-KHB did not change the pressure-rate product

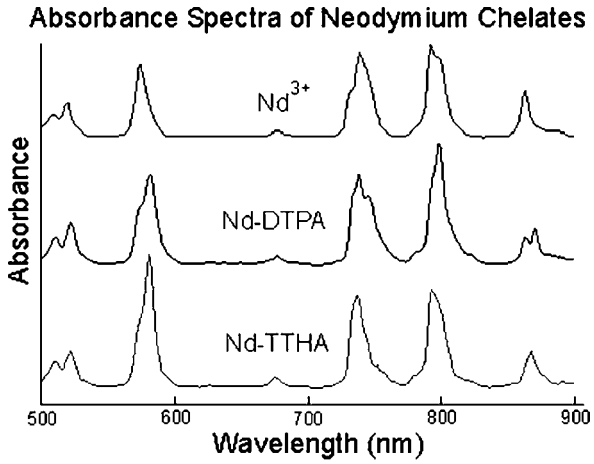

Fig. 1 Absorbance spectra of Nd in ionic form and chelated with TTHA and DTPA.

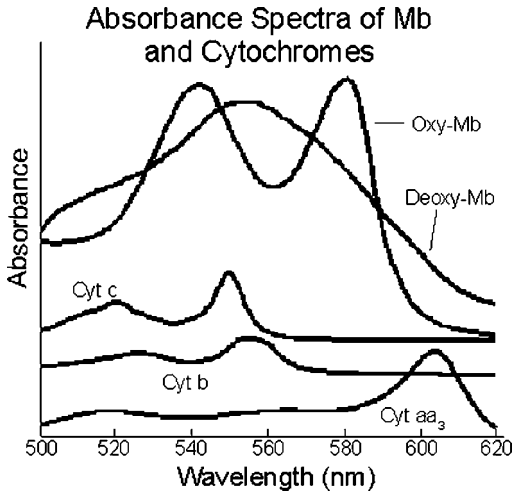

Fig. 2 Absorbance spectra of myoglobin and cytochromes. Only the spectra of cytochromes in their reduced forms are shown. The spectra of cytochromes in their oxidized forms show only very weak absorptions that make no substantial contribution to observed cardiac tissue spectra.

or left ventricular diastolic pressure and only marginally increased perfusion pressure and coronary resistance (Table 1). Infusion of digitonin, however, decreased the pressure-rate product by over 80%, mainly due to contracture development (insufficient muscular relaxation during diastole) and a decrease in heart rate (not shown). Simultaneously, perfusion pressure increased by over 50% and coronary resistance rose twofold to threefold. The increase in perfusion pressure was attenuated by a manual reduction in coronary flow by 30 to 50%, to avoid extremely high perfusion pressures and excessive cardiac edema. The increase in the coronary resistance resulted from compression of the capillaries due to contracture and possibly from vascular constriction caused by digitonin. Most likely, both cardiac contracture and vasoconstriction were induced by massive Ca^{2+} entry through the permeabilized cell membrane. The preceding changes occurred within the first 10 min of exposure to digitonin and cardiac functional parameters were stable by the end of digitonin infusion. The presence of Nd complexes did not affect the functional effects of digitonin (Table 1).

3.2 *Optical Tissue Spectra*

Both Nd chelates have two sharp absorptions at 740 and 800 nm and a weaker absorption at 870 nm (Fig. 1). There are also two bands in the visible region, but these are not readily apparent in tissue spectra measured *in situ*. The most prominent absorptions of Mb are in the visible region: dMb has a single strong band at 555 nm, whereas MbO_2 has a pair of overlapping peaks[14,21] at 542 and 581 nm.

These Mb bands were also overlapped by weaker cytochrome absorptions. Cytochromes b, c, c_1, and aa_3 (cyt c oxidase) are present in cardiac mitochondria in nearly equal proportions[22] (aa_3:b:c:c_1=2:2:2:1), and they are integral components of the electron transport chain crucial to adenosine triphosphate (ATP) production. The absorption profiles of the oxidized forms of these cytochromes are largely featureless [with the exception of a small band at 605 nm due to cyt aa_3 (Ref. 15)] but in their reduced forms, cytochromes c and c_1 (whose spectra are essentially identical) have a sharp band at 550 nm and the 605-nm band of cytochrome aa_3 becomes much more intense (Fig. 2).

