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Impact of hyperbilirubinemia on rat cardiomyocyte injury

Jiajia Zhao[1], Hui Ye[1*], Xiangjun Wu[1], Danying Wang[1], Yuanxiang Ke[1] and Weijie Fang[1]

Abstract

Background To investigate bilirubin-induced injury in rat myocardial cells at varying concentrations.

Methods The study utilized the rat cardiomyocyte cell line H9C2 and primary rat cardiomyocytes. Bilirubin-rich and control sera were prepared, and cells were cultured for 48 h with or without vitamin C. Cell viability was assessed using the CCK-8 assay, while superoxide dismutase (SOD), glutathione peroxidase (GPx), Na^+/K^+-ATPase, creatine kinase-MB (CK-MB), and cardiac troponin I (cTn-I) levels were measured using their respective assay kits.

Results Bilirubin treatment markedly reduced the viability of H9C2 cells and primary rat cardiomyocytes compared to the control group. Additionally, it elevated the levels of cardiac injury markers, including cTn-I and CK-MB in the culture supernatant. Conversely, bilirubin exposure led to a decline in the release of GPx, Na^+/K^+-ATPase, and SOD in the medium. Vitamin C supplementation demonstrated partial attenuation of bilirubin-induced effects: including enhanced viability of primary rat cardiomyocytes, partially restored GPx, Na^+/K^+-ATPase, and SOD levels, and reduced concentrations of CK-MB and cTn-I in bilirubin-treated cells.

Conclusions Hyperbilirubinemia induces concentration-dependent cardiotoxicity in rat models, while vitamin C supplementation partially mitigates bilirubin-induced myocardial damage.

Trial registration Not applicable.

Keywords Hyperbilirubinemia, Cardiomyocytes, Sodium-potassium-exchanging ATPase, Superoxide dismutase, Glutathione peroxidase

Background

Neonatal jaundice is a common clinical manifestation, occurring in over 80% of newborns during the first postnatal weeks [1–3]. The majority of neonatal jaundice cases are physiological, resulting from developmental immaturity in bilirubin conjugation/excretion pathways and increased bilirubin production. Clinically significant hyperbilirubinemia requiring phototherapy occurs in approximately 10% of term infants and 25% of near-preterm infants [4, 5]. The major risk factors for severe neonatal hyperbilirubinemia include: early-onset jaundice (observed within the first 24 h of life), prematurity or lower gestational age, hemolytic disease (confirmed or suspected), significant birth trauma (e.g., cephalohematoma or extensive bruising), requirement for phototherapy during the initial hospitalization, family history or genetic predisposition to erythrocyte disorders, Down syndrome, maternal diabetes (gestational or pre-existing) with resultant fetal macrosomia, and exclusive breastfeeding with inadequate intake [6]. Pathologic neonatal jaundice arises from three primary etiologies: hemolytic disorders, enzymatic deficiencies (particularly

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of UDP-glucuronosyltransferase), and hepatobiliary abnormalities [4, 5]. In term infants, total serum bilirubin concentrations exceeding 428–513 μmol/L (25–30 mg/dL) carry significant risk for kernicterus development [4, 5]. At these critical levels, unconjugated bilirubin demonstrates neurotoxic potential through multiple mechanisms: it readily crosses lipid membranes, disrupts cellular metabolism, impairs membrane polarization, compromises energy production, and interferes with neurotransmitter synthesis. These effects can lead to multiorgan dysfunction affecting cardiac, hepatic, renal, and neurological systems [7]. Notably, while the neurotoxic effects of hyperbilirubinemia are well-documented, its specific impact on myocardial tissue remains poorly characterized in current literature [8].

Cardiovascular disease remains a major global health burden, ranking among the most significant public health challenges worldwide [9, 10]. This clinical reality stems primarily from the adult mammalian heart's fundamental biological limitation - cardiomyocytes in postnatal mammals exhibit a remarkably limited capacity for intrinsic self-repair following injury due to their permanent withdrawal from the cell cycle [11]. This inherent deficiency in cellular proliferation mechanisms directly underlies the myocardium's compromised regenerative potential and inadequate wound healing responses after ischemic or traumatic damage. In stark contrast, neonatal cardiac tissue retains transient regenerative capacity during early postnatal development [12], though this reparative window closes rapidly and the underlying mechanisms remain evolutionarily constrained in mammals [10].

Bilirubin, a terminal metabolite of heme catabolism mediated by heme oxygenase-1 (HO-1), serves as both a biochemical marker of HO-1 enzymatic activity and an endogenous antioxidant with complex biological implications [13]. Emerging clinical evidence demonstrates that elevated systemic bilirubin concentrations correlate significantly with heightened cardiac troponin release and increased intracoronary thrombotic burden in acute coronary syndrome (ACS) patients manifesting myocardial injury [14–16]. Notably, recent multicenter studies have identified serum bilirubin as an independent predictor of COVID-19-associated myocarditis severity and adverse cardiovascular outcomes [17]. While these findings provide compelling pathophysiological rationale for investigating bilirubin's cardiotoxic potential, critical knowledge gaps persist regarding neonatal myocardial susceptibility particularly concerning the dose-dependent injury thresholds, temporal progression of cellular damage, and the potential for functional recovery in developing myocardium exposed to hyperbilirubinemia.

