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DUAL EFFECTS OF BILIRUBIN ON THE PROLIFERATION OF RAT RENAL NRK52E CELLS AND ITS ASSOCIATION WITH GAP JUNCTIONS

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□ **Objective:** The effect of bilirubin on renal pathophysiology is controversial. This study aimed to observe the effects of bilirubin on the proliferation of normal rat renal tubular epithelial cell line (NRK52E) and its potential interplay with gap junction function.

Methods: Cultured NRK52E cells, seeded respectively at high- or low- densities, were treated with varying concentrations of bilirubin for 24 hours. Cell injury was assessed by measuring cell viability and proliferation, and gap junction function was assessed by Parachute dye-coupling assay. Connexin 43 protein was assessed by Western blotting.

Results: At doses from 17.1 to 513μmol/L, bilirubin dose-dependently enhanced cell viability and colony-formation rates when cells were seeded at either high- or low- densities (all $p<0.05$ vs. solvent group) accompanied with enhanced intercellular fluorescence transmission and increased Cx43 protein expression in high-density cells. However, the above effects of BR were gradually reversed when its concentration increased from 684 to 1026μmol/L. In high-density cells, gap junction inhibitor 12-O-tetradecanoylphorbol 13-acetate attenuated bilirubin-induced enhancement of colony-formation and fluorescence transmission. However, in the presence of high concentration bilirubin (1026μmol/L), activation of gap junction with retinoid acid decreased colony-formation rates.

Conclusion: Bilirubin can confer biphasic effects on renal NRK52E cell proliferation potentially by differentially affecting gap junction functions.

Keywords: Bilirubin, NRK52E cells, gap junction, connexin, cell proliferation, kidney injury

INTRODUCTION

Bilirubin (BR), one of the main terminal metabolites of hemoglobin, increases in patients with liver dysfunction or perioperatively in patients with liver transplantation (Alvares-da-Silva *et al.* 1999; Hei *et al.* 2008). We and other scholars found that the apparent increase of BR is a major risk factor of acute kidney injury during liver failure or liver transplantation

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perioperatively (Lafayette *et al.* 1997; Alvares-da-Silva *et al.* 1999; Lima *et al.* 2003; Faenza *et al.* 2006). It is reported that alteration of gap junction (GJ) function had participated in the pathophysiologic processes of diseases and ischemic organ injuries, including heart and kidney (Harris 2001; Rodríguez-Sinovas *et al.* 2006; Mese *et al.* 2007; Desforges *et al.* 2011; Sorensen and Holstein-Rathlou 2011). However, it is unknown whether or not BR may exacerbate renal injury by alternating GJ function.

GJ channel proteins are composed of special channel protein –connexins (Cxs), which can combine with one another for form complexes that have diverse functions involving the regulation of cell proliferation, cell differentiation, and cell apoptosis (Harris 2001; Kalvelyte *et al.* 2003; Vinken *et al.* 2006; Mese *et al.* 2007; Harris 2008). Gap junctions, as intercellular protein channels directly connecting adjacent cells, ubiquitously present in human parenchymatous organs (such as kidney, heart and liver) and facilitate the functioning of groups of cells (Harris 2001). GJs directly link the cytoplasmic compartments of adjacent cells so that ions, signal molecules and metabolic substances with molecular weight no more than 1kDa may be exchanged, as such the functional intercellular reactions are coincidently and synchronously achieved based on the feature of rapid conduction and low impedance (Harris 2008).

Cx proteins are the major component of GJ. In kidney there exist several Cxs (including Cx 37, 40, 43, and 45), among which Cx43 was the main channel protein for renal tubule epithelial cells (Haefliger *et al.* 2006; Takenaka *et al.* 2008). Proper functioning of GJ is important for the maintenance of renal cell viability. Deficiency of Cx43 and ATP in kidney has been shown to be associated with renal tubule epithelium damage (Vergara *et al.* 2003). On the other hand, overexpression of Cx43 was seen in renal tubule epithelium in puromycin aminonucleoside-induced renal injury (Yaoita *et al.* 2002). Given that renal cell injury and kidney dysfunction often occurs in patients with liver diseases with concomitant significant increase of plasma BR and that GJ plays an important role in maintaining renal cell integrity, we hypothesized that BR may exacerbate renal cell injury by altering Cx protein expression and GJ function.

The hypothesis was tested in cultured normal rat renal tubular epithelial cell line (NRK52E), a major cell type of the kidney parenchyma that is most vulnerable to notorious stimuli, treated with varying concentrations of BR.

METHODS

Reagents

Bilirubin, NaOH, crystal violet**,** 12-O-tetradecanoylphorbol 13-acetate (TPA), and retinoid acid (RA) were purchased from Sigma (American). Cell culture reagents, fetal bovine serum, phosphate-buffererd saline

(PBS), Dulbecco's modified eagle's medium (DMEM)-F12, and Pentazyme digestive enzyme were from Gibco (USA). Calcein-acetoxymethyl ester (calcein-AM) and CM-Dil were from Invitrogen (USA). Dimethyl sulphoxide (DMSO) solvent and methyl thiazolyl tetrazolium (MTT) were from Amresco (USA).