Spectra acquired during equilibration with KHB, perfusion with Nd-KHB, and digitonin infusion clearly showed the characteristic absorbance bands of myoglobin and water (Fig. 3). On addition of Nd to the perfusate, the characteristic peaks at 740, 800, and 870 nm, became apparent with minimal effect on Mb oxygenation, as determined by its spectral bands near 550 nm. However, the Nd absorptions at 582 and 520 nm, while having absorptivity values comparable to the absorptions at 740 and 870 nm, respectively, are relatively very weak in the tissue spectra. Subtraction of baseline spectra (acquired before perfusion with Nd-KHB) from later spectra effectively eliminated the Mb contribution to the spectra, but the Nd bands in the visible region were still not apparent (data

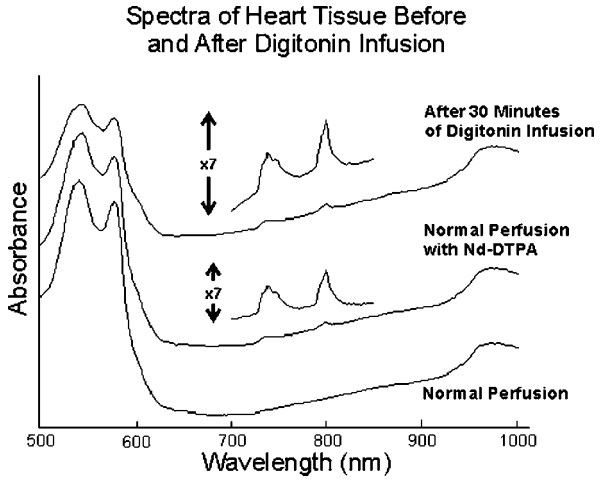

Fig. 3 Absorbance spectra taken of heart tissue *in vivo*. The insets show portions of the spectra magnified sevenfold along the vertical axis, highlighting the Nd absorptions. After digitonin infusion, the Mb absorption near 550 nm decreased in intensity and the Nd band intensities increased, while the water absorption at 970 nm was not substantially affected.

not shown). This is most likely a result of the much shorter effective optical path length in the visible region as compared to the near IR.

3.3 *Effects of Digitonin*

Infusion of digitonin provoked a sudden deoxygenation of Mb and reduction of cytochromes. However, after several more minutes of continued digitonin infusion, Mb oxygenation and cytochrome redox states returned to normal levels (although total Mb levels fell). These events can be explained by a massive influx of Ca^{2+} on membrane disruption, stimulating myofibrillar contraction and ATP consumption; these events, in turn, accelerated mitochondrial electron transport and oxygen uptake, resulting in the reduction of cytochromes and deoxygenation of myoglobin. As the enzymes and substrates necessary for glycolysis washed out into the perfusate, however, the cessation of glycolysis soon halted mitochondrial electron transport, resulting in reoxidation of cytochromes and reoxygenation of myoglobin.

Figure 4 shows changes in Nd chelate, tissue Mb, and tissue water concentrations through the experimental protocol. After 30 min of digitonin infusion, total Mb levels dropped to $46 \pm 14\%$ ($n=12$) of their initial values during normal perfusion. Nd concentrations (pooled for all three digitonin concentrations) rose to $157 \pm 18\%$ ($n=9$) of their levels prior to digitonin infusion. On return of perfusion to Nd-free KHB, the Nd concentrations fell to $12 \pm 6\%$ of their levels prior to digitonin infusion rather than to zero, suggesting that some Nd remained in the damaged tissue.

To investigate their dependence on digitonin concentration, mean Nd chelate and Mb levels were calculated throughout the protocol for each of the three digitionin concentrations (2.5, 5 and 10 μg/mL). For purposes of comparison, all Nd and Mb levels were normalized to the corresponding levels immediately prior to digitonin infusion. As illustrated by Fig. 4, lower digitonin concentrations resulted in markedly slower wash-in/washout than higher digitonin concentrations. After 5 min of digitonin infusion, myoglobin concentrations in trials corresponding to 10 μg/mL digitonin were already significantly different from baseline values prior to digitonin infusion ($p<0.01$). In the trials corresponding to 5 μg/mL digitonin, myoglobin concentrations were not significantly different from baseline values until after 10 min of digitonin infusion ($p<0.05$). Low-digitonin trials (2.5 μg/mL) did not exhibit significantly different Mb concentrations until after 30 min of digitonin infusion. Similarly, Nd concentrations in trials with both high and moderate digitonin levels were significantly different from predigitonin baseline values after 2 min of digitonin infusion ($p<0.05$), whereas Nd concentrations in trials with a low digitonin infusion rate were not significantly different from baseline until after 30 min of infusion ($p<0.05$).