Cardiovascular disease remains a leading global health burden, driven by the adult heart's limited regenerative capacity. Postnatal cardiomyocytes lose proliferative potential due to permanent cell cycle exit, impairing injury recovery [9–11]. While neonatal hearts exhibit transient regeneration via conserved mechanisms [12], this capacity is restricted in duration and evolutionary scope [10]. Our study investigates bilirubin-induced cardiotoxicity thresholds and vitamin C (a widely used antioxidant) [18]'s protective effects on neonatal cardiomyocytes, aiming to establish preventive strategies against hyperbilirubinemia-related cardiac damage.

Methods

Animals

All rats were maintained and bred in the animal facility of Hangzhou Hibio Technology Company. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Hangzhou Hibio Technology Company (Approval No. HB-20150608). The animals were housed under controlled environmental conditions, including a temperature range of 20–25 ℃, relative humidity of 40–70%, and a 12 h light/dark cycle, with ad libitum access to food and water. The Sprague-Dawley rats were obtained from Shanghai SLAC Experimental Animal Co., Ltd. [Animal Production License No. SCXK (Shanghai) 2017-0005; Certificate of Conformity No. 0351618; Animal Facility License No. SYXK (Zhe) 2015-0008].

Serum preparation

Since bilirubin is poorly soluble in water, a rat model was established to prepare bilirubin-containing serum [19, 20]. Five 6-week-old Sprague-Dawley rats were administered unconjugated bilirubin (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 mg/kg/day via oral gavage once daily for 7 consecutive days. Anaesthesia was induced with 1% sodium pentobarbital (0.2 ml/20 g body weight). On day 7, blood samples were collected 1 h post-gavage on a sterile surgical platform. Animals were euthanized in compliance with institutional animal ethics protocols through an approved anesthetic regimen involving gradual induction. Prior to euthanasia, deep anesthesia was confirmed by absence of pedal withdrawal reflex, corneal response, and tail pinch reflex. Following verification of the anesthetic state, blood was promptly collected via cardiac puncture using aseptic techniques. Serum was obtained after allowing blood to clot at room temperature for 2 h, followed by centrifugation at 2000 rpm for 20 min. Bilirubin-containing serum was adjusted to a concentration of 200.0 ± 10.0 μmol/L, while control serum was diluted to $\leq 10.0 \pm 2.0$ μmol/L bilirubin using saline. Bilirubin concentrations were quantified using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Samples underwent inactivation treatment in a 56

℃ water bath for 30 min and were stored in aliquots at -80 °C until further analysis.

Cell culture

The rat cardiomyocyte cell line H9C2, derived from embryonic BD1X rat heart tissue, was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, GIBCO, Carlsbad, CA, USA) at 37 ℃ in a 5% CO_2 incubator.

Primary rat cardiomyocytes were isolated from neonatal Sprague-Dawley rats (1–3 days old) in compliance with institutional animal ethics guidelines. Animals were euthanized by cervical dislocation. Ten hearts were transferred to a biosafety cabinet, rinsed three times in ice-cold phosphate-buffered saline (PBS), and placed in an ice bath for processing. Atria and ventricles were dissected, minced into small fragments in ice-cold PBS, and sequentially digested with 0.1% trypsin (Thermo Fisher Scientific, GIBCO, Carlsbad, CA, USA) for 5 min per cycle (5–7 cycles total). After each digestion, the supernatant was collected in DMEM supplemented with 10% FBS, with the first digestion fraction discarded to remove non-myocyte contaminants. Cell suspensions were filtered through a 100–200 mesh cell strainer, centrifuged at 800 rpm for 10 min, and resuspended in culture medium. The cells were incubated in a T75 culture flask for 2 h. Following incubation, non-adherent cells were collected, seeded into 96-well plates, and cultured for 48 h. Purity and viability of myocardial cells were assessed via α-actin immunohistochemical staining. Well-grown myocardial cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and endogenous peroxidases quenched with 3% H_2O_2. A rabbit polyclonal anti-α-actin primary antibody (Abcam, ab7817) was applied to the cells and incubated overnight at 4 ℃ or for 1 h at 37 ℃. Subsequently, a goat anti-rabbit IgG secondary antibody (Zhongshan Jinqiao Biotechnology Co., Ltd., China) was added and incubated at room temperature for 30 min. The staining reaction was visualized using 3,3'-diaminobenzidine (DAB), followed by microscopic observation and analysis (Fig. 1).