Bilirubin, TPA and RA treatment

100mg crystal BR protection from light was initially dissolved with 0.1 M NaOH (1mol/L NaOH 1ml and then topped up to 10 ml with 9 ml ultra-pure water) and was adjusted to pH 7.4 by adding equal amounts of 0.1 M HCl. NRK52E cells were seeded into 96-well plates and were cultured for 24 hours after the addition of BR at various concentrations (range from 0 which serve as solvent group, 17.1, 85.5, 171, 342, 513, 684, 855, and 1026μmol/L (5 wells for each concentration). Cells treated with 10% fetal bovine serum only without BR solvent (i.e., 0.1M NaOH) were viewed as medium control group to differentiate from the solvent group (BR concentration at 0μmol/L but with BR solvent 0.1M NaOH).

GJ inhibitor TPA was initially dissolved in DMSO at 14.1mg/mL (50mM), and was diluted with culture medium to a final concentration of 50 nM. The concentration of TPA (50nM) was chosen based on the study of Rakib and colleagues (Rakib *et al.* 2010), who demonstrated in human mammary epithelial cell line that TPA induced a dose- and time- dependent inhibition of GJ intracellular communication and the maximum inhibitory effect was obtained at the concentration of 50 nM. GJ agonist RA was dissolved in DMSO at 30.04mg/mL (10mM), and was diluted with culture medium to a final of concentration of 10μM in culture medium. The concentration of RA (10μM) was chosen based on the finding of Watanabe (Watanabe *et al.* 1999), who showed that RA at 10μM greatly enhanced renal epithelial cell GJ intracellular communication as well as our recent study which confirmed that RA at 10μM also most prominently enhanced GJ intracellular communication in cultured NRK52E cells (Hei *et al.* 2012, in press). Cells were pretreated with TPA for 1h and RA for 24h before performing parachute dye-coupling assay, and cells were continuously exposed to these reagents during parachute dye-coupling assay.

Cells culture

NRK52E was kindly provided by Prof. Xueqing Yu at the First Affiliated Hospital of Sun Yat-sen University (China). Cells were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO_2 (Hepac Class 100 incubator; Thermo Scientific, USA).

Standard MTT assay to assess cell survival rate

NRK52E cells were plated in 96-well plates and cultured in 37°C incubator with 5% CO_2. Rate of MTT uptake (by viable cells) was observed under an inverted microscope (LW200-37XB; Olympus, Japan) and quantitated by measuring absorbance (optical density, OD) at 490 nm on a microplate reader (ELX800; Bio-Tek, USA). Survival rate was calculated as: [(OD_{490} *experiment group* - OD_{490} blank) / (OD_{490} medium control group - OD_{490} blank) ×100%].

Standard Colony-formation Assay to detect Colony-formation rates for high- and low- density cells

Colony-formation rate was detected by standard colony-formation assay (Jensen and Glazer 2004) on the basis of the presence or absence of GJ channel formation in high- or low- density cells, respectively. Clonogenic cells, with continuous reproductive activity, could form later generations with colony up to 50cells/cm^2 after six generations. To avoid the potential confounding factor that cells at different growth stage may have different sensitivity to BR treatment, all cells were synchronized in generation 1 (G1) stage and were cultured in serum-free medium for 24h before the addition of BR. Quantitative analysis for clonogenic cells was done by counting the colony-formation to reflex the effects of BR on cellular proliferation activity. Colony-formation rate was calculated as: (*Colony counts experiment group/* Colony counts medium control group) ×100%.

Cells were seeded at different densities to obtain cells respectively with high rate of confluence (in high-density cells) or rarely have confluence (low-density cells), so that to facilitate the study of GJ function. For the high-density condition, cells were seeded at 8×10^5 cells/ml which could form 70% to 100% confluence, while cells seeded at 500cells/ml (low-density) did not have confluence formation, nor did they have GJ formation.

Parachute dye-coupling assay for fluorescence transmission detection

In a separate experiment, NRK52E cells cultured in high-density were firstly treated as above in various groups using 12-well plates. At the end of the treatment, cells were digested with trypsin, and then cell medium was taken from one of the twelve wells to wash with PBS once before the addition of 1ml DMEM-F12 (with fluorescent reagent calcein-AM 2.5μl and Dil-CM 5μl) to form donor cells, followed by incubation for 30min. Thereafter, donor cells were further prepared into cell suspension (800/ml) with DMEM-F12. The cell suspension (1 ml) was added into all the other wells and the medium was imbibed and washed with PBS to form receiver cells. The donor cells were then seeded onto the receiver

cells at a 1:150 donor/receiver ratio. The donor cells were allowed to attach to the monolayer of receiver cells 4 h at 37°C to facilitate the formation of gap junctions and then examined with a fluorescence microscope as previously described (Wang *et al.* 2010). For each experimental condition, the number of receiver cells to donor cell containing calcein was counted. Gap junction communication was assessed as the number of receiver cells receiving calcein from a labeled cell (He *et al.* 2009). Meanwhile, for each group, 12 different 200× visual fields on the diameter of every dish were photographed by a fluorescence microscope under the same conditions. In every field, all the donor cells including their receive cells were calculated by the same researcher to reduce the errors.

