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ORIGINAL RESEARCH

Bilirubin Nanoparticles Protect Against Cardiac Ischemia/Reperfusion Injury in Mice

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BACKGROUND: Ischemia/reperfusion (I/R) injury causes overproduction of reactive oxygen species, which are the major culprits of oxidative stress that leads to inflammation, apoptosis, myocardial damage, and dysfunction. Bilirubin acts as a potent endogenous antioxidant that is capable of scavenging various reactive oxygen species. We have previously generated bilirubin nanoparticles (BRNPs) consisting of polyethylene glycol–conjugated bilirubin. In this study, we examined the therapeutic effects of BRNPs on myocardial I/R injury in mice.

METHODS AND RESULTS: In vivo imaging using fluorophore encapsulated BRNPs showed BRNPs preferentially targeted to the site of I/R injury in the heart. Cardiac I/R surgery was performed by first ligating the left anterior descending coronary artery. After 45 minutes, reperfusion was achieved by releasing the ligation. BRNPs were administered intraperitoneally at 5 minutes before and 24 hours after reperfusion. Mice that received BRNPs showed significant improvements in their cardiac output, assessed by echocardiogram and pressure volume loop measurements, compared with the ones that received vehicle treatment. BRNPs treatment also significantly reduced the myocardial infarct size in mice that underwent cardiac I/R, compared with the vehicle-treatment group. In addition, BRNPs effectively suppressed reactive oxygen species and proinflammatory factor levels, as well as the amount of cardiac apoptosis.

CONCLUSIONS: Taken together, BRNPs could exert their therapeutic effects on cardiac I/R injury through attenuation of oxidative stress, apoptosis, and inflammation, providing a novel therapeutic modality for myocardial I/R injury.

Key Words: apoptosis ■ bilirubin nanoparticles ■ inflammation ■ ischemia reperfusion injury ■ reactive oxygen species

Early revascularization therapy via thrombolysis or percutaneous coronary intervention after myocardial infarction has significantly improved the mortality and morbidity of patients. However, ischemia/reperfusion (I/R) injury reduces the benefit of early revascularization therapy.[1,2] Myocardial I/R injury is associated with excessive reactive oxygen species (ROS) production, which contributes to myocyte apoptosis and inflammation, and ultimately leads to myocardial damage and dysfunction.[3,4] Therefore, ROS can be an attractive target for therapeutic intervention in myocardial infarction. Reducing ROS levels using an antioxidant has been proposed as a therapeutic strategy.[5] Various antioxidants have been investigated as therapeutic strategies to reduce the deleterious effects of excessive ROS.[6–8] However, the clinical application of antioxidant therapy for myocardial I/R injury remains a challenge.

Bilirubin is an endogenous antioxidant that scavenges various ROS. It has been shown to be a biomarker of cardiovascular health, and is inversely associated with the severity of cardiovascular diseases[9,10] and all-cause mortality[11] in the general population. Reduced risk of ischemic heart disease has been observed in Gunn rats, a mutant rat model with elevated bilirubin levels.[12–14] The beneficial effects of bilirubin may be because of their antioxidative properties.[12,13,15,16] Studies using the Langendorff heart

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CLINICAL PERSPECTIVE

What Is New?

- Therapeutic effects of bilirubin nanoparticles, which were generated to improve solubility of bilirubin, were examined in mouse model cardiac ischemia/reperfusion injury.
- Mice that received bilirubin nanoparticles showed significant improvements in their cardiac output, reduction of myocardial infarct size, suppression of reactive oxygen species and proinflammatory factor levels, and inhibition of cardiac apoptosis.

What Are the Clinical Implications?

- Bilirubin nanoparticles could exert their therapeutic effects on cardiac ischemia/reperfusion injury through attenuation of oxidative stress, apoptosis, and inflammation, providing a novel therapeutic modality for myocardial ischemia/reperfusion injury.

Nonstandard Abbreviations and Acronyms

BRNP-ICGs	indocyanine green–encapsulated bilirubin nanoparticles
BRNPs	bilirubin nanoparticles
HIF-1α	hypoxia-inducible factor 1-α
ICG	indocyanine green
I/R	ischemia/reperfusion
MCP-1	monocyte chemoattractant protein-1
NOX	nicotinamide adenine dinucleotide phosphate oxidase
PPAR-α	peroxisome proliferator-activated receptor-α
PV	pressure-volume
ROS	reactive oxygen species
TNF-α	tumor necrosis factor-α

perfusion approach to establish global cardiac ischemia models have demonstrated that bilirubin treatment may be protective against cardiac ischemia ex vivo.[13,17] Particularly, administration of bilirubin dilaurate before or after global ischemia showed greater protection after ischemia.[13]

To improve the solubility of bilirubin, we have previously developed bilirubin nanoparticles (BRNPs) using polyethylene glycol-modified bilirubin. We showed that BRNPs could be a simple and safe alternative to the parent bilirubin to effectively boost serum bilirubin concentrations without causing jaundice.[18] In a hepatic I/R injury mouse model, BRNPs preconditioning exerted protective effects against hepatocellular injury.[18] However, the effects of BRNPs on cardiac I/R models have not been reported. In this study, we investigated the potential therapeutic effects of BRNPs in a mouse model of cardiac I/R injury.

METHODS

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

Animal Model

C57BL/6 mice (8–10 weeks old, both male and female) (Charles River Laboratory, Wilmington, MA) underwent cardiac I/R surgery as previously described.[19] Briefly, mice were anesthetized with 2% inhalant isoflurane, intubated, and placed on a rodent ventilator (model 687; Harvard Apparatus). Meloxicam SR (4.0 mg/kg, ≈50 μL volume) was given subcutaneously once just before the procedure for pain control for all animals undergoing surgery. After thoracotomy, the left anterior descending artery was ligated with a 7-0 surgical silk suture tied around a specialized 30-gauge catheter. After 45 minutes, reperfusion was achieved by releasing the ligation. Sham-operated mice underwent the same procedure but without left anterior descending artery ligation or reperfusion.