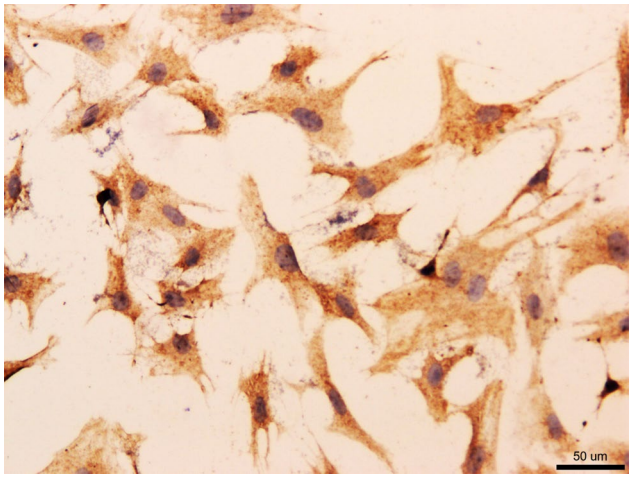

Fig. 1 Identification of cardiomyocytes. The phase-contrast images of cardiomyocytes in culture. Scale bar: 50 μm

Drug treatments

Primary rat cardiomyocytes were cultured in DMEM supplemented with bilirubin-rich or control serum at defined proportions and assigned to four experimental groups based on serum composition: control group (10% normal control serum), low bilirubin group (10% volume fraction of bilirubin serum, 20 μmol/L), medium bilirubin group (20% volume fraction of bilirubin serum,40 μmol/L), high bilirubin group (30% volume fraction of bilirubin serum, 60 μmol/L).

A vitamin C intervention group was established by supplementing the medium with vitamin C (China National Institutes for Drug Control, China) at a final concentration of 0.5 mmol/L. Cells were initially cultured for 48 h. Cardiomyocyte injury was observed under an inverted microscope (Nikon, Tokyo, Japan). After discarding the old medium, fresh medium was added according to the assigned grouping, and cells were further cultured for 48 h with six replicates per group.

CCK8 assay

The cell viability of H9C2 cells was assessed using the CCK-8 assay as previously described [21]. Cells were seeded in 96-well plates at a density of 1×10^4 cells/100 μl/well. Following removal of the original medium, 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, followed by incubation at 37 ℃ in the dark for 1 h. Absorbance was measured at 450 nm using a microplate reader (MD Spiro, Lewiston, ME, USA). Cell viability was quantified using the formula: viability (%) = (OD value of bilirubin-treated group/OD value of control group) × 100%.

Superoxide dismutase assay

The superoxide dismutase (SOD) detection kit (A001-1) was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Follow the kit instructions, 1 ml of Reagent 1 was added to reaction tubes followed by sequential addition of 0.1 ml sample (0.1 ml distilled water for control tubes), 0.1 ml Reagent 2, 0.1 ml Reagent 3, and 0.1 ml Reagent 4. The reaction mixture was thoroughly vortexed and incubated at 37 ℃ for 40 min. Colorimetric analysis was performed at 550 nm using a spectrophotometer, with SOD activity calculated according to the manufacturer's instructions.

Glutathione peroxidase assay

The glutathione peroxidase (GPx) detection kit was obtained from Nanjing Jiancheng Bioengineering Institute (A005, Nanjing, China). For the assay, 200 μl of sample, standard control, or distilled water (as the blank control) were added to separate tubes, followed by the addition of 100 μl reagent solution. The tubes were incubated in a 37 ℃ water bath for 5 min, then Reagent 2 was introduced. After centrifugation at 3500–4000 rpm for 10 min, the supernatant was collected for absorbance measurement at 412 nm using a spectrophotometer. GPx activity was calculated according to the manufacturer's protocol.

Na^+/K^+-ATPase detection

The Na^+/K^+-ATPase detection kit was obtained from Nanjing Jiancheng Bioengineering Institute (A070-2, Nanjing, China). For the assay, 100 μl of sample, standard control, or double-distilled water (as blank control) were added to separate tubes. After adding 420 μl matrix solution and 100 μl Reagent IV, the tubes were centrifuged at 3500–4000 rpm for 10 min. The supernatant was collected and absorbance measured at 626 nm using a spectrophotometer. Na^+/K^+-ATPase activity was calculated according to the manufacturer's instructions.

Creatinine kinase MB and cardiac troponin I

The determination of creatine kinase-MB (CK-MB) and cardiac troponin I (cTn-I) was performed using an enzyme-linked immunosorbent assay (ELISA) kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). 50 μl of diluted standard solutions and serum samples were added to the wells of a 96-well ELISA plate and incubated at 37 ℃ for 30 min. After discarding the liquid, the plate was washed five times with 300 μl of 1× washing buffer (2 min per wash). Subsequently, 50 μl of HRP-conjugated CK-MB or cTnI antibody was added to each well, followed by incubation at 37 ℃ for 30 min. The plate was then washed five times under identical conditions. For color development, 50 μl of TMB Substrate A and 50 μl of TMB Substrate B were added to each well. After gentle mixing, the plate was incubated at 37 ℃ in the dark for 15 min. The reaction was terminated by adding 50 μl of stop solution, and the optical density (OD) was measured at 450 nm within 15 min using a microplate reader. Protein concentrations were calculated based on the standard curve generated by serial dilutions of reference standards.