Western blotting

NRK52E cells were washed thrice with ice-cold PBS, and then added lysis buffer [50mmol/L NaH_2PO_4, 50mmol/L NaCl, 5mmol/L EDTA, 5mmol/L EGTA, 80mmol/L n-octyl β-D-glucopyranoside, 1mmol/L â-mercaptoethanol, 0.5mmol/L diisopropyl fluorophosphates (pH 7.5)] (7μl protease inhibitor was added into lysis buffer per ml) 50-100μl/well. Cells were scrapef off for porphyrization and centrifuged for 30 min at 4°C and then supernatant was taken for protein quantitation. After electrophoresis for 90min and trarsmembrane for 90min at 100V, polyvinylidene difluoride membrane was enveloped in 4% skimmed milk for 60min, and then blocked with 5% skim dry milk in wash buffer, incubated overnight with primary antibody (mouse anti-Cx43 clone Cx43 IgG, 1:2000 dilution; Sigma), washed with TBST thrice and then blocked with 5% skim dry milk, incubated for 30min with secondary antibody (1:3000 dilution; Sigma). The primary antibody for β-action was diluted at 1:10,000 (sigma, USA), and secondary antibody was diluted at 1:10,000, (Sigma, USA). The primary antibody for connexin was anti-Cx43 (Sigma; 1:3000), and secondary antibody was anti-mouse IgG (1:3000, Sigma).

All Western blot exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by Bio Imaging system (Gene Genius).

Data analysis and statistics

The data were expressed as mean ± standard deviation or as median (range), as appropriate. Comparison of more than two groups was determined by One-Way Analysis of Variance (ANOVA) followed by the LSD multiple-comparison test. Colony-formation rates between the high- and low-density cells were analyzed by the Student's *t*-test. A probability level of $p < 0.05$ was selected to indicate statistically significant differences.

RESULTS

Cell survival rate under BR challenge

Exposure to BR induced dose-dependent and biphasic effects on cell survival rates of NRK52E cells (Figure 1). BR at 17.1μmol/L did not affect cell viability as compared to solvent group (without BR). Of interest, cell viability rates increased gradually when BR concentrations were in the range from 85.5 to 513μmol/L (all $p<0.05$ or $P<0.01$, compared with solvent group). However, cell viability started to decline gradually when BR concentrations was higher than 684μmol/L ($P<0.05$ vs. 513μmol/L). Of note, at 1026μmol/L, BR reduced the cell viability to a level lower than that in the solvent group ($p<0.05$), which indicated cell toxicity.

Cell colony-formation rates in response to BR in low- and high-density cells

GJ formation was lacking in low-density cells. In this situation, colony-formation rate reflected the viability of low-density cells.

Colony-formation rates of NRK52E cells at low- and high- density were assessed after challenging with different doses of BR for 24 h. By this method we could also assess the cytotoxicity of BR on un-fused cells. And

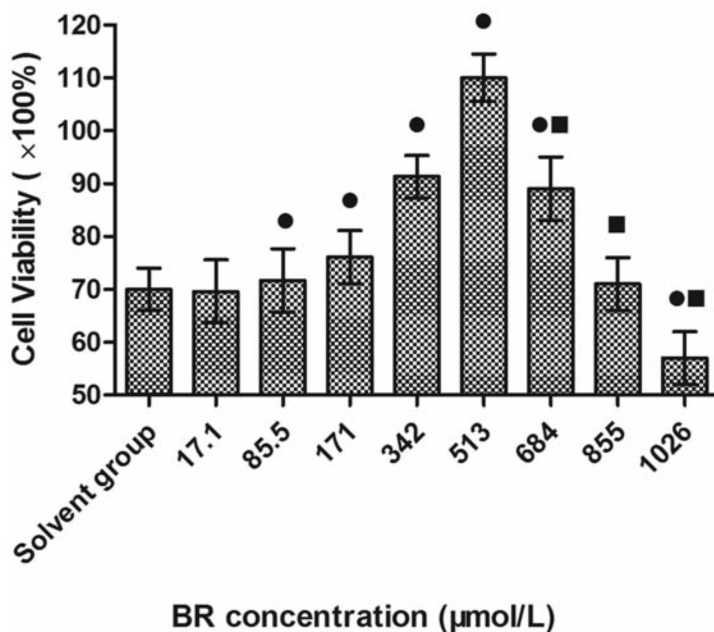

FIGURE 1. The survival rates of NRK52E cells after challenge with BR at varying concentrations for 24 h. Data are presented as mean ± SD of three independent experiments. ● $p < 0.05$ vs. solvent group, ■ $p < 0.05$ vs. BR 513μmol/L.

analyzing the disparity of colony-formation rates between low- and high-density cells can effectively reflect the influence of GJ formation on cell viability.

