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Curcumin ameliorates high glucose-induced neural tube defects by suppressing cellular stress and apoptosis

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Abstract

Objectives—Curcumin is a naturally occurring polyphenol present in the roots of the *Curcuma longa* plant (turmeric), which possesses antioxidant, anti-tumorigenic and anti-inflammatory properties. Here, we test whether curcumin treatment reduces high glucose-induced neural tube defects (NTDs), and if this occurs via blocking cellular stress and caspase activation.

Study Design—Embryonic day 8.5 mouse embryos were collected for use in whole embryo culture under normal glucose (100 mg/dl glucose) or high glucose (300 mg/dl glucose) conditions, with or without curcumin treatment. After 24 h in culture, protein levels of oxidative stress makers, nitrosative stress makers, endoplasmic reticulum (ER) stress makers, cleaved caspase 3 and 8 and the level of lipid peroxides (LPO) were determined in the embryos. After 36 h in culture, embryos were examined for evidence of NTD formation.

Results—Although 10 μM curcumin did not significantly reduce the rate of NTDs caused by high glucose, 20 μM curcumin significantly ameliorated high glucose-induced NTD formation. Curcumin suppressed oxidative stress in embryos cultured under high glucose conditions. Treatment reduced the levels of the lipid peroxidation marker, 4-hydroxynonenal(4-HNE), nitrotyrosine-modified protein, and LPO. Curcumin also blocked ER stress by inhibiting phosphorylated protein kinase ribonucleic acid (RNA)-like ER kinase (p-PERK), phosphorylated inositol-requiring protein-1α (p-IRE1α), phosphorylated eukaryotic initiation factor 2α (p-eIF2α), C/EBP-homologous protein (CHOP), binding immunoglobulin protein (BiP) and x-box binding

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protein 1 (XBP1) mRNA splicing. Additionally, curcumin abolished caspase 3 and caspase 8 cleavage in embryos cultured under high glucose conditions.

Conclusions—Curcumin reduces high glucose-induced NTD formation by blocking cellular stress and caspase activation, suggesting that curcumin supplements could reduce the negative effects of diabetes on the embryo. Further investigation will be needed to determine if the experimental findings can translate into clinical settings.

Keywords

High glucose; curcumin; neural tube defects (NTDs); oxidative stress; nitrosative stress; endoplasmic reticulum stress; caspase activation

Introduction

Maternal diabetes increases the risk of congenital birth defects, including neural tube defects (NTDs)[1, 2]. Glycemic control by insulin treatment reduces the incidence of birth defects in both humans and animal models[3]. However, glycemic control is difficult to achieve and maintain, and even transient exposure to high glucose can result in embryonic anomalies[4]. Offspring from diabetic women under modern preconception care still have a two- to five-fold higher incidence of birth defects, compared with offspring of mothers without diabetes[5]. Therefore, there is a great need for new therapeutics that inhibit the mechanisms underlying diabetic embryopathy.

Animal studies have shown that antioxidants, such as multivitamins, the tea polyphenol Epigallocatechin gallate (EGCG) and the naturally occurring disaccharide trehalose, effectively ameliorate maternal diabetes-induced NTD formation[6-8]. However, human clinical trials have not shown similar results[9, 10]. The beneficial effect of multivitamin in preventing birth defects in diabetic human pregnancies has not been clearly established[8]. EGCG use in patients with type 2 diabetes does not significantly affect the degree of hyperglycemia, insulin resistance and other altered metabolic indices associated with type 2 diabetes[11]. A clinical trial on the effect of trehalose, an autophagy-inducing sugar, on cardiovascular diseases, is ongoing. Therefore, it is unclear whether health benefits can actually be achieved by human consumption of trehalose. Because it is also uncertain if EGCG or trehalose can prevent diabetes-associated diseases, we need to identify new therapeutics that may work in human.

Curcumin is a phenolic compound present in the rhizomes of the turmeric spice plant that is used in traditional Indian medicine to treat a variety of diseases and conditions, including those of the skin, pulmonary, and gastrointestinal systems[12]. Curcumin is a potent antioxidant which has been shown to suppress diabetes-induced superoxide in vascular endothelial cells[13]. In addition to its antioxidant properties, curcumin appears to be able to modulate signal transduction and gene expression[14]. A previous study has demonstrated that curcumin blocks diabetes-induced inducible nitric oxide synthase (iNOS) expression in the adult heart[15]. Another study showed that curcumin improves diabetes-induced endothelial dysfunction by inhibiting protein kinase C (PKC) activation[13]. Additionally, others have observed that curcumin abrogates endoplasmic reticulum (ER) stress, caspase activation and

apoptosis induced by either high glucose or hypoxia in non-cancerous cells[16]. The antioxidant, anti-cellular organelle stress, signaling transduction and gene expression modulating effects of curcumin make it as an ideal candidate therapeutic to prevent diabetic embryopathy.

