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Research

The Potential Protective Effects of Ginkgo Biloba on Bilirubin Cytotoxicity in Newborn Rat

Yenidoğan Ratlarda Ginkgo Bilobanın Bilirubin Sitotoksisitesi Üzerindeki Potansiyel Koruyucu Etkisi

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ABSTRACT

Objective: The mechanism of neurotoxicity associated with high serum bilirubin concentrations is still not fully elucidated. The cytotoxic effect of bilirubin has been demonstrated in various cell types, including astrocytes and neurons. The protective effect of Ginkgo biloba (EGB-761), which has antioxidant, anti-inflammatory, and anti-apoptotic effects, against neurotoxicity due to hyperbilirubinemia is not known. This study aimed to investigate the effect of EGB-761 in neonatal rat astrocyte cell cultures with hyperbilirubinemia-induced cytotoxicity.

Methods: Astrocyte cell culture was obtained from one-day-old Wistar albino rats using the modified Cole and de Vellis method. Indirect bilirubin was found to be toxic to 50% of astrocyte cells at a dose of 10 μM (TC_{50}). Bilirubin-induced apoptotic cell death was evaluated using the TUNEL staining method. EGB-761 increased cell viability by 100% and 110% at 10 μg/mL and 0.5 μg/mL concentrations, respectively. No drug was administered to the control group. In the study group, for the protective effect, 10 μM bilirubin was administered to the astrocyte cell culture 4 hours after 10 μg/mL EGB-761 was administered in the ginkgo[10]+bilirubin[10] group, and for therapeutic effect, 10 μg/mL EGB-761 was administered 4 hours after 10 μM bilirubin was administered in the bilirubin[10]+ginkgo[10] group, for a duration of 48 hours. Cell viability and apoptosis were evaluated in both prophylaxis and treatment groups after the procedure.

Results: There was a 50% decrease in cell viability and a five-fold increase in apoptosis in the bilirubin[10] group compared with the control group (p<0.001, p<0.001). EGB-761 given for prophylaxis and treatment increased cell viability (p<0.001, p<0.001) and reduce apoptosis (p<0.001, p<0.001) compared with the control group.

Conclusion: In this *in vitro* study, it was shown that bilirubin has a cytotoxic effect on astrocyte cells, and EGB-761 used for prophylaxis and treatment reduced the cytotoxic effects of bilirubin.

Keywords: Bilirubin, Ginkgo biloba, neurotoxicity, newborn

ÖZ

Amaç: Yüksek serum bilirubin konsantrasyonu ile ilişkili nörotoksisitenin mekanizması günümüzde hala tam olarak açıklanamamıştır. Bilirubinin sitotoksik etkisi, astrositler ve nöronları da içeren değişik hücre tiplerinde gösterilmiştir. Antioksidan, antienflamatuvar, antiapoptotik etkileri olduğu bilinen Ginkgo bilobanın (EGB-761), hiperbilirubinemiye bağlı nörotoksisitedeki koruyucu etkisi bilinmemektedir. Bu çalışmada hiperbilirubinemiye bağlı sitotoksisite oluşturulmuş yenidoğan rat astrosit hücre kültüründe EGB-761'in etkisinin araştırılması hedeflendi.

Gereç ve Yöntem: Bir günlük Wistar albino ratlardan modifiye Cole ve de Vellis yöntemi ile astrosit hücre kültürü elde edildi. İndirekt bilirubinin 10 μM dozunda (TC_{50}) astrosit hücrelerinin %50'sine toksik etkili olduğu saptandı. TUNEL boyama yöntemiyle bilirubine bağlı apopitotik hücre

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ölümü değerlendirildi. EGB-761'in 10 µg/mL, 0,5 µg/mL konsantrasyonlarda hücre canlılığını sırasıyla %100 ve %110 artırdığı saptandı. Kontrol grubuna ilaç uygulanmadı. Çalışma grubunda, koruyucu etki için astrosit hücre kültürüne ginkgo[10]+bilirubin[10] grubunda 10 µg/mL EGB-761 uygulandıktan 4 saat sonra 10 µM bilirubin, tedavi edici etki için bilirübin[10]+ginkgo[10] grubunda 10 µM bilirubin uygulandıktan 4 saat sonra 10 µg/mL EGB-761 48 saat süreyle uygulandı. İşlem sonrasında hem profilaksi hem tedavi grubunda hücre canlılığı ve apoptozis değerlendirildi.