Similar to its effects on cell viability, BR conferred biphasic effects on colony-formation both in low- and in high-density cells (Figure 2A and 2B). Compared to solvent group, colony-formation rates increased gradually and significantly with the increase of BR concentration up to 513μmol/L) ($p<0.05$, compared with solvent group). But in both low- and high-density cells the rate of colony-formation decreased when BR concentration reached 684μmol/L and above ($P<0.05$ vs. 513μmol/L

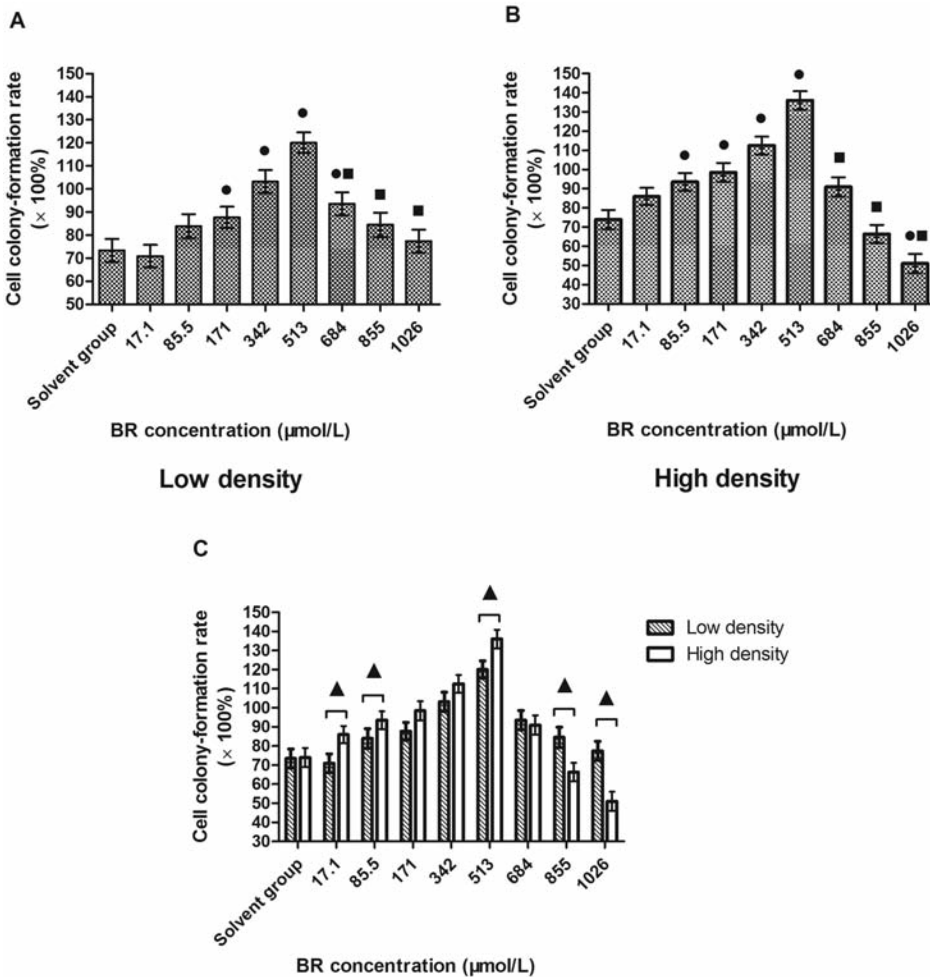

FIGURE 2. BR-induced a dose-dependently biphasic effects on colony-formation rates of (A) low- and (B) high-density NRK52E cells. (C) Comparison between low- and high- density groups. Data are presented as mean ± SD of three independent experiments. ● $p < 0.05$ *vs.* solvent group, ■ $p < 0.05$ *vs.* BR 513μmol/L, ▲ $p < 0.05$ compare high-density cells to low-density cells.

group). Of note, the rate of colony-formation in high-density cells was significantly lower than in the solvent group when BR reached 1026μmol/L.

Compared with low-density cells, BR exerted more prominent effects on rate of colony-formation in high-density cells (Figure 2C). When the BR dose range was between 17.1 and 513μmol/L, the rates of colony-formation in high-density cells were higher relative to that in the low-density cells ($P<0.05$ at 17.1, 85.5 and 513μmol/L). In contrast, when the BR dose range was between 855 and 1026μmol/L BR more prominently reduced rate of colony-formation in high-density cells were seen as compared to low-density cells ($P<0.05$).

Effects o f BR on Fluorescence transmission in high-density NRK52E cells

Parachute dye-coupling assay is a reliable method that can visualize GJ-mediated intercellular information transmission (Goldberg *et al.* 1995; Koreen *et al.* 2004).

As shown in Figure 3, the numbers of fluorescence transmission among NRK52E cells basically mirrored the GJ communicating function at various concentrations of BR treatments. At the BR dose range between 85.5 and 513μmol/L, BR gradually increased the average number of receiver cells around per donor cell by fluorescence transmission detection ($P<0.05$ vs. solvent group), but it subsequently gradually decreased average number of fluorescence transmission from donor cell to receiver cells when its concentration reached 684μmol/L and above ($P<0.05$ vs. BR 513μmol/L), and eventually the fluorescence transmissions at high concentration BR were lower than that in the solvent group ($P<0.05$).