We, and others, have demonstrated that oxidative stress is a central causal event in diabetic embryopathy[3, 17-22]. Oxidative stress-induced kinase signaling triggers ER stress in the developing embryo, leading to NTD formation[19]. iNOS expression and its associated nitrosative stress are induced by maternal diabetes, and deletion of the *iNos* gene alleviates NTD formation in diabetic pregnancies[23]. Maternal diabetes-induced specific PKC isoform activation is a key component of the causal events in NTD formation[18, 24]. It is possible that curcumin can target all critical events that lead to diabetic embryopathy. Thus, we propose that curcumin ameliorates high glucose-induced NTD formation by suppressing oxidative stress and ER stress.

In the present study, we assessed the effect of curcumin on NTD formation in murine embryo culture under high glucose conditions, and revealed its impact on high glucose-induced cellular stress and apoptosis in the developing embryo.

Materials and Methods

Animals and Whole-embryo culture

Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. The procedure of whole-embryo culture has been previously described[6, 25]. C57BL/6J mice were paired overnight. The next morning was designated embryonic day E0.5 if a vaginal plug was present. Mouse embryos at E8.5 were dissected out of the uteri in PBS (Invitrogen, La Jolla, CA). The parietal yolk sac was removed using a pair of fine forceps, and the visceral yolk sac was left intact. Embryos (four per bottle) were cultured in 25% Tyrode's salt solution and 75% rat serum that is freshly prepared from male rats. The embryos were cultured at 37°C in 30 revolutions/min rotation in the roller bottle system. The culture bottles were gassed 5% O_2/ 5% CO_2/ 90% N_2 for the first 24 h and 20% O_2/ 5% CO_2/ 75% N_2 for the last 12 h.

Embryos were cultured for 24 h or 36 h with 100 mg/dl glucose, a value close to the blood glucose level of non-diabetic mice, or 300 mg/dl glucose, which is equivalent to the blood glucose level of diabetic mice, in the presence or absence of curcumin (Sigma-Aldrich). We started our whole-embryo culture experiments using 0, 10 and 20 μM curcumin. At the end of 24 h, embryos were dissected from the yolk sac for biochemical and molecular analyses. At the end of 36 h, embryos were dissected from the yolk sac and examined under a Leica MZ16F stereomicroscope (Leica Microsystems, Bannockburn, IL) to identify embryonic malformations.

Images of the embryos were captured by a DFC420 5 MPix digital camera (Leica Microsystems). Normal embryos were classified as possessing a completely closed neural tube and no evidence of other malformations. Malformed embryos were classified as

showing evidence of failed neural tube closure or of an NTD. NTDs were verified by histological sections.

Lipid hydroperoxide quantification

The degree of lipidperoxidation was quantitatively assessed by the LPO assay, as previously described[26], and using the Calbiochem Lipid Hydroperoxide Assay Kit (Milliproe, Bedford, MA), following the manufacturer's instructions. Briefly, embryos cultured for 24 h under normal and high glucose conditions were homogenized in HPLC-grade water. The lipid hydroperoxides of the embryonic tissue were extracted by deoxygenated chloroform, and then measured by the absorbance of 500 nm after reaction with chromogen. The results were expressed as μM lipid hydroperoxides per microgram protein. Protein concentrations were determined by the BioRad DC protein assay kit (BioRad, Hercules, CA).

Immunoblotting

Immunoblotting was performed as described by Yang *et al.*[17, 20]. To extract protein, a protease inhibitor cocktail (Sigma) in lysis buffer (Cell Signaling Technology) was used. Equal amounts of protein and the Precision Plus Protein Standards (Bio-Rad) were resolved by SDS-PAGE and transferred onto Immunobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated in 5% nonfat milk for 45 minutes, and then were incubated for 18 hours at 4 °C with the following primary antibodies at dilutions of 1:1000 in 5% nonfat milk: p-PERK; PERK; p-eIF2α; eIF2α; CHOP; BiP; IRE1α nitrotyrosine (cell signaling); p-IRE1α (Abcam); 4-HNE(Millipore); caspase 8 (mouse specific) (Alexis Biochemicals) and caspase 3 (Millipore). Membranes were exposed to HRP-conjugated goat anti-rabbit or goat anti-mouse (Jackson ImmunoResearch Laboratories) or goat anti-rat (Chemicon) secondary antibodies. Signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific), and chemiluminescence emitted from the bands was directly captured using a UVP Bioimage EC3 system. Densitometric analysis of chemiluminescence signals was performed using VisionWorks LS software (UVP). To ensure that equivalent amounts of protein were loaded among samples, membranes were stripped and incubated with β-actin (Abcam). All experiments were repeated in triplicate with the use of independently prepared tissue lysates.