Bulgular: Kontrol grubuna göre bilirubin[10] grubunda hücre canlılığında yaklaşık %50 oranında azalma, apoptozisde beş kat artış saptandı (p<0,001, p<0,001). Profilaksi ve tedavi amacıyla verilen EGB-761'in kontrol grubuna göre hücre canlılığını artırdığı (p<0,001, p<0,001); apoptozisi azalttığı saptandı (p<0,001, p<0,001).

Sonuç: *In vitro* olarak yapılan bu çalışmada bilirubinin astrosit hücrelerine sitotoksik etkili olduğu, profilaksi ve tedavi amacıyla verilen EGB-761'in bilirubinin sitotoksik etkilerini azalttığı gösterildi.

Anahtar Kelimeler: Bilirubin, Ginkgo biloba, nörotoksisite, yenidoğan

INTRODUCTION

Despite the developments in neonatology, neurotoxicity caused by hyperbilirubinemia is still an important problem in newborns. High bilirubin levels cause encephalopathy and kernicterus, low bilirubin levels oxidative damage in newborns. In newborns, the increase in bilirubin and insufficient enterohepatic circulation cause high serum levels of indirect bilirubin, which is dissolved in fat and can easily pass cross the blood-brain barrier. Many mechanisms have been suggested to explain the neurotoxic effect of bilirubin. The basic cellular mechanism is the inhibition of oxidative phosphorylation in neurons (1-3). High bilirubin levels induced oxidative stress by increasing the formation of free radicals in the brain. The harmful effects of the free radicals continuously formed in biological systems are prevented by neutralizing the effect of antioxidant defense mechanisms. In newborns the insufficient antioxidant defense mechanisms contribute to the development of cerebral ischemia, excitotoxicity and neurodegenerative processes in the nervous system (3). Several mechanisms have been reported for bilirubin toxicity. High bilirubin levels have been shown to increase apoptosis (2).

In bilirubin toxicity, the primary targets are glial cells and neurons. Among nerve cells, neurons were shown to be more susceptible to the toxic effects of bilirubin than astrocytes (3,4). Astrocytes, the most intense cell group in the brain, are critically important in protection of the central nervous system as they provide metabolic and trophic support to neurons, which also contribute to form blood-brain barrier. Astrocytes are the first cells effected by bilirubin, which eventually causes blood-brain barrier damage (4,5). Astrocytes have been reported to be more resistant to bilirubin-associated oxidative damage, firstly increased expression of the pump, which removes bilirubin out of the cell, and by their high antioxidant capacities (4). Astrocytes have been used in many studies as an experimental kernicterus model (3,4,6). Astrocytes are also thought to play an important role in encephalopathy developed during severe hyperbilirubinemia, and are potential targets in the future treatment models. In this study, we used astrocytes to assess the toxic effects of bilirubin.

Ginkgo biloba is an agent derived from the dried leaves of this plant. Ginkgo biloba has been traditionally used in China and Western countries for the treatment of cerebrovascular diseases. The neuroprotective effects of plants have were shown in numerous *in vivo* and *in vitro* studies. Ginkgo biloba and its metabolites can cross the blood-brain barrier, which provides healing in different types of neurological damages, without side effects (7-10). Ginkgo biloba extract shows its effect through its flavonoid (22-27%) and terpenoid (5-7%) content (8). Many mechanisms explaining the neuroprotective effect of Ginkgo biloba were suggested in *in vivo* and *in vitro* studies; these mechanisms are protection of mitochondrial ATP synthesis, inhibition of apoptotic damage, suppression of hypoxia induced membrane damage in the brain, and increased expression of mitochondrial DNA encoding COX III subunitin of cytochrome c oxidase and NF I subunitin of NADH dehydrogenase (11,12). In animal studies, Ginkgo biloba extract (EGB-761) was reported to have protective effects against oxidative damage by removing free radicals. Moreover, Ginkgo biloba increases the activities of antioxidant enzymes such as superoxide dismutase, catalase by the flavonoid fraction of the EGB-761 extract. EGB-761 was reported that nitric oxide (NO) production decrease by suppressing inducible NO synthase as well as inhibiting malondialdehyde (MDA) (7,8,12).