BRNPs were prepared from polyethylene glycol–modified bilirubin by using a film formation and rehydration method as previously described.[18,20] Stock solution of BRNPs was prepared by dissolving BRNPs in PBS at a concentration of 1 mg BRNPs/1 mL PBS. BRNPs were administered intraperitoneally at 2 time points, once at 5 minutes before and once at 24 hours after reperfusion. Tissues were collected at 24 hours after surgery to assess the markers for apoptosis, ROS, and inflammation. Cardiac functions were measured at 2 weeks after surgery. The animal care standards were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

In Vivo and Ex Vivo Fluorescence Imaging and Biodistribution Assay

In vivo and ex vivo fluorescence imaging was performed using a near-infrared imaging system. To assess the biodistribution of BRNPs, indocyanine green (ICG), a widely used near-infrared fluorophore, was encapsulated in BRNPs (BRNP-ICGs), and the complex was intravenously administered in mice via tail-vein injection. One hour after administration, mice were euthanized, and the hearts, lungs, livers, and kidneys

were harvested. Tissues were washed with PBS before imaging. For the in vivo near-infrared imaging, mice were first anesthetized by intraperitoneal injection of ketamine/xylazine solution. The animals were then placed in supine position and underwent cardiac I/R procedure with BRNP-ICGs being administered intravenously upon initiating reperfusion.

Cardiac Functional Analysis

Cardiac function was measured by echocardiography and pressure-volume (PV) loop 2 weeks after cardiac I/R as previously described.[19] Using a high-resolution small-animal echocardiogram (Vevo2100; Visual Sonics, Toronto, Canada) with an MS400 (18–38 MHz) transducer, 2-dimensional guided M-mode echocardiography was performed to assess anterior and posterior wall thickness, left ventricle (LV) dimensions, ejection fraction, and fractional shortening. Echocardiography was performed at baseline and 2 weeks after cardiac I/R.

The PV loop parameters were measured using a 1.4F microtip, pressure-volume catheter (Scisense, Ontario, Canada). While the mouse was anesthetized at 2% isoflurane, the catheter was inserted into the LV via the right common carotid artery to obtain LV hemodynamic parameters. Data were recorded using Powerlab (AD Instruments, Colorado Springs, CO). Beat-by-beat PV parameters including heart rate, stroke work, LV systolic contractility, LV diastolic relaxation, and cardiac output were measured and analyzed using CardioSoft Pro software (CardioSoft, Houston, TX).

Infarct Size Measurement

Myocardial infarct size was measured as previously described.[19] After thoracotomy, the left anterior descending artery was ligated, and 50 μL of green fluorescent FluoSpheres (Molecular Probe, Carlsbad, CA) were injected into the LV of the heart to delineate the area at risk (FluoSpheres-negative area). After 24 hours, excised hearts were cut into ≈1-mm-thick slices from apex to base and then incubated in 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO) solution. Afterward, all slices were placed in a 10% (v/v) formaldehyde solution to improve contrast between stained (viable) and unstained (necrotic) tissues. The infarction area and area at risk were determined of each slice using ImageJ (National Institutes of Health, Bethesda, MD).

ROS Detection by Dihydroethidium Staining

Frozen heart sections were incubated in 5 μmol/L dihydroethidium (Sigma-Aldrich) at 37 °C for 30 minutes, and mounting medium with 4′,6-diamidino-2-phenylindole (H1200; Vector Laboratories) was then applied for each slide. Images were acquired by confocal fluorescence microscopy, and the fluorescence intensity was analyzed by Image 1.57 software.

Apoptosis Detection by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Staining

Apoptosis in myocardial tissues was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using an in situ cell death detection kit (Roche, Mannheim, Germany) and fluorescein (Roche Applied Science, Indianapolis, IN) as described previously.[19] Frozen heart sections were incubated in TUNEL reaction mixture at 37 °C for 60 minutes. The percentage of TUNEL-positive cells was calculated by the ratio of stained TUNEL-positive (apoptotic) cells to the total number of cells. Cell nuclei were then counterstained with 4′,6-diamidino-2-phenylindole. Images of the sections were acquired using confocal microscopy.

Reverse Transcriptase-Polymerase Chain Reaction Analysis for mRNA Expression

Twenty-four hours after cardiac I/R, heart tissues of the mice were collected for molecular analyses. Reverse transcriptase-polymerase chain reaction was performed as previously described.[21] 18S ribosomal RNA primer was used as an internal control. All reverse transcriptase-polymerase chain reaction signals were normalized to the 18S ribosomal RNA signal of the corresponding reverse transcriptase products to eliminate the measurement error from uneven sample loading and to provide a semiquantitative measure of the relative changes in gene expression.

Statistical Analysis

The results were expressed as mean value±SEM. Statistical analysis of the data was performed using a nonparametric 1-way ANOVA (the Kruskal-Wallis test). All statistical analyses were performed using Prism 5.0 statistical software (GraphPad, San Diego, CA). *P* values of <0.05 were considered significant.

RESULTS

Targeting of BRNPs to the I/R Injury Site in the Heart

A schematic illustration of the preparation of BRNPs is shown in Figure 1A. Using a film formation and subsequent rehydration process in aqueous medium, polyethylene glycol–modified bilirubin self-assembles into BRNPs at an average size of ≈100 nm.[18,20,22,23] We have shown in our previous reports that BRNPs possess high colloidal stability under physiological conditions and can circulate in the bloodstream with a half-life of