Detection of XBP1 mRNA splicing

The mRNA of XBP1 was extracted from 24-h cultured embryos and reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen). The PCR primers for XBP1 were as follows: forward, 5′-GAACCAGGAGTTAAGAACACG-3′ and reverse, 5′-AGGCAACAGTGTCAGAGTCC-3′. If no XBP1 mRNA splicing occurred, a 205 bp band was produced. When XBP1 splicing occurred, a 205 bp band and a 179 bp main band were produced.

Statistical analyses

Data were presented as means ± SE. Embryonic samples from each replicate were from different dams. Statistical differences were determined by one-way analysis of variance (ANOVA) using SigmaStat 3.5 software. In one-way ANOVA analysis, *Tukey* test was used

to estimate the significance of the results ($P < 0.05$). The *Chi square* test was used to estimate the significance of the NTD rates.

Results

Curcumin ameliorates high glucose-induced NTD formation

Mouse embryonic neurulation takes place during embryonic day 8.5 (E8.5) to E10.5. To assess whether curcumin treatment reduced high glucose-induced NTD formation, E8.5 mouse embryos were cultured under normal glucose (100 mg/dl glucose) or high glucose (300 mg/dl glucose) conditions, with or without 10 or 20 μM curcumin. As shown in Table 1, the NTD rate of embryos cultured under high glucose conditions was significantly higher than that of embryos cultured under normal glucose conditions. Although treatment with 10 μM curcumin slightly reduced NTD formation in embryos cultured under high glucose conditions (Table 1), the NTD rate of embryos given 20 μM curcumin was significantly lower than that of embryos without curcumin treatment, both cultured in high glucose conditions (Fig. 1A, Table 1). However, treatment with 20 μM curcumin did not completely prevent high glucose-induced NTDs because the NTD rate in the high glucose plus 20 μM curcumin group was still higher than that in the normal glucose group (Table 1). Because 20 μM is an effective dose of curcumin in inhibiting high glucose-induced NTD formation, this concentration was used hereafter.

Curcumin alleviates high glucose-induced oxidative stress and nitrosative stress

Oxidative stress is a key mechanism underlying maternal diabetes-induced NTD formation[3, 18, 19]. Maternal diabetes-induced ROS react with iNOS-induced nitric oxide to generate RNS, which cause to a severe form of oxidative stress, nitrosative stress[23]. To explore whether curcumin treatment blocks high glucose-induced oxidative and nitrosative stress, we assessed the levels of 4-HNE, a lipidperoxidation marker and nitrotyrosine-modified protein. 4-HNE levels in embryos cultured under high glucose conditions were significantly higher than those in embryos under normal glucose conditions, with or without 20 μM curcumin (Fig. 1B).

Elevated levels of nitrotyrosine-modified protein also are indicative of nitrosative stress. Levels of nitrotyrosine-modified proteins in embryos exposed to high glucose were significantly higher than those in embryos under normal glucose conditions, in the presence or absence of curcumin (Fig. 1C). Treatment with 20 μM curcumin reduced high glucose-induced nitrotyrosine protein modification (Fig. 1C). In addition, high glucose induced high LPO levels, and 20 μM curcumin blocked high glucose-increased LPO levels (Fig. 1D). These findings support the hypothesis that curcumin treatment abrogates high glucose-induced oxidative stress and nitrosative stress.

Curcumin treatment abrogates high glucose-induced ER stress

To investigate whether curcumin treatment abolishes high glucose-induced ER stress, we detected the protein levels of ER stress markers. Protein levels of p-PERK, p-eIF2α, p-IRE1α, CHOP and BiP were significantly increased by high glucose (Fig. 2A, B, C, D, E).

Treatment with 20 μM curcumin significantly suppressed high glucose-induced ER stress marker expression (Fig. 2A, B, C, D, E).

XBP1 mRNA splicing is another index of ER stress. To determine whether curcumin treatment blocks high glucose-triggered XBP1 mRNA splicing, we used reverse transcription-PCR. Embryos exposed to high glucose exhibited robust XBP1 splicing, with the PCR products showing two bands at 205 bp and 179 bp (Fig. 3), whereas embryos under normal glucose conditions did not have any spliced XBP1 mRNA. Treatment with 20 μM curcumin diminished high glucose-induced XBP1 mRNA splicing (Fig. 3).