Effects of GJ inhibition or activation on BR-induced changes in colony-formation rate

Cell proliferation and GJ transmission were significantly enhanced by BR at concentrations up to 513μmol/L. To further investigate the effects of GJ inhibition or activation on BR-induced changes in cell viability, we pretreated NRK52E cells at high- and low- density with either 50 nM TPA (a GJ inhibitor) for 1h or with 10 μM RA (a GJ activator) for 24h, and then challenged TPA or RA pre-treated cells with 513μmol/L or 1026μmol/L BR.

Neither TPA nor RA per se had significant effects, nor did they significantly affects the effects of BR on colony-formation rate in low-density cells, all $P>0.05$, Figure 4A and 4C. In high-density cells, TPA significantly attenuated the effect of BR in enhancing colony-formation rate at 513μmol/L, while RA exacerbated BR mediated reduction of colony-formation rate at 1026μmol/L (Figure 4B and 4D). These results indicated that under 513μmol/L BR stimulation, the intercellular GJs may function to transmit protective signals to promote cell proliferation. However while under notorious stimulations such as under high concentration of

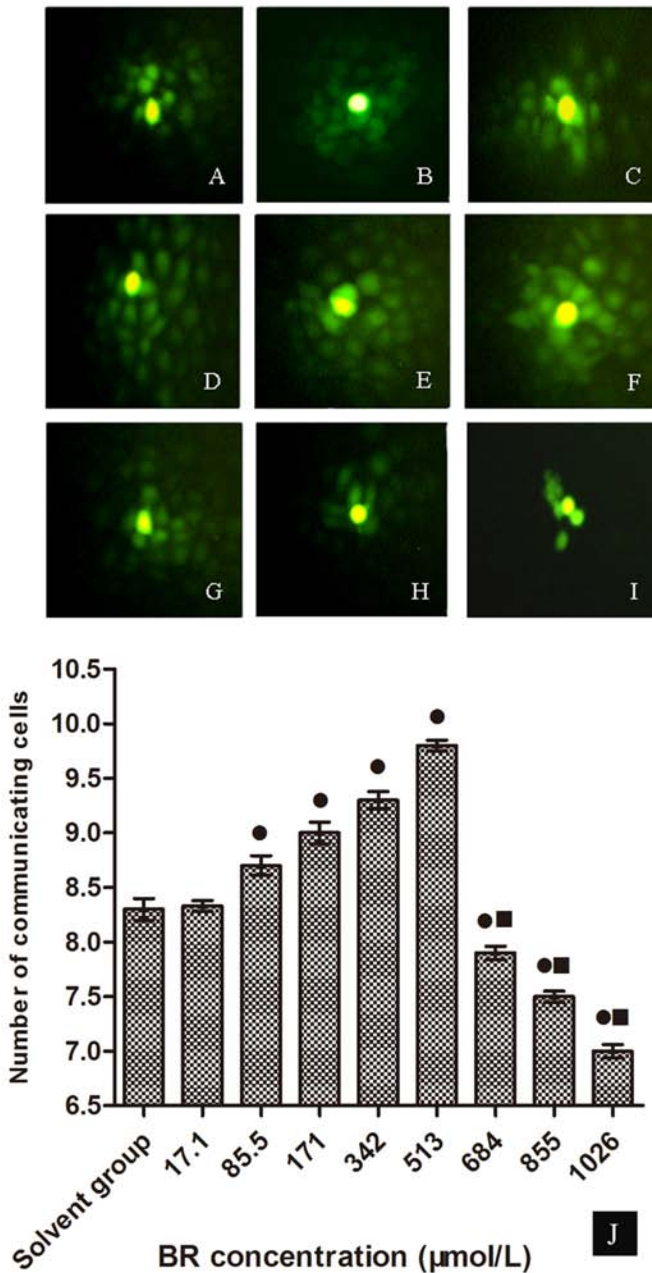

FIGURE 3. BR influenced fluorescence transmission of high-density cells through gap junctions. Fig 3A-3I were the representative images from the parachute dye-coupling assay in which cells were exposed to BR at 0, 17.1, 85.5, 171, 342, 513, 684, 855, and 1026μmol/L respectively (obtained by fluorescence microscope, magnification ×200). Dye spread through GJ was assessed by the average number of communicating cells (number of receiver cells containing calcein from each donor cell). Bar graph 3J was quantified for the communicating cells after BR exposure at different concentrations mentioned above. Data are presented as means ± SD of three independent experiments. ● $p < 0.05$ *vs.* solvent group, ■ $p < 0.05$ *vs.* BR 513μmol/L.