Therefore, discovery of new agents that will decrease the toxic effects of bilirubin has gained importance. Although, most of these agents have given favorable results in experimenter studies, only a few of them could be used for clinic use. Although the antioxidant, antiapoptotic, vasorelaxant, antiaggregant, anti-inflammatory effects of EGB-761 have been shown, its effect on bilirubin toxicity in astrocytes remain unclear. We started this study with the hypothesis that Ginkgo biloba might be effective against bilirubin neurotoxicity. This study investigates the effects of Ginkgo biloba extract on newborn rat primary astrocyte cell culture by modeling hyperbilirubinemia-associated neurotoxicity.

METHODS

Study Groups

Group I, the control group (n=6): No drug was administered.

Group II, bilirubin[10] group (n=6): 10 μM bilirubin (Sigma Aldrich, B 4126-1G, St. Louis, MO, USA) was applied to the astrocyte cell culture for 48 hours.

Group III, ginkgo[10] group (n=6): 10 μg/mL ginkgo alkaloid (EGB-761) (Ginkgo biloba Hevert injekt. Dil. D3 2 mL, Hevert-Arzneimittel GmbH & Co. KG Nussbaum, Deutschland) was applied to the astrocyte cell culture for 48 hours.

Group IV, ginkgo[0.5] group (n=6): 0.5 μg/mL EGB-761 was applied to astrocyte cell culture for 48 hours.

Group V, four hours after 10 μM bilirubin was added to the astrocyte cell culture, 10 μg/mL EGB-761 was added and applied for 48 hours.

Group VI, bilirubin[10]+ginkgo[0.5] group (n=6): Four hours after 10 μM bilirubin was added to the astrocyte cell culture, 0.5 μg/mL EGB-761 was added and applied for 48 hours.

Group VII, ginkgo[10]+bilirubin[10] group (n=6): Four hours after the addition of 10 μg/mL EGB-761 to the astrocyte cell culture, 10 μM bilirubin was added and administered for 48 hours.

Group VIII, ginkgo[0.5]+bilirubin[10] group (n=6): Four hours after 0.5 μg/mL EGB-761 was added to the astrocyte cell culture, 10 μM bilirubin was added and administered for 48 hours.

Cell Cultures

The study was launched with the Pamukkale University Ethics Committee's approval, dated 19.08.2011, and numbered 2011/031. The study was conducted in accordance with the Declaration of Helsinki. Astrocyte cell cultures were prepared from the brains of 1-day-old Wistar albino rat pups using a modified version of Cole and de Vellis' shake off method (13,14). Following decapitation, brains were extracted and meninges were fully trimmed. The brains were mechanically minced and dissociated before being sieved through a nylon mesh (pore size of 70 m; Millipore). Cells were spun at 1500 rpm/min for 5 minutes using a benchtop centrifuge, and the cell pellets were resuspended in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (DMEM/Ham's F-12 1:1, 500 mL, Biochrom, Berlin, Germany), which contained 10% heat inactivated fetal bovine serum (FBS) (Hyclone, 100 mL, Thermo Scientific, Cromlington, UK), 500 μl gentamisin (Gentamisin, 10 mg/10 mL, Sigma Aldrich, St Louis, USA) and 5 mL fungisone (Gibco Antibiotic-

Antimycotic, 25 μg/mL amphoterisin B, 100x/100 mL, Invitrogen, New York, USA). For primary neuron cell culture, the resuspended cells were seeded in 75-cm^2 flasks previously coated with 10 μg/mL of poly-D-lisine (Sigma-Aldrich, St Louis, USA). Cells were incubated in a humidified CO_2 incubator at 370C, 5% CO_2, and 95% humidity, with medium changes every 3 days (Figure 1). Macrophages and loosely attached cells were removed from the astrocyte monolayer after 8-10 days of culture by shaking cultures at 150 rpm for 1 hour (Figure 1). The oligodendrocytes on top of a confluent monolayer of astrocytes were then dislodged by orbital shaking at 150 rpm for 24 hours. The media containing floating cells, microglia, and oligodendrocytes were transferred to separate flasks. At the bottom of the flasks, astrocyte cells were collected and the cell pellets were resuspended in astrocyte medium [DMEM/F12 containing 500 μL gentamisin, 5 mL fungizone, 15% FBS, 5 mL L-glutamine (Gibco L-Glutamine-200 mM, 100x/100 mL, Invitrogen, New York, USA)] and 500 μL insulin (Human insulin <rh>, 0.5 mg/mL, Biochrom, Berlin, Germany). The method for preparing astrocytes is been estimated to produce cultures with a purity of approximately 95% astrocytes. Stock unconjugated bilirubin (UCB) was prepared in 0.1 N NaOH and stored at 40 °C in the dark before being used. Under sterile conditions, the stock UCB solution was further diluted with astrocyte medium and added to cultures at various concentrations. Ginkgo biloba (EGB-761) was purchased from Biochrom.