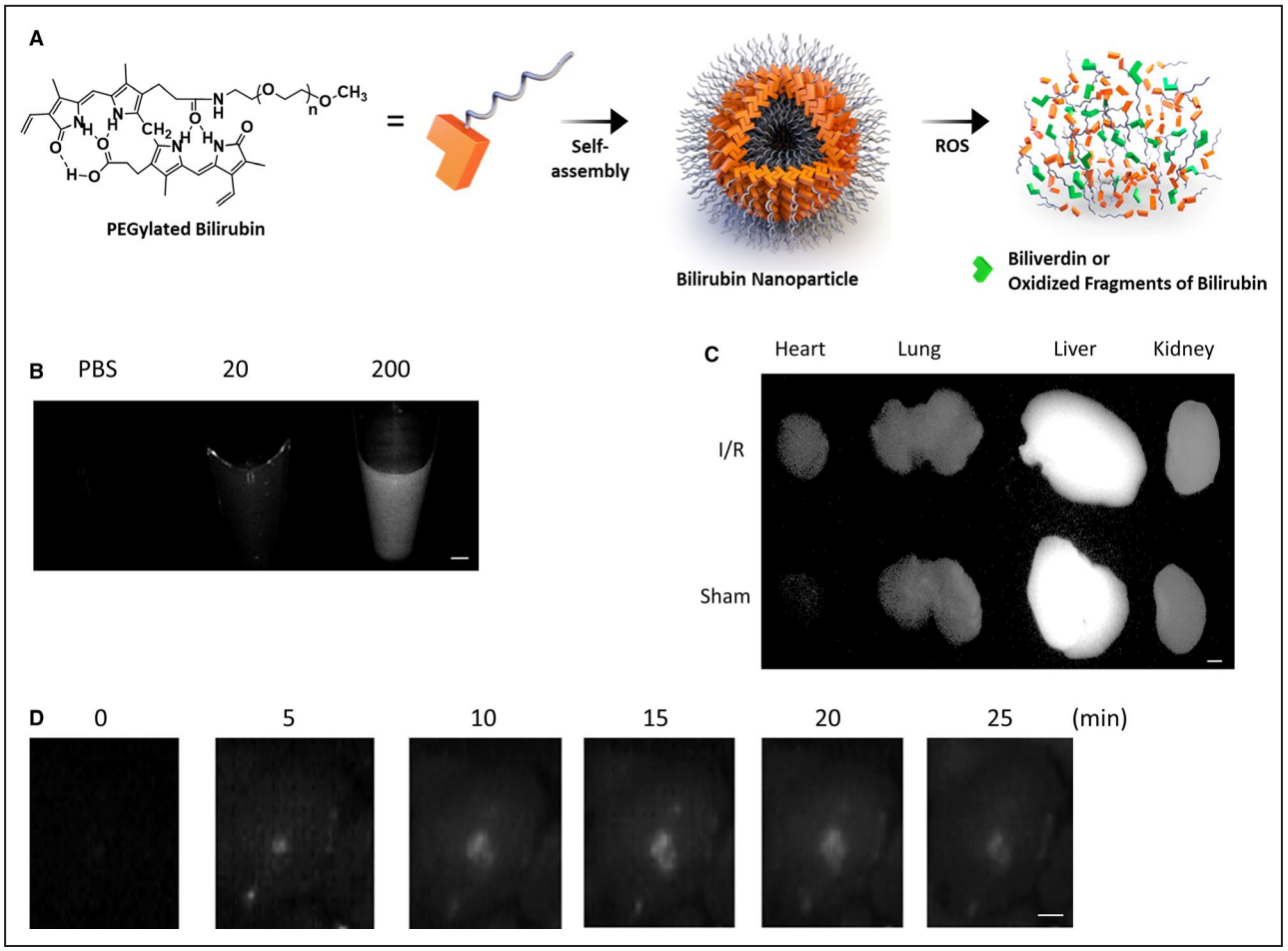

Figure 1. Synthesis and biodistribution of bilirubin nanoparticles (BRNPs) in mice after cardiac ischemia/reperfusion (I/R). **A**, BRNPs were synthesized from bilirubin and polyethylene glycol (PEG). Reactive oxygen species (ROS) causes the oxidation of bilirubin to biliverdin. **B**, Fluorescent intensity of the solution containing PBS only, 20 μg/mL BRNPs-indocyanine green (ICG), and 200 μg/mL BRNP-ICGs. Scale bar=2 mm. **C**, Biodistribution of BRNP-ICGs after intravenous administration in the heart, lung, liver, and kidney with (top panel) or without (bottom panel) cardiac I/R surgery. Scale bar=1 mm. **D**, Time-lapse imaging showing the accumulation of BRNP-ICGs in the heart after cardiac I/R surgery. Scale bar=1 mm.

≈6 hours, suggesting dramatic increase in its overall therapeutic effect compared with the parent bilirubin.[18,23] To analyze the biodistribution of BRNPs, ICG-encapsulated BRNPs (BRNP-ICGs) were administered intravenously. To determine the optimal concentration of BRNP-ICGs for the near-infrared imaging system, we compared the image of 20 μg/mL and 200 μg/mL BRNP-ICGs (Figure 1B). We found that 200 μg/mL BRNP-ICGs showed significantly brighter fluorescence than the lower concentration. As a result, we selected 200 μg/mL BRNP-ICGs to examine the biodistribution of BRNPs. At baseline, we observed that BRNP-ICGs mostly accumulated in the liver (Figure 1C). Kidneys and lungs also showed considerable BRNP-ICG accumulation, but the heart did not show any significant distribution of BRNP-ICGs at baseline. We then examined whether BRNPs were targeted to the site of I/R injury in the heart. Upon the initiation of reperfusion after 45 minutes of ischemia, mice were administered BRNP-ICGs intravenously. As expected, the majority of BRNP-ICGs accumulated in the liver, and some accumulated in the lungs and the kidneys (Figure 1C). However, compared with sham-operated mice, cardiac I/R resulted in significantly increased fluorescence intensity in the heart. Time-lapse video of in vivo fluorescence–captured images showed that the fluorescence signal appeared 5 minutes after reperfusion, reaching the peak intensity at ≈15 minutes after perfusion, and gradually faded after 25 to 30 minutes after perfusion (Figure 1D). These results suggest that BRNPs preferentially target to the injured heart tissues after cardiac I/R surgery in mice.