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Normality of continuous variables was assessed via the Shapiro-Wilk test. Normally distributed data are presented as mean ± standard deviation (SD), with intergroup comparisons performed using Student's t - test for two-group comparisons and one-way analysis of variance (ANOVA) for multiple group comparisons. Graphical representations were generated with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). For normally distributed data, Pearson's correlation analysis was employed to evaluate associations between variables. Statistical significance was defined as $P < 0.05$.

Results

Changes in the viability of H9C2 cardiomyocytes and primary rat cardiomyocytes after treatment with different concentrations of bilirubin

The CCK-8 assay was utilized to evaluate the effects of bilirubin on the viability of both H9C2 cardiomyocytes and primary rat cardiomyocytes. Our results demonstrated a concentration-dependent cytotoxic effect of bilirubin on H9C2 cells. Compared to controls (100.44 ± 2.33%), cell viability progressively decreased with increasing bilirubin concentrations (79.12 ± 2.66%, 60.39 ± 7.50%, and 33.47 ± 3.10% for low, medium, and high concentrations, respectively; all $P < 0.01$ vs. control). Statistical analysis revealed significant viability reductions between consecutive concentration groups: low vs. medium ($P < 0.05$) and medium vs. high ($P < 0.01$), confirming the dose-dependent nature of bilirubin's cytotoxic effects.

Similarly, in the primary rat cardiomyocyte culture experiments, bilirubin treatment significantly reduced cell viability in a concentration-dependent manner compared to the control group. The high-concentration bilirubin group exhibited the most pronounced decrease in viability (control vs. low, medium, or high bilirubin: 99.3 ± 2.2% vs. 57.7 ± 6.4%, 43.8 ± 9.0%, and 20.2 ± 11.0%, respectively; all $P < 0.01$). However, co-treatment with vitamin C markedly attenuated this effect. Notably, all bilirubin-treated groups supplemented with vitamin C demonstrated significantly higher cell viability compared to their bilirubin-only counterparts (low bilirubin: 57.7 ± 6.4% vs. 93.9 ± 4.2%; medium bilirubin: 43.8 ± 9.0% vs. 87.8 ± 6.8%; high bilirubin: 20.2 ± 11.0% vs. 73.9 ± 14.2%; all $P < 0.01$) (Fig. 2). These findings indicate that bilirubin exerts a concentration-dependent cytotoxic effect on cardiomyocytes, while vitamin C effectively counteracts this detrimental impact.

Changes in biomarkers of primary rat cardiomyocyte injury after treatment with different concentrations of bilirubin

Primary rat cardiomyocytes were exposed to varying concentrations of bilirubin in cell culture medium. Biochemical parameters, including SOD, GPx, CK-MB, cTn-I, and Na^+/K^+-ATPase activity, were measured in the culture supernatant.