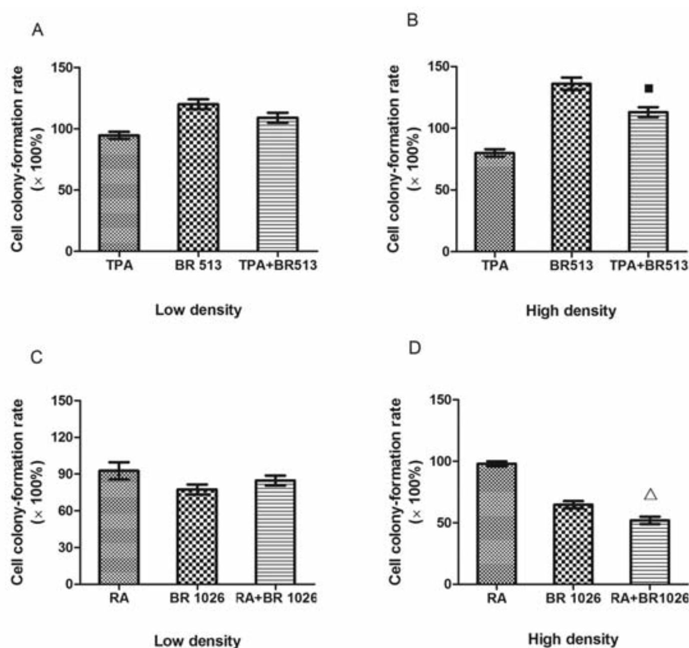

FIGURE 4. Effects of GJ inhibition or activation on BR-induced alteration of colony-formation rates. (A and B) Cells at low- and high-density were pretreated with the GJ inhibitor TPA before BR 513μmol/L. (C and D) Cells at low- and high-density were pretreated with the GJ agonist RA before BR 1026μmol/L. ■ $p < 0.05$ *vs.* BR 513μmol/L. △ $p < 0.05$ *vs.* BR 1026μmol/L. The data are presented as mean ± SD of three independent experiments.

BR at 1026μmol/L, further enhancement of GJ function may exacerbate cell injury.

Effects of GJ inhibition or activation on BR-induced changes in intercellular fluorescence transmission

As shown in Figure 5, as compared to solvent control, the GJ inhibitor TPA at the concentration used significantly reduced the number of intercellular fluorescence transmissive cells (P<0.05), and cancelled BR (at 513μmol/L) mediated enhancement of intercellular fluorescence transmission. In contrast, the GJ activator RA significantly enhanced the number of intercellular communicating cells as compared to solvent control (P<0.05) and reverted BR1026μmol/L induced reduction in intercellular fluorescence transmission.

BR effects on Connexin43 protein expression in NRK52E cells

Corresponding to its effects on intercellular fluorescence transmission mentioned above, BR dose-dependently increased connexin43 protein expression at concentrations ranging from 171 to 513μmol/L (all

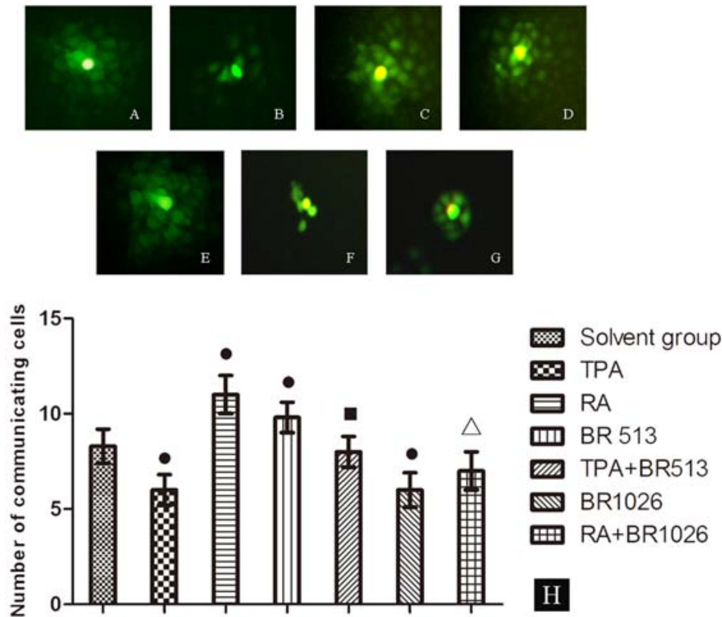

FIGURE 5. GJ function altered in response to varying BR concentrations following GJ inhibitor or GJ agonist for high-density cells. Fig 3A-3G were the representative images from the parachute dye-coupling assay in which cells were exposed to Solvent group, TPA group, BR 513μmol/L group, (TPA+BR 513μmol/L) group, RA group, BR 1026μmol/L group, and (RA+BR1026μmol/L) group respectively (obtained by fluorescence microscope, magnification ×200). Dye spread through GJ was assessed by the average number of communicating cells (number of receiver cells containing calcein from each donor cell). Bar graph 3H was quantified for the communicating cells treated with mentioned above. Data are presented as means ± SD of three independent experiments. ● $p < 0.05$ vs. Solvent group, ■ $p < 0.05$ vs. BR 513μmol/L, △ $p < 0.05$ *vs*. BR 1026μmol/L.

P<0.05 vs. solvent control), while it significantly reduced connexin43 protein expression at concentrations above 684μmol/L (P<0.01 vs. solvent control) (Figure 6).