BRNPs Attenuated I/R-Induced Cardiac Dysfunction

Reperfusion of the ischemic myocardium triggers an overproduction of ROS, causing myocardial damage

and cardiac dysfunction. Our previous studies showed that the cardiac functions of the mice were decreased 2 weeks after I/R injury.[24–26] To examine the potential beneficial effect of BRNPs after cardiac I/R, we first performed pathologic analysis 2 weeks after I/R surgery. No mouse died after I/R injury in either the vehicle- or BRNPs-treated (2 doses: 10 and 30 mg/kg) group in this study. In the vehicle-treated group, there was a significant increase in both heart weight/body weight and lung weight/body weight ratios, which are indirect assessments of left and right heart failure, respectively (Figure 2A and 2B). However, mice treated with 30 mg/kg of BRNPs showed no significant increase in heart weight/body weight or lung weight/body weight ratios compared with the vehicle-treated mice after I/R surgery.

We further evaluated the cardiac functions of the mice 2 weeks after cardiac I/R injury using echocardiography and PV loop analysis. Sham-operated animals treated with BRNPs did not show significant changes in cardiac function. Cardiac I/R injury led to a significant decrease in LV ejection fraction and fractional shortening measured by echocardiography in vehicle-treated mice compared with sham-operated mice (Figure 2C through 2E, Table S1). Mice treated with BRNPs demonstrated significant improvement in cardiac functions compared with vehicle-treated mice after I/R surgery. Moreover, the beneficial effect of BRNPs was dose dependent (Figure 2D and 2E).

To validate the echocardiography results, PV loop measurements were performed. Consistent with the echocardiogram findings, cardiac I/R injury led to a significant decrease in LV function, as demonstrated by significant decrease in cardiac output and stroke work compared with sham-operated mice (Figure 2F through 2H, Table S2). There was also a significant decrease in systolic contractility and stroke volume, which suggest a decrease in systolic function compared with

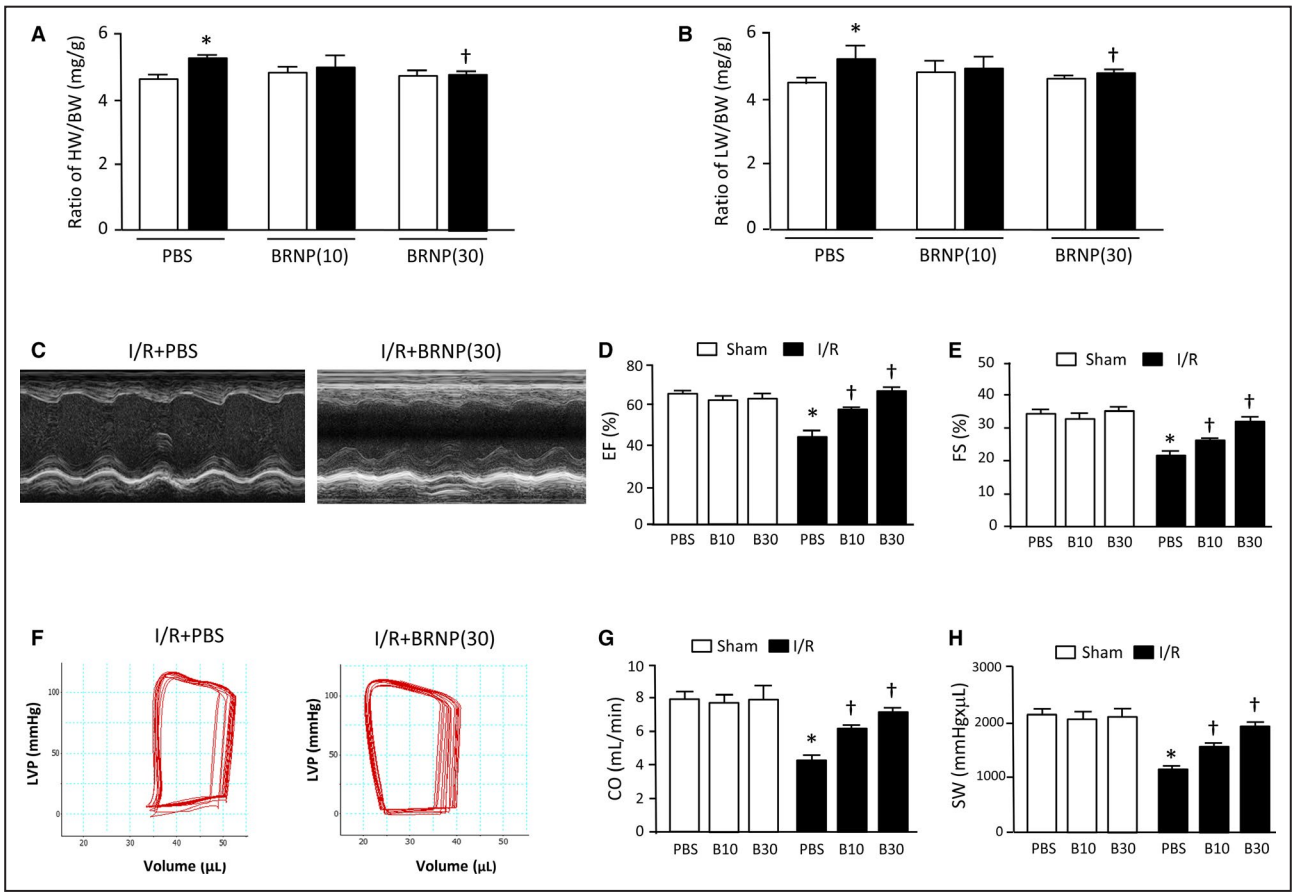

Figure 2. Effect of bilirubin nanoparticles (BRNPs) on cardiac function 2 wk after cardiac ischemia/reperfusion (I/R) surgery.