Although low digitonin concentration produced slower Nd accumulation and Mb washout than high digitonin concentration, total washin/washout after 30 min of digitonin infusion was not significantly different among the three groups of trials. Although the mean normalized Mb and Nd concentrations after 30 min of digitonin infusion were more extreme for the low-digitonin trials than trials with higher digitonin levels, the ranges of values are highly overlapping. This is illustrated by Fig. 5, which depicts the normalized Mb, Nd, and LDH values after 30 min of digitonin infusion; while the mean values do indeed indicate greater Mb washout and Nd accumulation for 2.5 μg/mL digitonin than for higher concentrations, the differences are not significant.

By comparison, integrated perfusate LDH release levels are presented in Fig. 5(c) for each digitonin concentration. While total LDH release in the low-digitonin trials was lower than those in the moderate- and high-digitonin trials, LDH release in baseline samples for those trials were also substantially lower than those for the higher digitonin trials. The same is true for the trials in which no Nd was added to the perfusate. Given the small sample sizes, no reliable conclusions regarding the digitonin dependence of LDH release can be made.

4 Discussion

4.1 *Contrast Agents*

These data validate the hypothesis that digitonin-induced membrane disruption can be monitored spectroscopically by Mb washout and accumulation of an IR-absorbing contrast agent. The use of a contrast agent with optical spectroscopy is not new, and indocyanine green (ICG) is another such agent that has been used extensively with near-IR spectroscopy to monitor hepatic function, having an absorption peak near[23,24] 800 nm. However, the absorptions of ICG are broader than those of Nd, and the concentration dependence of the ICG absorptivity spectrum in blood[24,25] (due to binding with plasma proteins which results in shifting of the bands) complicates analysis.

In order to consider the physiological impact of adding neodymium to the perfusate in the absence of digitonin, isolated pig hearts ($n=4$) were perfused in a separate study[26] with a similar Nd-KHB solution (the Nd concentration of 5 mM was the same as that used here). After 40 min of normal perfusion without any infusion of digitonin, tissue Mb concentrations (determined spectroscopically) rose by $5 \pm 4\%$ from values measured shortly after addition of Nd to the perfusate. Similarly, Nd concentrations rose by $12 \pm 11\%$ of initial values over 40 min of perfusion. These results, in comparison with the changes already reported, confirm the assertion that Nd is nontoxic to cardiac tissue and that its addition to the perfusate does not by itself result in significant changes to tissue Mb or Nd levels in the absence of digitonin.

Nd is not the only lanthanide that is suitable for this application. Gadolinium, dysprosium, and europium are also nontoxic when chelated and remain outside the cellular volume in healthy tissue when added to the perfusate. Nd was selected for this study because it has the most prominent near-IR absorptions. While other lanthanides exhibit near-IR absorptions, e.g., dysprosium (756, 808, and 908 nm), erbium (650, 800, 976 nm), thulium (684 and 780 nm), and ytterbium (972 nm), these absorptions are weaker and/or fewer in number than the Nd bands. Since lanthanides (e.g., gadolinium, dysprosium) are also employed as contrast agents in magnetic resonance (MR) studies, it may be feasible and useful to have a single lanthanide chelate serving as a contrast agent in MR and optical studies simultaneously. Although the low paramagnetic moment of Nd limits its applicability as a magnetic resonance contrast agent, dysprosium has multiple near-IR ab-