Determination of Astrocyte Cell Viability and UCB and Ginkgo Biloba Concentrations

Cells were seeded in 96-well plates (3x10^4 cells/well). After 24 hours, the cells were treated with UCB at the following concentrations: 400 μM, 200 μM, 100 μM, 80 μM, 60 μM, 40 μM, 20 μM, 10 μM, 8 μM, 5 μM, 4 μM, 2 μM, 1 μM, and 0.5 μM for 48 hours. Astrocyte cells were also treated with EGB-761 at the following concentrations: 60 μg/mL, 40 μg/mL, 20 μg/mL, 10 μg/mL, 8 μg/mL, 6 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL, and 0.5 μg/mL. Cell viability was measured using the luminetric method by Becerir et al. (15).

Apoptosis Evaluation

To investigate any protective and/or curative effects, cells were treated with IC_{50} values of bilirubin before or after 4-h treatment with EGB-761. The cells were washed with phosphate-buffered saline and trypsinized after 24 hours of incubation. Apoptosis was determined using the deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method. TUNEL staining was performed using the ApopTag plus Peroxidase *in situ*

Apoptosis Detection (Millipore) kit as per the manufacturer's instructions. Each sample had at least five random microscopic fields counted, and the mean values were expressed as a percentage of apoptotic nuclei (16).

Statistical Analysis

Statistical packages for social sciences (SPSS) (SPSS for Windows 17.0; SPSS, Chicago, Illinois, USA) software was used to computation the data on a computer. The statistical significance of the study groups was determined using both parametric (paired samples t-test) and non-parametric tests (Kruskal-Wallis and Mann-Whitney U). Data were tested for conformity to the normal distribution. ANOVA and posthoc tests were used among the parametric tests when comparing means in the data conforming to a normal distribution. All data were presented in the form of mean ± standard deviation. Statistical significance was set at $p < 0.05$.

RESULTS

Determining the Bilirubin and Ginkgo Alkaloid Concentrations for Testing

Cell vitality was measured in 48-hour astrocyte cultures after different concentrations of bilirubin were administered. Figure 2 depicts the results as a percentage of cell vitality ± standard deviation at the studied bilirubin concentration. The results showed that increasing bilirubin concentrations concentration-dependently reduced cell viability. The concentration of indirect bilirubin that has a toxic effect on 50% of astrocyte cells (TC_{50}) was determined to be 10 µM and was used in cytotoxicity and apoptosis tests.

Cell vitality was determined after administration of different concentrations of EGB-761 in 48 hour-cultured astrocytes. Figure 3 depicts the results as a percentage of cell vitality ± standard deviation for each EGB-761 concentration administered. The concentrations of ginkgo alkaloid
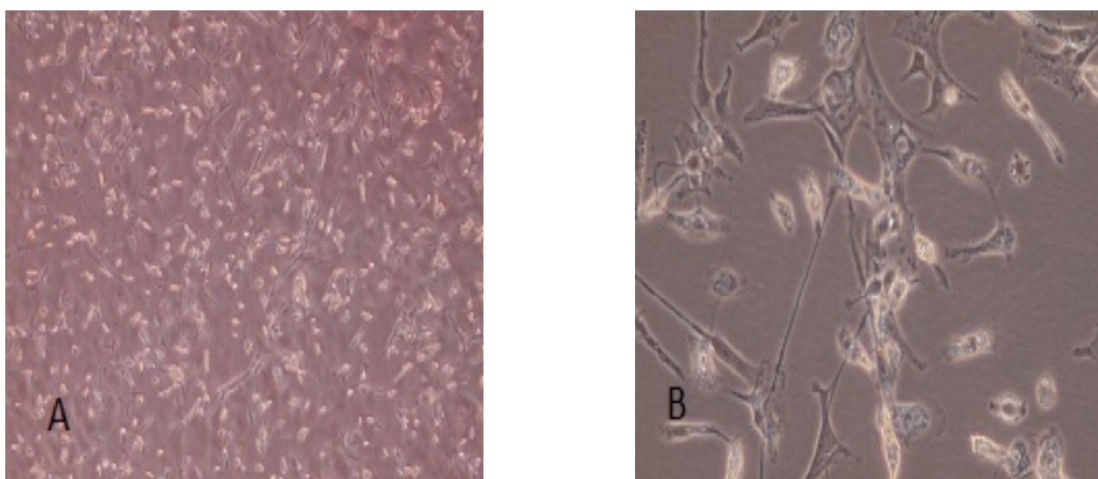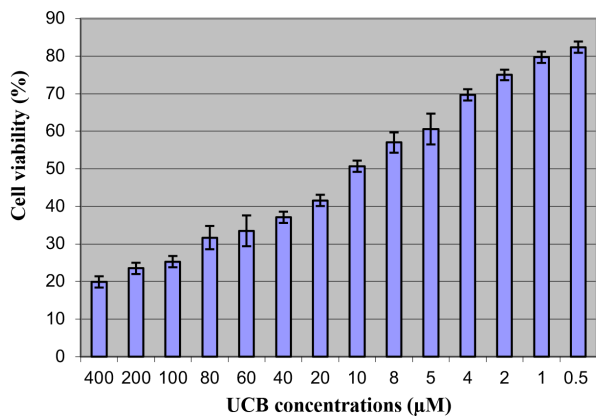