A and **B**, Heart weight (HW)/body weight (BW) ratios (**A**) and lung weight (LW)/BW ratio (**B**) 2 wk after cardiac I/R surgery. **C**, Representative 2-dimensional echocardiographic images after cardiac I/R with or without BRNPs treatment. **D** and **E**. Ejection fraction (EF) (**D**) and fractional shortening (FS) (**E**) after cardiac I/R with or without BRNPs treatment. **F**, Representative pressure-volume loop images after cardiac I/R with or without BRNPs treatment. **G** and **H**. Cardiac output (CO) (**G**) and stroke work (SW) (**H**) after cardiac I/R with or without BRNPs treatment. **P*<0.05 vs sham, †*P*<0.05 vs PBS I/R; n=5 mice/group (2 female, 3 male) in the sham group, 7 mice/group (3 female, 4 male) in the I/R group. LVP indicates left ventricular pressure.

sham-operated mice (Table S2). We observed modest decrease in diastolic relaxation, an indication of impaired diastolic function, but the findings were not significant. BRNPs-treated mice showed significant improvement in cardiac output and stroke work compared with vehicle- treated mice after cardiac I/R surgery (Figure 2F through 2H). In addition, improvement in systolic contractility and stroke volume was noted with BRNPs treatment, suggesting improved systolic function (Table S2).

BRNPs Attenuate Myocardial Damage Via Inhibition of Apoptosis After I/R

Inhibition of apoptosis is an important target for therapeutic intervention against I/R-induced tissue damage and organ dysfunction. To assess the antiapoptotic effect of BRNPs after I/R injury, we first evaluated the myocardial damage by measuring the infarct size using FluoSpheres and 2,3,5-triphenyltetrazolium chloride staining 24 hours after cardiac I/R. There was a significant increase in the infarct size (non–2,3,5-triphenyltetrazolium chloride–stained myocardium)/area at risk (non-FluoSpheres area) after I/R surgery (Figure 3A and 3B). Consistent with the cardiac functional data, BRNPs significantly reduced the infarct size compared with the vehicle group after I/R surgery. We then used TUNEL staining to quantify cardiac apoptosis. There was a significant increase in the number of apoptotic cells in the heart after cardiac I/R compared with sham-operated hearts (Figure 3C and 3D). Treatment with BRNPs significantly reduced the number of TUNEL-positive cells in the heart compared with the vehicle-treated group after cardiac I/R.

BRNPs Reduced Inflammation Induced by I/R

Elevated oxidative stress caused by ROS overproduction is a known factor that causes apoptosis and tissue

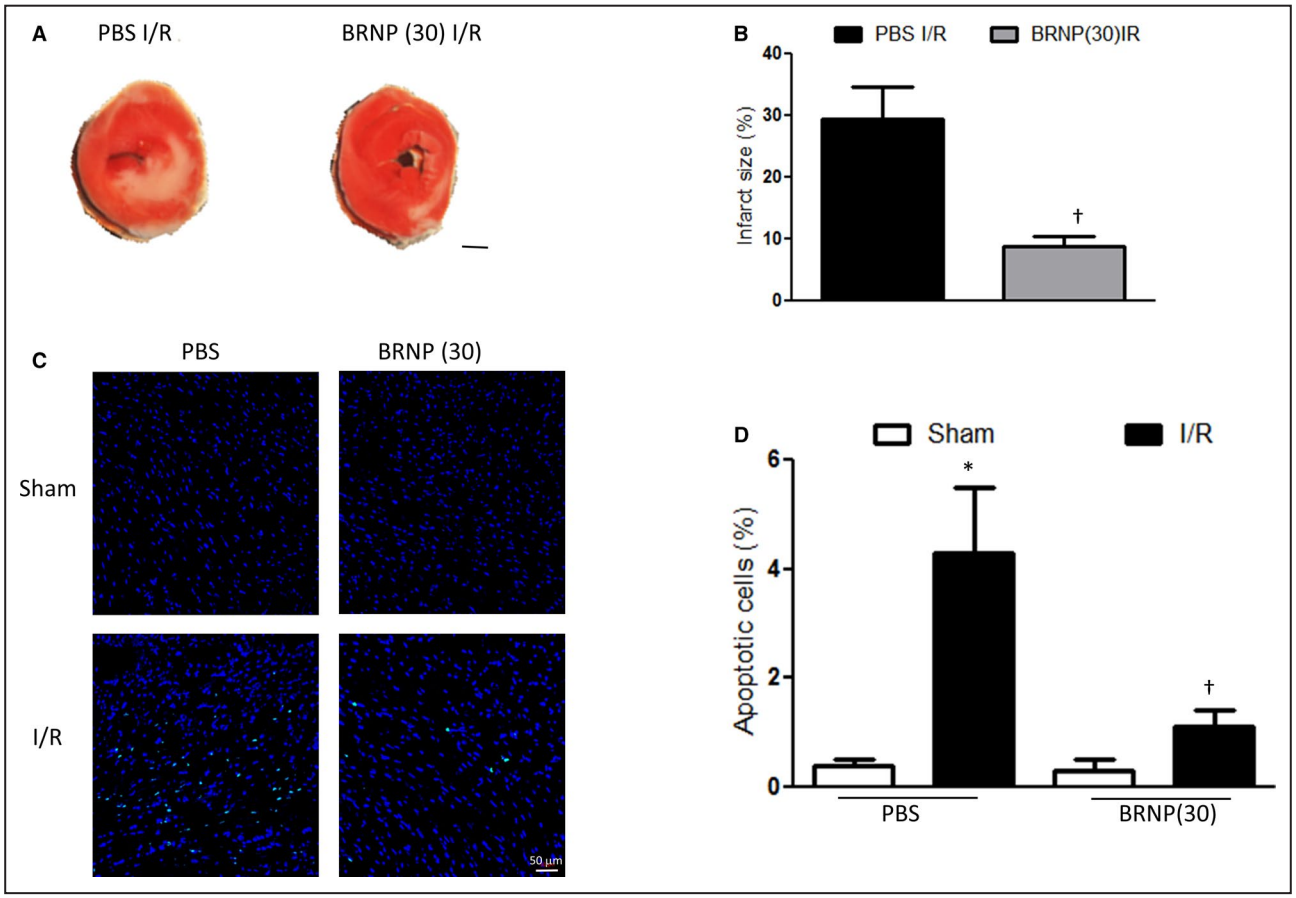

Figure 3. Effect of bilirubin nanoparticles (BRNPs) on ischemia/reperfusion (I/R)-induced cardiac apoptosis and infarct size 24 h after cardiac I/R surgery.