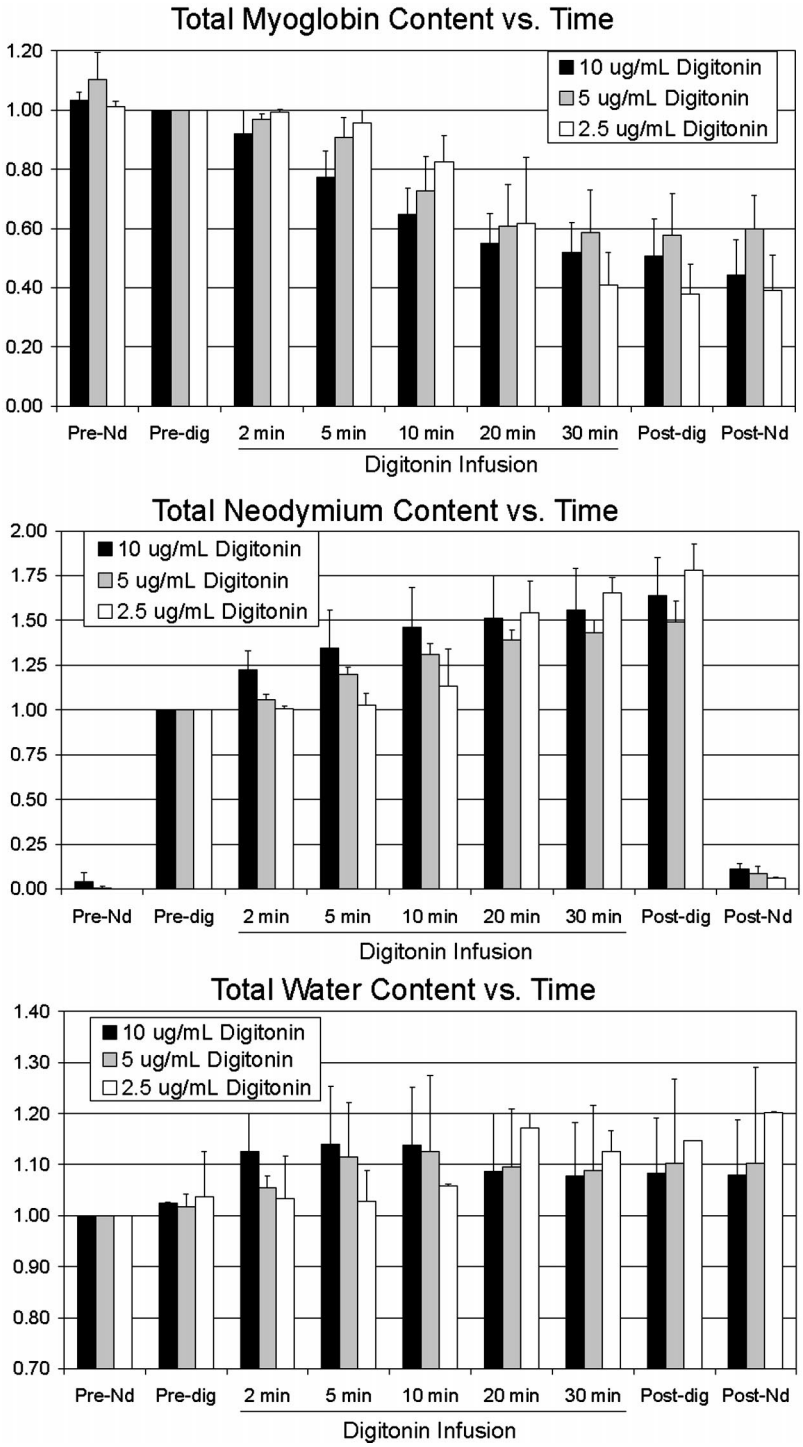

Fig. 4 Tissue myoglobin, neodymium, and water levels (normalized to predigitonin levels) at various timepoints during the experiment for each digitonin concentration.

sorptions and a reasonable paramagnetic moment, potentially lending itself well to such studies.[27]

4.2 *Quantitative Analysis*

It is of interest to note that while tissue Mb concentrations in this experiment fell substantially with digitonin infusion, they consistently stabilized at nonzero values ($46 \pm 14\%$). This is consistent with previous observations that a 30-min treatment of rat hearts with 10-μg/mL saponin (digitonin analog) caused washout of approximately 60% of total nuclear MR (NMR)-visible intracellular phosphates[28] (phosphocreatine + ATP + P_i). Nevertheless, since even low concentrations of digitonin have been reported to result in complete membrane disruption in isolated myocytes,[12] it is unclear why residual Mb levels were so high. Since membane damage (induced by the calcium paradox) has been reported to result in almost com-