Figure 1. Neuron cell (A), astrocyte cell (B)

Figure 2. Concentration-response curve of UCB
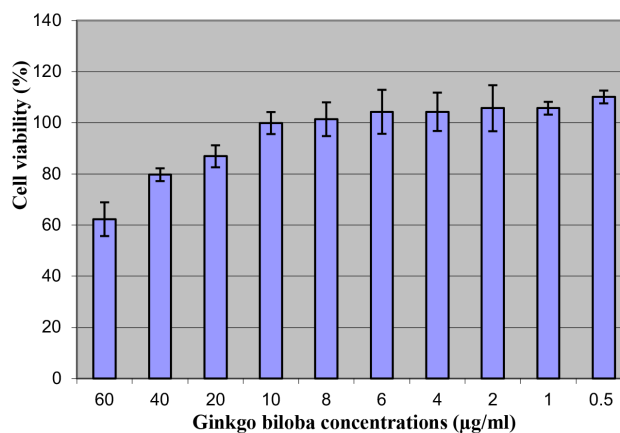UCB: Unconjugated bilirubin

Figure 3. Concentration-response curve of Ginkgo biloba

that increase cell vitality by 100% and most (110%) were determined to be 10 µg/mL and 0.5 µg/mL, respectively, and these concentrations were used in cytotoxicity measurements. EGB-761 at 10 µg/mL concentration was found to be the most effective ginkgo alkaloid concentration for apoptosis and was used in apoptosis tests.

Cytotoxicity Assessment

Cell vitality of the groups was evaluated in 48-hour cultures. At the outset, cell vitality was assumed to be 100%. Cell vitality reached 147.1±25.2% in the control group, and 69.9±5.7% in the B[10] group compared to the outset. The reduction in the cell vitality in the B[10] group compared to the control group was found to be statistically significant (p<0.001). Cell vitality was observed as 151.5±14.8% in the G[10] group, and 162.7±10.3% in the G[0.5] group. Increases in cell vitality in the control, G[10] and G[0.5] groups compared with the B[10] group were found to be statistically significant (p<0.001). Compared to the onset, cell vitality was observed as 117.9±16.4% in the B[10]+G[10] group, 105.5±12.3% in B[10]+G[0.5] group, 134.4±18.8% in G[0.5]+B[10] group, and 147.2±10.2% in the G[10]+B[10] group. In G[10]+B[10] and G[0.5]+B[10] groups, the increase in cell vitality was not different compared to the control groups (p>0.05). A significant reduction was observed in cell vitality in the B[10]+G[10] and B[10]+G[0.5] groups (p=0.039, p=0.001, respectively) compared to the control group (Figure 4).

Apoptosis Detection

Apoptosis in astrocyte cells was assessed in 48-hour cultures. Apoptosis was found to be 4.1±0.6% in the control group (Figure 5), 19.1±2.3% in the B[10] group (Figure 5), 5.2±0.9% in the G[10] group (Figure 5), 9.2±1.6% in the G[10]+B[10] group (Figure 5), and 10.7±1.6% in the B[10]+G[10]

group (Figure 5). Apoptosis was significantly higher in the B[10] group than in the other groups (p<0.001). Apoptosis was also higher with combined bilirubin and Ginkgo biloba administration compared to Ginkgo biloba alone.