A and **B**, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of heart tissue section (**A**) and quantification of the infarct size (**B**) 24 h after cardiac I/R surgery with or without BNRPs treatment. **C** and **D**, Representative TUNEL staining of heart tissue section (**C**) and the quantification of apoptosis (**D**) 24 h after cardiac I/R surgery with or without BNRPs treatment. Scale bar=50 μm. **P*<0.05 vs sham, †*P*<0.05 vs PBS I/R; n=5 mice/group (2 female, 3 male) in sham group, 7 mice/group (3 female, 4 male) in I/R group.

damage. To determine whether the beneficial effects of BRNPs treatment is associated with their antioxidative and anti-inflammatory properties during cardiac I/R, we first performed dihydroethidium staining on cryosections of the heart 24 hours after cardiac I/R. There was a significant increase in dihydroethidium fluorescence in vehicle-treated hearts compared with sham-operated hearts (Figure 4A), suggesting a significant overproduction of ROS in the heart after cardiac I/R. BRNPs treatment significantly reduced the dihydroethidium fluorescence in hearts compared with the vehicle-treated group after cardiac I/R.

Previous studies showed that increased nicotinamide adenine dinucleotide phosphate oxidase (NOX) expression level and NOX-dependent ROS production influence several key components in cardiac remodeling, such as apoptosis, contractility, and fibrosis.[27] Thus, we measured the level of NOX-2 and NOX-4 mRNA in the hearts after cardiac I/R. The mRNA levels of both NOX-2 and NOX-4 were upregulated in the heart in response to I/R compared with sham operation (Figure 4B through 4D). BRNPs treatment significantly attenuated the mRNA level of NOX-2 compared with vehicle treatment after cardiac I/R surgery. However, there was no statistically significant difference between BRNPs-treated and vehicle-treated mouse hearts after I/R surgery on NOX-4 mRNA expression level (Figure 4B through 4D). This finding suggests that BRNPs treatment attenuates myocardial damage via inhibition of oxidative stress.

In addition, we evaluated the inflammatory response in the heart after cardiac I/R by measuring the mRNA expression of inflammatory markers, MCP-1 (monocyte chemoattractant protein-1), and TNF-α (tumor necrosis factor-α). Both were significantly increased in the heart after cardiac I/R compared with the sham-operated group (Figure 5A through 5C). BRNPs treatment significantly attenuated the increase in TNF-α and MCP-1 mRNA levels compared with the vehicle-treated heart after cardiac I/R. These results indicated that BRNPs can effectively prevent I/R-induced tissue damage by inhibiting apoptosis and inflammation.

DISCUSSION

In this study, we showed that BRNPs could specifically target to the injury site in the heart after cardiac I/R. This targeting and ROS-scavenging ability of BRNPs resulted in significantly reduced infarct size after I/R. In addition, cardiac function measured by echocardiography and PV loop showed BRNPs administration significantly attenuated I/R-induced cardiac dysfunction. The mechanism of the BRNPs' protective effects was most likely because of the antioxidant effect of bilirubin. BRNPs treatment resulted in inhibition of ROS-induced cardiomyocyte apoptosis and inflammation.

Nanoparticle-based therapeutic systems have been examined in the treatment of various diseases because of their ability to effectively target diseased areas.[28]

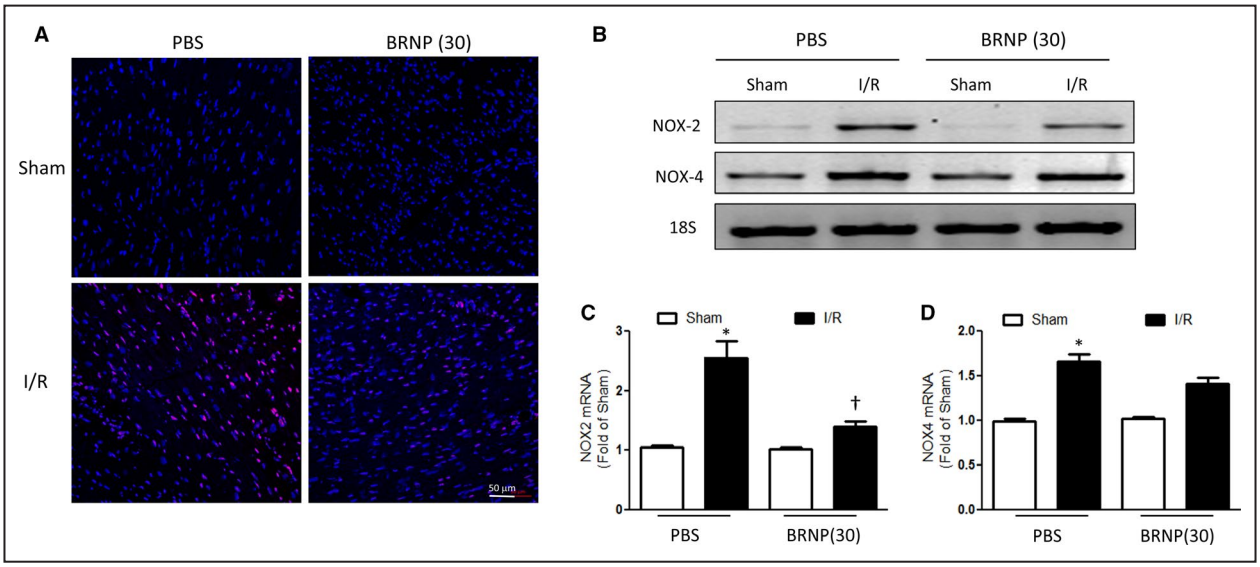

Figure 4. Effect of bilirubin nanoparticles (BRNPs) on ischemia/reperfusion (I/R)-induced reactive oxygen species overproduction 24 h after cardiac I/R surgery.