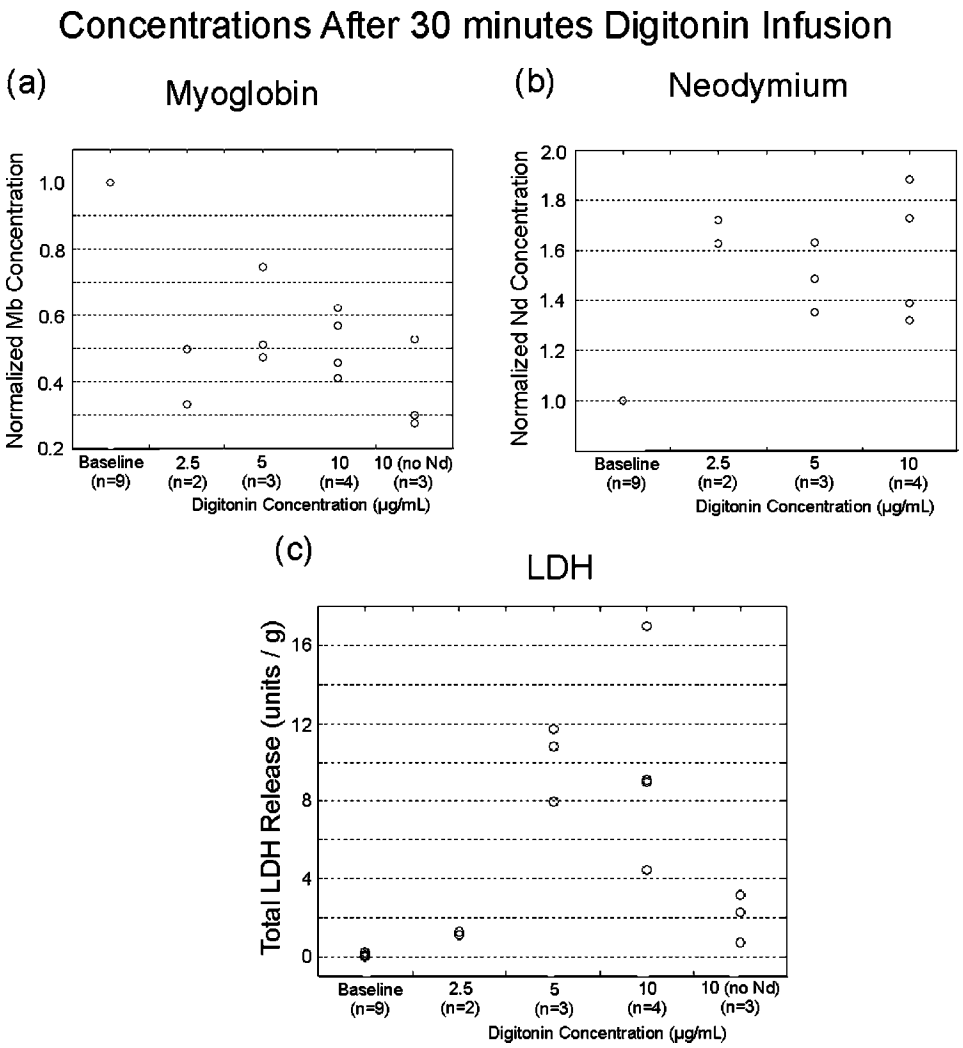

Fig. 5 Effect of 30-min digitonin infusion on three indicators of membrane disruption: (a) tissue Mb concentration, (b) tissue Nd concentration, and (c) perfusate LDH levels.

plete washout of Mb in isolated hearts,[29] it is improbable that in experiments reported here, Mb remained in permeabilized cells. Therefore, some myocyte membranes may have remained intact. One mechanism that may account for the high residual Mb levels is that digitonin damaged the vasculature sufficiently to cut off some domains from perfusion, preventing further loss of Mb or accumulation of Nd. Indeed, the fact that Nd washout was not complete, falling to 12% of predigitonin levels after reperfusion with Nd-free KHB, supports the claim that some tissue was cut off from the circulating perfusate; however, it also indicates that this no-perfusion zone only comprised roughly 10% of the heart. Therefore, some other mechanism must be invoked to account for much of the residual Mb.

One factor to consider is that the spectroscopic results do not reflect Mb and Nd levels in the entire heart. Since the emitting and collecting fibers were only 2 mm apart, some of the detected photons would pass through only the outer layers of the ventricular wall. To estimate the tissue depth probed by the detected photons, a small balloon containing a concentrated Nd solution was placed inside the left ventricle at the end of the Nd-free experiments, filling the cavity. The fact that spectra acquired with the balloon in place exhibited characteristic Nd absorptions indicates that the full thickness of the ventricular wall was sampled by the near-IR spectra. However, tissue on the side of the heart opposite to the probe was not likely monitored. Another factor to consider is that since the probe tip was placed against the ventricular wall distal to the major arteries, it is possible that the no-perfusion zone contributed disproportionately to the spectroscopic results. Membrane permeabilization, and hence Mb washout, may have been more pronounced in domains closer to the major arteries. While this may explain in part the high residual Mb values observed here, it is insufficient to simultaneously account for both the high residual Mb and the comparatively low residual Nd concentration.