DISCUSSION

In the current study, we conducted series assessment of cell proliferation and colony-formation rates and showed that BR conferred biphasic effects on NRK52E cells viability and cell proliferation in a dose-dependent manner. Of note, BR mediated changes in cell viability and rates of colony-formation were well in accordance with changes in GJ function and Cx43 protein level, and the effects of moderate concentration BR-mediated enhancement in cell viability and intercellular fluorescence transmission in high-density cells can be attenuated by GJ inhibition, while GJ agonist exacerbated the severely high concentration BR-mediated decrease of the above two parameters. These results indicated that BR level was an important factor for cell proliferation, and that BR exerted

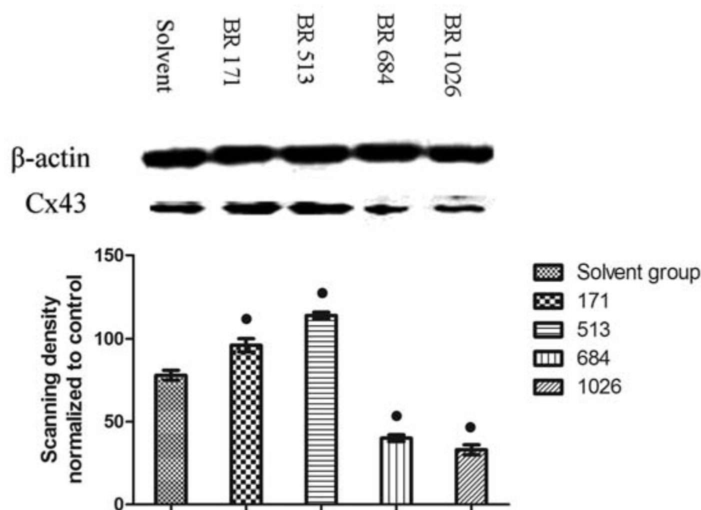

FIGURE 6. BR affected the expression of Connexin43 in a dose-dependent biphasic manner for NRK52E cells. Cells were either solvent group or treated with BR at 171μmol/L, 513μmol/L, 684μmol/L, or 1026μmol/L and Cx43 protein expression were detected by Western blot. ● $p < 0.05$ *vs.* Solvent group.

its effects on NRK52E cell proliferation at least in part by affecting GJ function.

Controversy exists regarding the influence of BR on renal cellular injury and kidney function. Different reports have distinct results which might be resulted from disparities in animal models or cell types used in various experiments or due to differences in BR concentrations applied. It has been reported that BR at a concentration as low as 10nM could protect tissues against high concentration H_2O_2-mediated injury in the presence of biliverdin reductase (Baranano *et al.* 2002). BR, at concentrations ranging from 0.05 to 10μmol/L, also had been shown to have strong protective effects against renal tubular epithelial cell injury (Adin *et al.* 2005). However, Silva *et al.* reported that BR caused damage to astrocytes and neurons in rats and dose- and time-dependently decreased astrocyte viability and enhanced apoptotic cell death (Silva *et al.* 2001). It is interesting to note that most of the protective effects of BR were observed in vitro when the BR was applied at low to moderate concentrations, while the harmful effect often appeared in bile duct ligation models in which higher BR concentration was presented (Lanone *et al.* 2005; Cheriyath *et al.* 2010; Ajja *et al.* 2011).

Usually moderate increase of BR during liver disease would not result in kidney injury, while kidney dysfunction did occur in patients with severe liver dysfunction and serious hyperbilirubinemia (Rivera-Huizar *et al.* 2006). The normal value of BR in human serum is below 17.1μmol/L;

however, it may increase to more than 100-folds of its normal value in patients with hepatic dysfunction. In our study, based on the concentration range of clinical hyperbilirubinemia and our preliminary experiment, we applied the BR concentration from 17.1 to 1026μmol/L, which covered the normal physiological condition and conditions with liver dysfunction when the BR concentration can be dramatically increased. We found that BR conferred biphasic effects in a dose-dependent manner. BR, at dose from 85.5 to 513μmol/L, increased cell viability and promoted colony-formation rates with concomitant enhancement in intercellular transmission, while these effects were gradually reverted when BR concentration was raised from 684 to 1026μmol/L. These results suggest that mild to moderate increases of serum bilirubin level, despite that it is higher than the normal values in control subjects, may not only do no harm to renal cells but can even be beneficial to promote renal cell proliferation. This is a novel finding that may have potential clinical implications in guiding the treatment of serious hyperbilirubinemia. However, it should be noted that highly abnormal increases in BR concentration may be one of the main reasons leading to renal dysfunction in patients with severe liver dysfunction. Findings from our current study provide rational explanation for the relationship between severe hyperbilirubinemia during liver failure and hepatic kidney injury.

Gap junctions between adjacent cells are composed of connexin protein subunits, which create a channel between the cells through which molecules may pass and functionally direct intercellular communications and synchronize cellular processes. Several Cxs have been identified in the kidney, while Cx43 is the main channel protein for renal tubule epithelial cells. We found that Cx43 is expressed in NRK52E cells and is tightly related to the GJ formation or function in fused cells. We further found that BR dose-dependently enhanced Cx43 expression at concentrations up to 513μmol/L that was correspondent to the increases in intercellular fluorescence transmission. In contrast, Cx43 expression significantly decreased when bilirubin dose was above 684μmol/L that was coincident with decreases in intercellular fluorescence transmission and cell viability. These results indicated that BR affected the renal tubular cells proliferation at least in part by altering connexin43 protein level and GJ function.