A, Representative dihydroethidium staining of heart tissue 24 h after cardiac I/R surgery with or without BNRPs treatment. Scale bar=50 μm. **B**, Representative mRNA levels of nicotinamide adenine dinucleotide phosphate oxidase (NOX)-2 and NOX-4 24 h after cardiac I/R surgery with and without BNRPs treatment. **C** and **D**, Quantification of NOX-2 (**C**) and NOX-4 (**D**) mRNA levels 24 h after cardiac I/R surgery with or without BNRP treatment. **P*<0.05 vs sham, †*P*<0.05 vs PBS I/R; n=5 mice/group (2 female, 3 male) in sham group, 7 mice/group (3 female, 4 male) in I/R group. 18S indicates 18S ribosomal RNA.

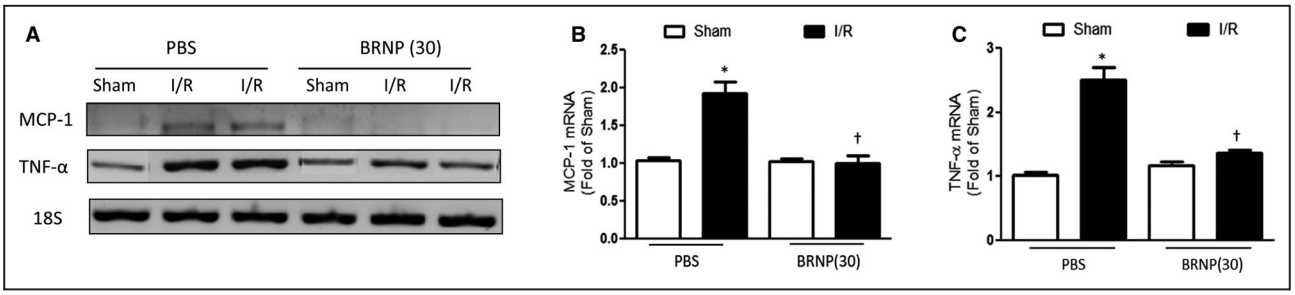

Figure 5. Effect of bilirubin nanoparticles (BRNPs) on ischemia/reperfusion (I/R)-induced inflammation 24 h after cardiac I/R surgery.

A, Representative mRNA levels of molecules associated with inflammation 24 h after cardiac I/R surgery with or without BNRPs treatment. 18S ribosomal RNA (18S) was used as an internal loading control. **B** and **C**, Quantification of MCP-1 (monocyte chemoattractant protein-1) (**B**) and TNF-α (tumor necrosis factor-α) (**C**) mRNA levels 24 h after cardiac I/R surgery with or without BNRP treatment. **P*<0.05 vs sham, †*P*<0.05 vs PBS I/R; n=5 mice/group (2 female, 3 male) in sham group, 7 mice/group (3 female, 4 male) in I/R group.

Previous studies demonstrated that nanoparticle drug delivery system could release drugs specifically to the affected area after cardiac I/R.[19,29] Recently, different classes of nanoparticles have been developed for targeted drug delivery.[30] However, low biocompatibility, low biodegradability, and potential toxicity caused by their accumulation in nontargeted organs limit the beneficial effects of these nanoparticles, hindering their application in the clinical setting.

Bilirubin is a final metabolite of heme catabolism.[31,32] Although increased circulating bilirubin levels is considered a sign of liver disease, bilirubin is inversely correlated with many cardiovascular disease risk factors, such as hypertension, type 2 diabetes, metabolic syndrome, and obesity.[33] Free bilirubin and unconjugated bilirubin are effective scavengers of peroxyl radicals against various oxidative stresses.[34–36] However, their poor solubility can lead to toxic waste deposition in various tissues. Our small-sized polyethylene glycol–modified BRNPs are soluble in physiological conditions. We also found that the antioxidant effect of bilirubin in the form of BRNPs was similar to unconjugated free bilirubin. In addition, BRNPs can be cleared out of the body through the hepatobiliary and renal excretory system, reducing the risk of toxicity when accumulated in nontarget organs.[18] Our previous study showed that BRNPs were effective in the targeted treatment to hepatic I/R injury.[18,37] Thus, BRNPs could be a safe alternative to purified bilirubin to effectively boost serum bilirubin concentrations without causing undesired side effects such as jaundice in mice.[18]

Previous studies showed that bilirubin is a potent antioxidant capable of scavenging various ROS, thereby playing a key role in protecting cells and tissues from oxidative stress-induced damage.[13,15,16] Our previous study showed that BRNPs effectively attenuated ROS generation in H_2O_2-stimulated mouse hepatocytes, and the antioxidant effect of BRNPs was far superior to that of an approved antioxidant drug, N-acetylcysteine.[18] Specific to the heart, the role of bilirubin in cardiac protection has been studied by other groups using various models of cardiac ischemia.[13,17,38,39] Using isolated Langendorff-perfused hearts, Bakrania et al showed that elevated bilirubin in Gunn rats was cardioprotective during ischemia,[38] and pre- and postischemic treatment with bilirubin improved oxidative tissue damage and improved cardiac function, especially the diastolic parameters.[39] In our study using the cardiac I/R injury model in vivo, bilirubin therapy demonstrated improvement in cardiac functional parameters, which are consistent with the previous data, further demonstrating the potentially promising role of bilirubin therapy in cardiac I/R injury.

Oxidative stress triggers inflammatory response and cellular apoptosis, which are important events in the development of myocardial I/R injury.[40] Therefore, inhibiting these responses may be an effective therapeutic strategy. Our previous study demonstrated that H_2O_2-responsive antioxidant polymer nanoparticles dramatically reduced ROS-mediated oxidative stress, inflammatory response, and apoptosis.[21] The present study also showed that the protective effects of BRNPs against I/R may be because of the antioxidative, anti-inflammatory, and antiapoptotic properties of BRNPs. Although ROS production and NOX 2 and 4 expression are reported, there is no direct assessment of oxidative damage. However, the decreased levels of apoptosis in mice treated with BRNPs support the beneficial effects of BRNPs.