DISCUSSION

Primary cell culture systems are very useful for toxicological and neurotoxicological studies, and have been used in the assessment of susceptibility of different neuron cells to toxins (17). Astrocytes, which provide metabolic, trophic support to the neurons, which are of critical importance in protection of the central nervous system, and are more resistant to oxidative damage than neurons, protect neurons from toxic damage in case of damage of blood-brain barrier (4,5). Astrocytes are thought to play an important role in encephalopathy developing during severe hyperbilirubinemia, and to be potential targets in the future treatment models (5,17). In bilirubin toxicity, main target is glial cells and neurons (3,4).

It was shown that bilirubin-associated damage is more persistent in neurons compared to astrocytes, of which the damage is mostly reversible. Silva et al. (4) reported that neurons are more susceptible to bilirubin toxicity than astrocytes.

In *in vitro* studies, threshold value for the neurotoxic effect of UCB was shown to be within a broad range, starting from as low as 70 nM (1,18,19). The fact that differences in toxic bilirubin concentrations are likely resulted from different methods, cell function and maturation and variation in the duration of bilirubin exposure (1). In the study by Tastekin et al. (20), TC_{50} concentration in primary cerebellar cell culture was found as 10 µM. In our study, indirect bilirubin
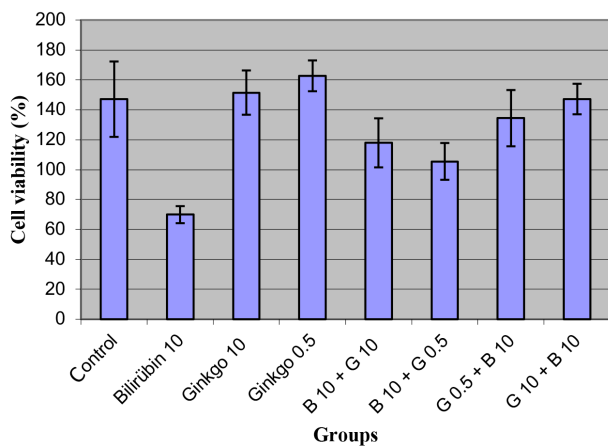

Figure 4. Alterations of the astrocyte cell viability in the groups (%)
*Decrease of the cell viability of the group B[10] compared to the control, G[0.5], G[10] group (p<0.001)
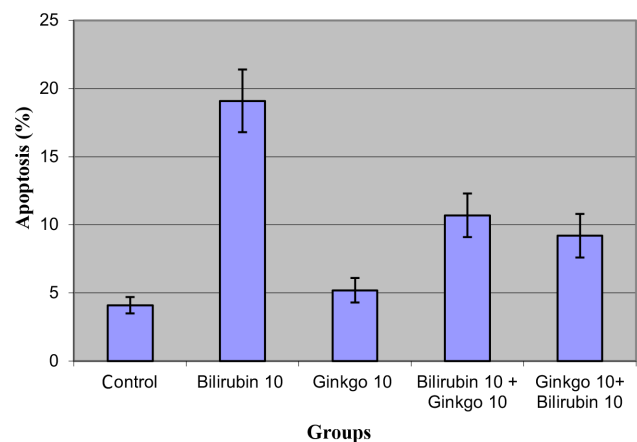

Figure 5. Evaluation of apoptosis in the groups
*Apoptosis of the B[10] compared to the other group (p<0.001)

was administered at the concentrations of 0.5-400 µM to primary astrocyte cell culture, and TC_{50} was found at 10 µM, as in the studies by Berns et al. (21) and Becerir et al. (15). Hence, bilirubin at 10 µM concentration was used in cytotoxicity and apoptosis tests. In our experiments, the cell death rate was higher at increased bilirubin concentrations as was reported by Ostrow et al. (1), Kumral et al. (5) and Becerir et al. (15).

Our study found that EGB-761 administered both prophylactically and therapeutically decreased bilirubin cytotoxicity by leading to a significant increase in cell vitality and a significant decrease in apoptosis in astrocyte cell culture. Many studies have been conducted for the prevention of bilirubin neurotoxicity using different agents NMDA channel antagonist MK-801, L-carnitin, glycoursodeoxicolic acid, taurine acting by blocking intracellular calcium increase, minocycline (20,22,23) that all have protective effects in neurons against oxidative damage associated with bilirubin.