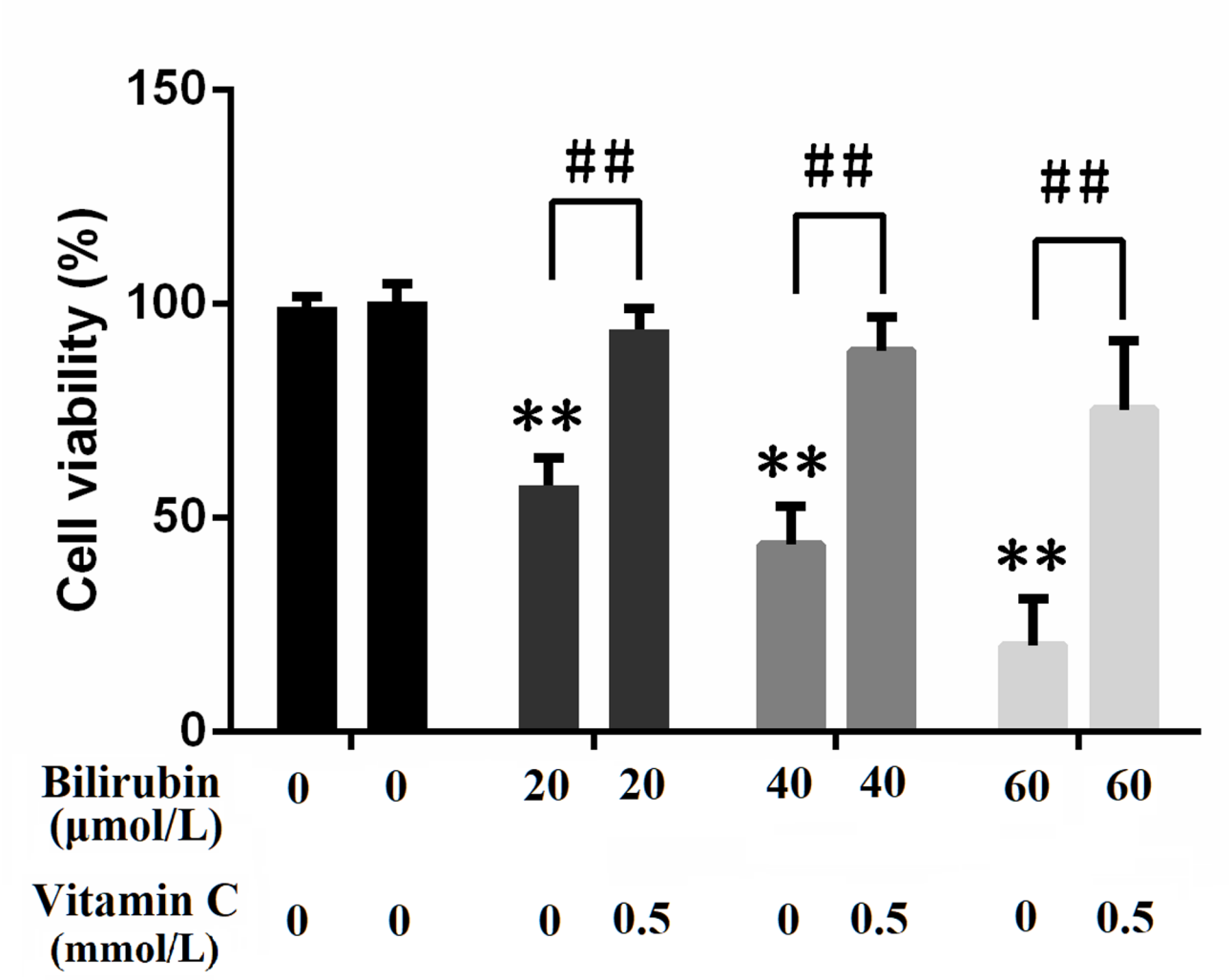

Fig. 2 Vitamin C attenuates bilirubin-induced viability reduction in primary rat cardiomyocytes. CCK-8 assay showing concentration-dependent viability reduction following 48-hour bilirubin exposure (0, 20, 40, 60 μmol/L; $^{**}P < 0.01$ vs. control), co-treatment with 0.5 mmol/L vitamin C significantly restores cellular viability ($^{\#\#}P < 0.01$ vs. corresponding bilirubin groups)

Compared to the control group, the concentrations of CK-MB and cTn-I in the culture medium increased significantly in a concentration-dependent manner [control vs. low, medium, or high bilirubin: (1) CK-MB: 1.68 ± 0.11 vs. 2.26 ± 0.10, 2.48 ± 0.01, and 3.73 ± 0.13 U/L; (2) cTn-I: 139.13 ± 8.01 vs. 223.70 ± 14.12, 315.00 ± 33.73, and 378.67 ± 39.93 pg/ml; All $P < 0.01$]. Specifically, the increase in CK-MB levels from the low to medium bilirubin group was significant ($P < 0.05$), and from the medium to high bilirubin group also reached significance ($P < 0.01$). A similar concentration-dependent elevation was observed for cTn-I. Notably, the addition of 0.5 mmol/L vitamin C significantly attenuated the elevation of CK-MB levels in the low and high bilirubin groups (low bilirubin: 2.26 ± 0.10 vs. 1.92 ± 0.07 U/L; high bilirubin: 3.73 ± 0.13 vs. 2.24 ± 0.50 U/L; all $P < 0.01$) and reduced cTn-I levels in the medium and high bilirubin groups (medium bilirubin: 315.00 ± 33.73 vs. 243.73 ± 19.98 pg/ml; high bilirubin: 378.67 ± 39.93 vs. 278.43 ± 16.30 pg/ml; all $P < 0.01$) (Fig. 3A, B). However, no significant differences were detected in CK-MB levels within the medium bilirubin group or in cTn-I levels within the low bilirubin group compared to controls. These findings collectively suggest that vitamin C exerts a partial concentration-dependent protective effect against bilirubin-induced cardiomyocyte injury by suppressing the release of cardiac injury biomarkers.

GPx levels in cardiomyocytes exposed to low, medium, or high bilirubin concentrations exhibited a significant decrease compared to the control group (control vs. low, medium, or high bilirubin: 44.02 ± 4.32 vs. 29.32 ± 5.02, 27.71 ± 6.80 and 22.98 ± 6.78 U/L; all $P < 0.01$). The reduction in GPx levels followed a concentration-dependent trend, with progressively greater suppression observed at higher bilirubin concentration. However, supplementation with 0.5 mmol/L vitamin C significantly elevated