Because the family of NOX proteins plays an important role in the generation of ROS during reperfusion, NOXs have been considered as therapeutic targets in ROS-related injury.[41] Our study showed that both NOX-2 and NOX-4 were upregulated in the heart in response to I/R injury, consistent with previous findings.[42] This elevation of NOX-2 during I/R was significantly reduced by BRNPs without significant

reduction of NOX-4. Although it is reasonable to inhibit NOX activities to decrease ROS production during I/R injury, a complete inhibition of the NOX family is likely not favorable, because NOX is also responsible for the physiological production of ROS.[43] A minimal amount of ROS is essential to prevent I/R injury via metabolic adaptations involving HIF-1α– (hypoxia-inducible factor 1-α) and PPAR-α– (peroxisome proliferator-activated receptor-α) dependent mechanisms.[42] Therefore, selective blockage of NOX of BRNPs may be desirable.[42,44]

It has been shown that intraperitoneal administration of bilirubin could increase plasma levels of bilirubin, but is quickly eliminated from the circulation.[45] In the serum, ≈99% of the bilirubin is bound to plasma protein and hence unavailable for intracellular actions.[46] Therefore, it has been suggested that redosing or chemical modification of the molecule would be necessary to maintain a sustained effect. Our BRNPs may be an effective strategy to deliver bilirubin to the targeted sites to increase the efficacy of bilirubin. Specifically, intravenous administration before reperfusion would be more clinically applicable to facilitate prophylactic treatment of individuals that are at risk of myocardial infarction. Because our study was a proof-of-concept study, we did not perform the pharmacokinetics of BRNPs. Of note, we showed that BRNPs preferentially target to the ischemic cardiac tissues. Thus, the blood level may not truly reflect the actual bilirubin concentration at the site of ischemic tissues. Further pharmacokinetic analysis will be needed for the circulating and local concentrations of BRNPs before clinical use.

In conclusion, these findings support the physiological importance of bilirubin in protecting the heart against I/R injury, and suggest the potential of BRNPs to be used as a new therapeutic option for various I/R injury-related diseases.

ARTICLE INFORMATION

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Disclosures

Dr Jon is a coinventor of BRNPs technology and has a patent for the use of BRNPs (Korea Patent 1681299). Dr Jon declares a financial interest in BiliX. This company did not support the aforementioned research and currently has no rights to any technology or intellectual property developed as part of this research. The remaining authors have no disclosures to report.

Supplementary Material

Table S1–S2

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SUPPLEMENTAL MATERIAL

Table S1. Echocardiographic analyses after cardiac I/R with or without BRNP treatment in mice.

	Sham			I/R		
	PBS	BRNP(10)	BRNP(30)	PBS	BRNP(10)	BRNP(30)
AWd (mm)	0.77±0.03	0.77±0.04	0.73±0.02	0.63±0.05*	0.81±0.04†	0.79±0.03†
PWd (mm)	0.81±0.03	0.79±0.05	0.84±0.05	0.78±0.03	0.76±0.03	0.79±0.04
LVDd (mm)	3.74±0.11	3.57±0.08	3.41±0.15	4.00±0.12	3.77±0.06	3.76±0.10
LVDs (mm)	2.46±0.09	2.41±0.11	2.33±0.11	3.11±0.13*	2.60±0.07	2.35±0.11†
EF (%)	64.0±1.6	61.8±2.5	62.7±2.9	44.8±3.9*	59.4±1.0†	68.5±2.3†
FS (%)	34.3±1.4	32.7±1.9	35.6±1.6	22.1±2.5*	28.7±0.9†	31.8±1.7†

HR=heart rate, AWd=anterior wall thickness in diastole, PWd=posterior wall thickness in diastole, LVDd=left ventricular dimension in diastole, LVDs=left ventricular dimension in systole, FS=fractional shortening. Results are presented as mean±SEM. *$P < 0.05$ vs. Sham; †$P < 0.05$ vs. PBS I/R; n=5 mice/group (2 female, 3 male) in sham group, 7 mice/group (3 female, 4 male) in I/R group.

Table S2. Left ventricular pressure-volume hemodynamic measurements after cardiac I/R with or without BRNP treatment in mice.

	Sham			I/R		
	PBS	BRNP(10)	BRNP(30)	PBS	BRNP(10)	BRNP(30)
HR (beat/min)	484±13.6	497±8.6	488±14.8	433±6.4	476±5.8	485±10.7
LVSP (mmHg)	121.5±2.3	119.0±2.6	119.9±3.8	108.4±2.3*	115.1±1.8	121.1±2.0†
LVEDP (mmHg)	3.0±0.41	2.7±0.45	2.8±0.74	4.1±0.70*	3.4±0.44	2.9±0.36†
dP/dt_{max} (mmHg/s)	12547±452	12600±477	12564±467	8760±479*	10731±283†	12470±364†
dP/dt_{min} (mmHg/s)	-8032±252	-8264±150	-8250±135	-7869±248	-7915±274	-7980±203
SV (µl)	16.4±0.94	15.6±1.06	16.2±1.54	9.6±0.68*	12.8±0.83	15.1±0.84†
SW (mmHgxµl)	2128±89	2033±102	2086±164	1194±69*	1563±73†	1959±76†
CO (mL/min)	7.9±0.34	7.7±0.38	7.9±0.56	4.2±0.29*	6.1±0.28†	7.2±0.30†

LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure, dP/dt_{max} and dP/dt_{min}, maximum and minimum first derivative of ventricular pressure with respect to time; SV, stroke volume; SW, stroke work; CO, cardiac output. *$P < 0.05$, vs. Sham; †$P < 0.05$, vs. PBS I/R; n=5 mice/group (2 female, 3 male) in sham group, 7 mice/group (3 female, 4 male) in I/R group.