This study investigated the bilirubin anti-neurotoxicity and therapeutic effects of Ginkgo biloba, which has previously shown to have neuroprotective effects on bilirubin neurotoxicity (7,9). In our study, approximately 100% cell vitality was attained with 10 µg/mL dose of Ginkgo biloba, and 110% with 0.5 µg/mL. This dose is reported in the literature to be within the effective dose interval (7). Bastianetto et al. (9) showed that, in beta amyloid-induced neurotoxicity in mixed hippocampal cell culture, 10 µg/mL and 100 µg/mL doses of EGB-761 prevented apoptosis. In our study, Ginkgo biloba increased cell vitality up to 162.7±10.3% while bilirubin decreased cell vitality to (69.9±5.7%). Pre and post applications of EGB-761 increased cell viability in bilirubin-treated cells. Preadministration of Ginkgo biloba had showed slightly better cell viability compared to the treatment group. In conclusion, EGB-761 was shown to provide a neuroprotective effect through administration both prophylactically and therapeutically.

The study by Oyama et al. (24) demonstrated decreased formation of hydrogen peroxide and reactive oxygen radicals in Ginkgo biloba treated cerebellar neuron cells in dose-dependent way. It is reported that Ginkgo biloba decreases intracellular calcium concentrations associated with the glutamate receptor agonist kainate in rat cerebellar neurons (25) and decreases calcium dependent oxidative metabolism (26). Ginkgo biloba substantially improves cell viability in hydrogen peroxide applied neuron cells (27).

Bilirubin-associated apoptotic cell death is thought to develop due to excitotoxicity occurring as a result of NMDA receptor activation, the disruption of mitochondrial functions, proapoptotic Bax translocation, decrease in Na-K ATPaz activity, increase in intracellular calcium level, intracellular cytochrome c increase, and disruption of cytoskeleton, lipid peroxidation and protein oxidation associated with oxidative stress (2,4,6,18,20,28). EGB-761 prevents bilirubin-associated neurotoxicity by protecting mitochondrial functions, preventing the cells from oxidative damage by increasing antioxidant enzyme activities, and decreasing proapoptotic caspase-3, Bax, c-Myc, and p-53, increasing antiapoptotic Bcl-2 activity (10,11,29,30). In our study, Ginkgo biloba was found to reduce bilirubin-induced apoptosis by half prophylactically and therapeutically.

EGB-761 may have neuroprotective effects by preventing cell neurons from oxidative, nitrosative damage and by its antiapoptotic properties.

This study clearly shows that bilirubin has neurotoxic effects on astrocytes *in vitro* and that Ginkgo biloba prophylactically and therapeutically substantially decreases the neurotoxic effects of bilirubin. The protective effects of Ginkgo biloba may occur through its antioxidant, antiapoptotic, anti-inflammatory, anti-nitrosative and anti-excitotoxicity effects. Further studies must elucidate the exact mechanisms of Ginkgo biloba, which may have future potential in use of treatment of bilirubin-associated neurotoxicity in newborns.

CONCLUSION

Despite the developments in neonatology, neurotoxicity caused by hyperbilirubinemia is still an important problem in newborns. The mechanism of neurotoxicity associated with high serum bilirubin concentrations is still not fully elucidated. The cytotoxic effect of bilirubin has been demonstrated in various cell types, including astrocytes and neurons. Our study found that EGB-761 administered both prophylactically and therapeutically decreased bilirubin cytotoxicity by leading to a significant increase in cell vitality and a significant decrease in apoptosis in astrocyte cell culture. Further studies must elucidate the exact mechanisms of Ginkgo biloba, which may have future potential in use of treatment of bilirubin-associated neurotoxicity in newborns.

ETHICS

Ethics Committee Approval: The study was launched with the Pamukkale University Ethics Committee's approval, dated 19.08.2011, and numbered 2011/031.

Informed Consent: Animal experiment study.

Authorship Contributions

Concept: Ö.Ş., H.E., A.D., M.B.Ö., H.A., Ç.Y., Design: Ö.Ş., H.E., A.D., M.B.Ö., H.A., Ç.Y., Data Collection or Processing: Ö.Ş., A.D., Analysis or Interpretation: Ö.Ş., H.E., A.D., H.A., Literature Search: Ö.Ş., H.E., Writing: Ö.Ş., H.E., A.D., H.A.

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