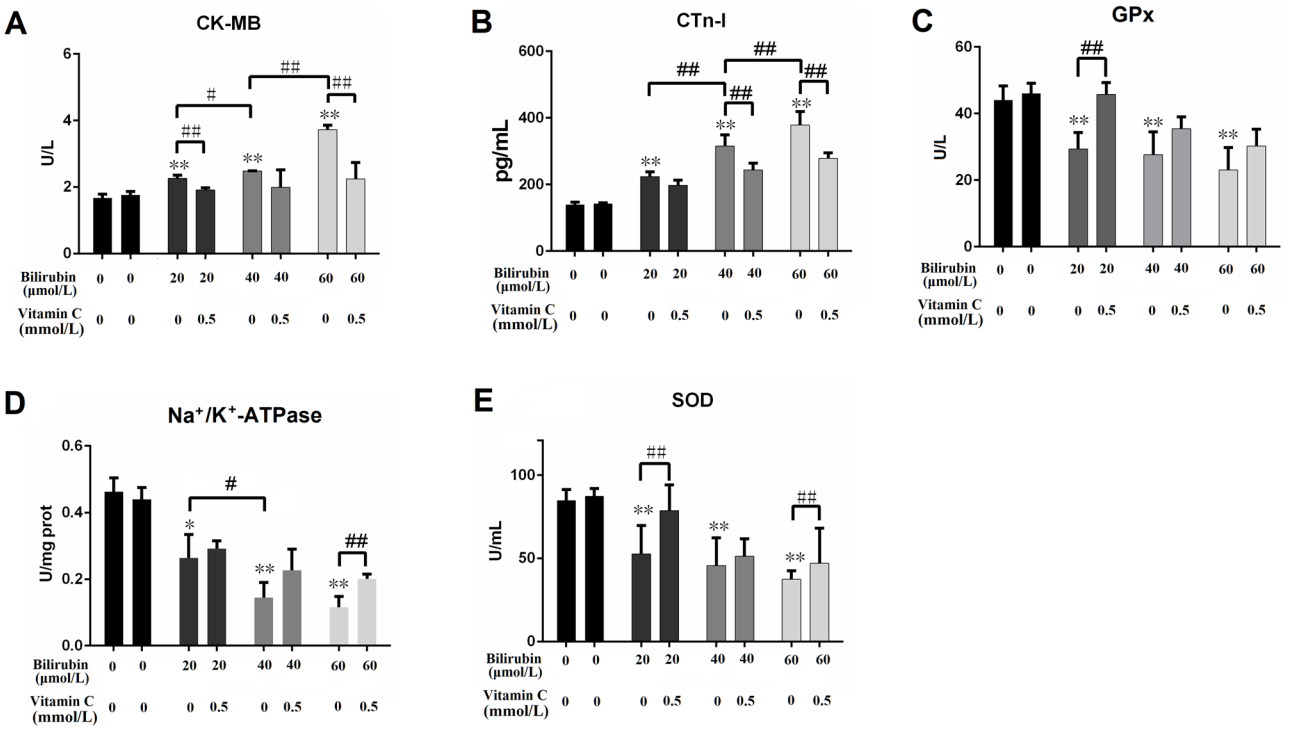

Fig. 3 Concentration-dependent bilirubin toxicity versus vitamin C intervention: cardiac biomarker release and antioxidant defense in primary rat cardiomyocyte models. (**A**) Creatine kinase-MB (CK-MB), (**B**) cardiac troponin-I (cTn-I), (**C**) glutathione peroxidase (GPx), (**D**) Na+/K+-ATPase activity, and (**E**) superoxide dismutase (SOD) levels following 48-hour bilirubin exposure(0, 20, 40, 60 μmol/L), and 0.5 mmol/L vitamin C co-treatment partially mitigates bilirubin-induced biochemical alterations (**$P < 0.01$ vs. control group; ##$P < 0.01$ for intergroup comparisons)

GPx levels in the low bilirubin group (29.32 ± 5.02 vs. 45.82 ± 3.49 U/L, $P < 0.01$) (Fig. 3C), highlighting its protective effect in preserving antioxidant enzyme function.

Similarly, Na^+/K^+-ATPase activity in cardiomyocytes exposed to low, medium, and high bilirubin concentrations exhibited a dose-dependent decline compared to the control group (control vs. low, medium, or high bilirubin: 0.463 ± 0.041 vs. 0.264 ± 0.070, 0.145 ± 0.045 and 0.115 ± 0.032 U/mgprot; all $P < 0.01$ or $P < 0.05$). Specifically, the reduction in enzyme activity was statistically significant from the low to medium bilirubin group ($P < 0.05$). Notably, supplementation with 0.5 mmol/L vitamin C significantly restored Na^+/K^+-ATPase activity in the high bilirubin group (0.115 ± 0.032 vs. 0.200 ± 0.014 U/mgprot, $P < 0.01$) (Fig. 3D), demonstrating its capacity to mitigate bilirubin-induced impairment of this critical membrane enzyme.

SOD activity assays demonstrated that exposure of cardiomyocytes to low, medium, and high bilirubin concentrations significantly suppressed SOD activity in a concentration-dependent manner compared to the control group (control vs. low, medium, or high bilirubin: 84.87 ± 6.44 vs. 52.65 ± 17.22, 45.68 ± 16.79 and 37.51 ± 5.04 U/ml, all $P < 0.01$). The degree of SOD activity reduction correlated positively with increasing bilirubin levels. Notably, vitamin C supplementation at 0.5 mmol/L significantly elevated SOD activity in both the medium and high bilirubin groups (medium bilirubin: 52.65 ± 17.22 vs. 78.64 ± 15.52 U/ml; High bilirubin: 37.51 ± 5.04 vs. 47.05 ± 21.21 U/ml; all $P < 0.01$) (Fig. 3E), demonstrating its capacity to counteract bilirubin-induced suppression of this key antioxidant enzyme.