The disparities of colony-formation rates between high- and low- density cells may serve as an indirect indicator of GJ function (Jensen and Glazer 2004; Barabas *et al.* 2008; He *et al.* 2009), given that GJ function basically did not exist in low-density cells. Our results showed that, with BR concentration from 17.1 to 513μmol/L, the colony-formation rates for both low- and high-density cells were enhanced, but compared to low-density cells, high-density cells generated more colony-formation units, which suggested that intercellular protective signals were transmitted by

GJs to enhance cell viability in high-density cells. In contrast, when BR was above 684μmol/L, the colony-formation in both high- and low- density cells were inhibited. What is more, compared to low-density group, colony-formation in high-density cells was even more apparently inhibited when BR was above 684μmol/L, which suggested that high concentration BR induced cell injurious information may be transmitted intercellularly in the high-density cells, as such to further reduce cell viability. Indeed, GJ activator RA to enhance intercellular transmission further exacerbated BR 1026μmol/L-mediated reduction of the colony-formation rate in high-density cells. By comparison, pretreatment with the GJ inhibitor TPA decreased or cancelled 513μmol/L BR mediated enhancement of the colony-formation rate in high-density cells, while TPA had no significant influence on cell viability and the colony-formation rate in low-density cells. In accordance with its effects on BR induced colony formation, TPA also decreased 513μmol/L BR induced enhancement in fluorescent transmission in high-density NRK52E cells. These findings collectively suggest that BR conferred biphasic effects on renal cell proliferation or injury at least in part by differentially altering Cx protein expression and GJ function.

Due to its highly specialized membrane structures and powerful function of intercellular transport of electrical coupling and biologically active substances, GJ was reported to play central roles in modulating pathologies in diseased conditions. Associated clinical researches have been or being performed to modulate GJ function in an effort to treat several diseases, including ischemic preconditioning induced protection against myocardial necrosis and arrhythmias (Miura *et al.* 2010), brain injury following hypoxia-ischemia (Sahores and Mendoza-Naranjo 2008), cell injury and death during myocardial ischemia-reperfusion (García-Dorado *et al.* 2004), cancer chemoprevention (Chipman *et al.* 2003), etc.. Moreover, since toxic effects of bilirubin have been demonstrated on erythrocytes, lymphocytes, and lung in a dose and time dependent manner (Elias *et al.* 1987; Alexandra Brito *et al.* 2006), the specific safe dose response effects should be considered in individual situations during its utilisation.

Our current study shows that GJ communication function plays an important role both in bilirubin mediated cytoprotection when its concentration moderately increased and in bilirubin induced toxicity on NRK-52E cells. Studies have shown that GJ communication play crucial roles in cell proliferation and differentiation, immunoregulation, and pathophysiological process of tumor and drug-induced liver toxicity (Bernzweig, *et al.* 2011; Haku *et al.* 2011). Our study further illustrated that application of GJ inhibitor may have potential clinical implications in combating renal injury induced by liver transplantation or severe liver diseases that were associated with severely increased bilirubin production.

Finding from the current study also suggests that proper intervention of GJ function might provide new opportunities for drug development and therapy for a variety of bilirubin- and GJ-related disorders. It should be noted that bilirubin may have also conferred its protection, in part via its antioxidant properties (Stocker 2004). However, we think that this should not be the major mechanism of bilirubin cellular protection in the current experimental settings. This is because that its antioxidant capability should increase with concentration, but in high concentrations bilirubin is toxic despite of its potential antioxidant capability. This suggests that bilirubin conferred its protection or toxicity mainly via mechanisms (such as GJ in the current experimental settings) others than its antioxidant property. Further study is needed to confirm the relative roles of GJ and oxidative stress and their interplay in bilirubin-mediated cytoprotection.

The current studies have some limitations that need to be addressed. Retinoic acid (RA) has been used in the current study and in other studies as a GJ activator, however it is known to have antioxidant capacity which may enhance GJ communication via cell protection subsequent to potential attenuation of oxidative stress induced cell injury. On the other hand, TPA not only could interfere with GJ function by regulating the activity of protein kinase C (PKC), it has also been shown to induce antigen-nonspecific inflammation (Hirasawa *et al.* 2009) which may have potential effect on GJ function. As such, the specificities of TPA or RA respectively as a GJ inhibitor or an activator are now particularly high. Therefore, the mechanisms contributed to the biphasic effects of bilirubin on cell viability/proliferation of renal tubular epithelial cells should be multiple, and further study is needed to confirm the relative role of GJ in BR mediated cytoprotection or injury. Nevertheless, our study incorporating the use of standard colony-formation assay for high-density (GJ existence) or low-density (no GJ existence) to investigate the relationship between GJ and cell proliferation and the assessment of Cx protein, a subunit of GJ protein that most highly presents in NRK52E cell line, should have provided strong evidence that GJ is one of the key players in BR cytoprotection or toxicity.

In summary, our results showed that BR when its concentrations mild to moderately increased could promote NRK52E cells proliferation, and that enhancement of GJ function played an important role in BR-mediated cytoprotection. However, severely high concentration BR may cause or exacerbate renal cell injury and the deleterious effects of high concentration BR could be further exacerbated in the presence of GJ function activation.

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CONFLICT OF INTEREST

The authors have no conflicts of interests, financial or otherwise, related to the publication of this study or its findings.

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