Another factor that may be of relevance is the compartmentalization of tissue water. Extra- and intracellular water make up approximately 35 and 45% of the total myocardial tissue volume respectively in crystalloid-perfused rat hearts.[30] Thus, prior to digitonin infusion, Nd was present in 35% of cardiac tissue volume. The intracellular space is further compartmentalized: mitochondria, whose membranes are not permeabilized by digitonin, comprise 35% of the intracellular

volume.[31] After cell membrane disruption, then, the Nd perfusate will equilibrate with the nonmitochondrial intracellular volume of permeabilized cells. If all cells were permeabilized, this added space would comprise 29% of total tissue volume ($65\% \times 45\% = 29\%$). If 90% of cardiac cells were permeabilized, the myocardial volume accessible to neodymium would increase by 26% ($90\% \times 29\% = 26\%$) of tissue volume, resulting in a relative increase of 74% (26%/35%) over predigitonin levels. On the other hand, if only 50% of the cells were permeabilized, then the anticipated relative increase in Nd would be 42%. These estimates are consistent with the range of values derived from the spectroscopic data (39 to 75%). Thus, it is possible that the size and relative contribution of the no-perfusion zone varied significantly among the individual hearts.

Implicit in the preceding discussion is the assumption that total tissue volume remains constant during digitonin treatment, which is unlikely to be strictly true because of increased perfusion pressure. The magnitude of changes in tissue water content was gauged by monitoring water absorptions in the near-IR spectra (Fig. 4). Water content (as determined by fitting the spectra between 900 and 980 nm) varied very little over the course of the experiment. After 30 min of digitonin infusion, it rose by only $11 \pm 12\%$ ($n = 12$) relative to predigitonin values, confirming that the expansion of extracellular space can not fully account for the observed increases in overall Nd concentration.

4.3 *Future Directions*

The central aim of this study was to apply near-IR spectroscopy as a means to gauge cell damage due to digitonin infusion. Implementing the same technique to assess cell damage resulting from prolonged ischemia must take certain potentially confounding factors into account. First, it would appear more challenging to use Mb as a marker of cellular damage in blood-perfused tissue since the Hb spectrum closely overlaps that of Mb. However, even in blood-perfused hearts, Mb contributes significantly to the overall absorption profile,[17] so that Mb washout should give rise to measurable spectroscopic consequences. A second challenge relates to the fact that the digitonin model leads to membrane disruption while the tissue is still perfused with the contrast agent, whereas in an ischemia-reperfusion model, the wash-in or washout of contrast agents would not occur until reperfusion. It may therefore be difficult to distinguish between tissue damage resulting from ischemia and any tissue damage resulting from reperfusion injury. Finally, contrast agent accumulation and Mb washout would not occur in damaged cells in the no-reflow zones.

A very exciting prospect arising from this study is the possibility of visualizing localized regions of cell damage with optical spectroscopic imaging. The equipment for such an investigation is available; we have recently published a study combining near-IR spectroscopy with imaging equipment to monitor regional oxygenation in isolated porcine hearts.[17] By adding Nd to the perfusate and inducing regional myocardial ischemia, this spectroscopic imaging technique might simultaneously monitor variations in oxygenation and regional cell damage (infarction) that may occur as a result. We are currently investigating this line of research and will report it separately.

5 Conclusion

We presented two ways in which optical spectroscopy can be exploited to gauge cell membrane disruption. In particular, cardiac membrane damage, as induced by digitonin infusion, was revealed by monitoring spectroscopically both decreases in Mb levels and increases in accumulation of an extracellular near-IR contrast agent (Nd complexes in this case). Membrane damage resulted in washout of $54 \pm 14\%$ of total myoglobin and an increase of $57 \pm 18\%$ in tissue Nd complex concentration. Some of the tissue was likely cut off from perfusion during digitonin treatment, preventing further Mb washout and Nd accumulation, but the exact proportion of tissue affected remains unclear. There appeared to be no significant dependence of the extent of membrane disruption on perfusate digitonin concentration over the range of 2.5 to 10 μg/mL, although higher concentrations permeabilized membranes more quickly.

Acknowledgments

This work was supported in part by a grant from the Canadian Institutes of Health Research. Mr. Nighswander-Rempel was funded by a studentship from the Natural Sciences and Engineering Research Council.

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