Table 1 Correlational analysis between serum bilirubin concentrations and cardiomyocyte pathophysiological indices: cardiac injury biomarkers (CK-MB, cTn-I), antioxidant enzymes (GPx, SOD), and Na^+/K^+-ATPase activity in primary rat cardiomyocytes

Bilirubin	CK-MB	CTn-I	GPx	SOD	Na^+/K^+-ATPase
r value	0.948	0.969	-0.787	-0.804	-0.913
P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Correlation analysis

Pearson's correlation analysis revealed significant associations between bilirubin concentrations and key myocardial injury biomarkers in primary rat cardiomyocytes. Specifically, CK-MB and cTn-I exhibited positive correlations with bilirubin levels ($r = 0.948–0.969$, $P < 0.01$), whereas GPx, Na^+/K^+-ATPase, and SOD demonstrated negative correlations (r =– 0.787 to– 0.913, $P < 0.01$) (Table 1), indicating a clear inverse relationship between

antioxidant enzyme activity and bilirubin-induced cardiac damage.

Discussion

Elevated bilirubin levels are known to cause neonatal cellular damage [4, 5]. This study investigated bilirubin-induced injury in neonatal rat cardiomyocytes exposed to varying bilirubin concentrations. Results demonstrated that hyperbilirubinemia causes dose-dependent cardiomyocyte injury, with bilirubin levels correlating with cellular damage markers. These findings highlight potential clinical implications for preventing permanent myocardial damage in infants with neonatal jaundice.

Neonatal hyperbilirubinemia with jaundice represents a common clinical phenomenon, observed in up to 80% of newborns during the initial postnatal weeks [1–3]. This transient condition typically arises from a physiological imbalance between bilirubin production and elimination, with levels generally normalizing as hepatic maturation progresses and metabolic homeostasis is established [22]. However, excessive bilirubin accumulation poses significant biological risks by disrupting critical cellular processes (including membrane potential regulation, energy metabolism, and neurotransmitter synthesis), potentially leading to multi-organ dysfunction and kernicterus in severe cases [4, 5, 7]. Notably, while bilirubin's neurotoxic effects are well-characterized, its cardiotoxic potential in neonates remains insufficiently understood [8]. Current diagnostic paradigms for myocardial injury, particularly myocardial enzyme assays and electrocardiographic monitoring developed for adults, demonstrate limited sensitivity in neonatal populations [1]. This diagnostic gap becomes clinically consequential given evidence suggesting that transient bilirubin elevation may induce irreversible cardiomyocyte damage, even after serum bilirubin normalization. Such subclinical myocardial injury could manifest progressively as cardiac functional impairment, cellular necrosis, or catastrophic cardiovascular events [23].

While developing preventive strategies requires clarifying bilirubin's dose-cardiotoxicity relationship, our study exposed H9C2 and primary rat cardiomyocytes to graded bilirubin concentrations. Results demonstrated dose-dependent cardiomyocyte viability loss, likely via oxidative stress-mediated disruption of mitochondrial energy metabolism [24, 25]. Contrary to bilirubin's known antioxidant effects at physiological levels, these findings reveal its cytotoxicity at supra-physiological concentrations, emphasizing the need for precise bilirubin monitoring in neonatal hyperbilirubinemia management.

cTn-I and CK-MB serve as gold-standard biomarkers for myocardial injury, exhibiting diagnostically relevant sensitivity and specificity that correlate with injury severity [26]. As a cardiomyocyte-specific structural protein, cTn-I is exclusively expressed in cardiac tissues and undergoes sustained release during cardiomyocyte necrosis. CK-MB is a tissue-specific enzyme predominantly found in cardiomyocytes; when the myocardium is damaged, CK-MB is rapidly released into the extracellular space through the compromised cell membrane [27]. In this study, elevated bilirubin levels were found to cause significant injury to primary rat cardiomyocytes compared with the control group ($P<0.01$). Moreover, the degree of cardiomyocyte damage was positively correlated with increasing bilirubin concentrations. These findings suggest that excessive bilirubin may disrupt the metabolic function of cardiomyocytes by altering cellular structure or membrane potential, thereby impairing normal metabolism, inhibiting glycolysis and protein synthesis, and ultimately leading to myocardial injury [28].

The levels of Na^+/K^+-ATPase in the low, medium, and high bilirubin groups were significantly reduced compared to those in the control group ($P<0.05$ or $P<0.01$). Na^+/K^+-ATPase, predominantly located in the inner cell membrane, plays a key role in catalyzing ATP hydrolysis to generate energy required for ion transport [29]. Elevated bilirubin levels have been shown to impair mitochondrial structure in cardiomyocytes, which may ultimately lead to heart failure in severe cases [30]. Na^+/K^+-ATPase functions by actively transporting three sodium ions out of the cell and two potassium ions into the cell per ATP molecule cleaved, thereby maintaining the resting membrane potential, ensuring cellular excitability and electrical conduction, and influencing myocardial contractility [31, 32]. Therefore, the observed decrease in Na^+/K^+-ATPase activity following bilirubin exposure suggests impaired cardiomyocyte function and a potential disruption of normal cardiac physiology.

SOD, GPx, and other antioxidant enzymes play a crucial role in maintaining the balance between oxidative and antioxidative factors within cardiomyocytes. Excessive oxidase activity or reduced antioxidant enzyme function can lead to oxidative stress [33], which may cause irreversible oxidation of intracellular DNA, proteins, and lipids, resulting in mitochondrial dysfunction, increased accumulation of reactive oxygen species (ROS), and ultimately, cellular damage [34, 35]. In the present study, SOD levels in all bilirubin-treated groups were significantly lower than those in the control group. Similarly, GPx levels were markedly decreased in the bilirubin-exposed groups compared to the control group. These findings are not entirely consistent with those reported by Fiorelli et al. [36]. This discrepancy may be attributed to a compensatory decrease in SOD levels in response to altered oxidative stress conditions. Alternatively, differences in the establishment of the oxidative-antioxidative imbalance model in cardiomyocytes between studies might account for the variation. As a well-established

antioxidant, vitamin C has long been recognized for its therapeutic potential in clinical antioxidant interventions. Zhang et al. [37]. suggested that incorporating vitamin C into the treatment regimen for myocardial injury could offer protective benefits. In the present study, vitamin C was added to cell cultures to evaluate its potential protective effects against bilirubin-induced myocardial injury. The results suggested limited protective efficacy of vitamin C, with partially improved outcomes observed in specific biomarker-group combinations: Na^+/K^+-ATPase activity(high bilirubin group), GPx activity (low bilirubin group), CK-MB & SOD levels (low and high bilirubin groups), cTnI levels (medium and high bilirubin groups).

This study revealed significant correlations between bilirubin levels and myocardial injury markers, including SOD, GPx, CK-MB, cTnI, and Na^+/K^+-ATPase, suggesting a potential association between bilirubin and myocardial injury. However, further investigations, particularly in vivo studies, are warranted to elucidate the direct effects of bilirubin on cardiomyocyte damage. Additionally, clinical correlation studies involving human blood biomarkers could provide valuable insights into the relationship between bilirubin and cardiac injury in patients.

This study underscores the importance of early screening for myocardial injury in neonates with hyperbilirubinemia, particularly in those presenting with severe jaundice. Incorporation of cardiac biomarkers (CK-MB and cTn-I) into standardized neonatal screening protocols could improve early identification of subclinical myocardial injury, enabling prompt therapeutic strategies including phototherapy intensification or exchange transfusion to mitigate progression toward irreversible cardiac compromise. Clinicians should also consider implementing individualized bilirubin management strategies, carefully balancing the neuroprotective benefits of bilirubin reduction with potential cardiac risks, especially in vulnerable populations such as preterm infants or those with comorbid conditions affecting bilirubin metabolism [38].

Further in vivo studies utilizing neonatal animal models are crucial for validating the dose-dependent mechanisms of bilirubin-induced cardiac injury and assessing long-term functional outcomes. Human cohort studies should aim to correlate bilirubin levels, cardiac biomarkers (such as CK-MB and cTn-I), and echocardiographic findings in jaundiced neonates to establish risk thresholds. Comparative trials investigating the outcomes of early biomarker-guided therapy versus standard care could help optimize clinical protocols. Additionally, research focusing on the mechanisms underlying bilirubin-induced mitochondrial dysfunction or antioxidant depletion may uncover potential therapeutic targets, such as mitochondrial protectants or targeted antioxidants [39].

Conclusions

In conclusion, by examining changes in the activity of myocardial injury markers in rat cardiomyocytes, this study demonstrated that elevated bilirubin levels can induce varying degrees of injury in primary rat cardiomyocytes. These findings may offer new insights for the clinical management of patients with hyperbilirubinemia, particularly neonates with jaundice. Early detection and intervention strategies such as monitoring cardiac biomarkers and administering antioxidants like vitamin C could potentially help prevent or mitigate cardiomyocyte damage. However, further in vivo studies and human clinical trials are necessary to confirm these results and to explore more effective preventive and therapeutic approaches.

Abbreviations

SOD	Superoxide dismutase
GPx	Glutathione peroxidase
CK-MB	Creatine kinase MB
cTn-I	Cardiac troponin I
HO-1	Heme oxygenase-1

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Not applicable.

Author contributions

HY and JJZ carried out the studies, participated in collecting data, and drafted the manuscript. XJW and DYW performed the statistical analysis and participated in its design. YXK and WJF participated in acquisition, analysis, or interpretation of data and draft the manuscript. All authors read and approved the final manuscript.

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None.

Data availability

All data generated or analysed during this study are included in this published article.

Declarations

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Ethics approval

Al methods were carried out in accordance with relevant guidelines and regulations. The animal experiment was executed with the agreement from the Institutional Animal Care and Use Ethics Committee of Hangzhou Hibio Technology Company (approval No. HB-20150608),all methods are reported in accordance with ARRIVE guidelines.

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