Curcumin treatment suppresses high glucose-induced caspase activation

To determine whether curcumin treatment blocks high glucose-induced caspase activation, we measured the protein levels of cleaved caspase 8 (an initiator of apoptosis) and cleaved caspase 3 (an index of the degree of apoptosis). Levels of cleaved caspase 3 and 8 were significantly up-regulated by high glucose, when compared with those in embryos under normal glucose conditions (Fig. 4A, B). Curcumin treatment significantly suppressed high glucose-induced caspase cleavage (Fig. 4A, B).

Comment

The present study demonstrates that curcumin can suppress high glucose-induced oxidative stress in the developing embryo. This appears to occur by reduction of LPO levels and diminished expression of the cellular lipidperoxidation marker, 4-HNE, and nitrosative stress marker, nitrotyrosine. Our previous studies have indicated that oxidative stress is a causal event in the induction of diabetic embryopathy[3, 18]. This present work confirms previous observations that curcumin inhibits diabetes-induced superoxide production, a major agonist of oxidative stress[13].

Our current study also reveals that curcumin treatment can block high glucose-induced ER stress markers in the neural tube. This observation is consistent with previous findings that curcumin treatment inhibits lipid-induced ER stress[14] and diabetes-induced ER stress in the adult heart[27]. In our previous studies, we have elucidated a critical role of ER stress in hyperglycemia-induced NTD formation[20, 28]. We have observed that both maternal diabetes *in vivo* and high glucose *in vitro* trigger the unfolded protein responses (UPR) by activating the IRE1a and the PERK signaling pathways, leading to ER stress in the developing embryo[19, 20, 28-30]. Additionally, we have shown that an ER stress inhibitor, 4-phenylbutyric acid, ameliorates high glucose-induced NTD formation[20]. Because ER stress appears to be a causal event in diabetic embryopathy [19, 20, 31], the present study confirms that suppressing ER stress reduces NTD incidence under high glucose conditions.

The mechanism underlying curcumin-blocked oxidative stress may involve in its stimulatory effect on endogenous antioxidant expression. It has been shown that curcumin restores hypoxia-suppressed expression of an endogenous antioxidant enzyme, peroxiredoxin 6, through blockage of the NF-κB signaling pathway[16]. Peroxiredoxins (Prx) are members of a family of peroxidases that can detoxify hydrogen peroxide, lipid peroxides and RNS[32], all of which have been detected in embryos exposed to maternal diabetes *in vivo* and high glucose

in vitro[23, 31]. While it is unknown whether Prx1-6 expression is affected in diabetic embryopathy, the expression of other endogenous antioxidant enzymes, including glutathione peroxidase and catalase, are inhibited by maternal diabetes[33].

Maternal diabetes-induced ER stress is the result of c-Jun-N-terminal kinase (JNK) 1/2 activation because deletion of either the JNK1 or the JNK2 gene abolishes embryonic ER stress[20, 31]. A recent study described an inhibitory effect of a potent curcumin analog on diabetes-induced JNK1/2 activation in cardiac tissues[27]. Therefore, it is possible that curcumin suppressed ER stress in the present study through inhibiting JNK1/2 activation.

Another possible mechanism underlying curcumin-blocked ER stress may rely on the antioxidant properties of this compound. Previous work has demonstrated that oxidative stress causes ER stress[19]. The antioxidant protein Prx6 prevents ER stress by detoxifying cellular ROS in lens cells[34]. In diabetic embryopathy, overexpressing antioxidants abrogates diabetes-induced UPR and ER stress in the developing embryo[19]. Therefore, curcumin resolves high glucose-induced oxidative stress leading to ablation of ER stress.

Oxidative and ER stress induce apoptosis, and inhibition of either stress blunts maternal diabetes-induced neuroepithelial cell apoptosis and, consequently, reduces NTD formation[19, 20, 31]. Maternal diabetes-induced embryonic neuroepithelial cell apoptosis is caspase 3- and 8-dependent. We observed that curcumin blocks high glucose-induced caspase 3 and 8 cleavage, which is likely due to its antioxidant and anti-ER stress properties. The protective effect of curcumin against diabetic embryopathy appears to converge on the inhibition of apoptosis.

Studies in humans have employed doses of curcumin as low as 10 or 20 μM [35], the concentrations we used in our experiments, and doses as high as to 8,000 mg/day without causing adverse events[36-38]. In our study, both doses reduced NTD incidence when used under high glucose conditions, suggesting that a low concentration of curcumin may be effective. However, curcumin at high doses exerted teratogenic effects on zebrafish embryo in one study[39], and high doses of curcumin caused adverse effects on early stage mouse embryos *in vitro* [40]. Because of these conflicting results related to the safety of curcumin, and the fact that additional precautions must be taken when giving a therapeutic to pregnant women, the *in vitro* effects of curcumin, both beneficial effects and potentially harmful effects, will need to be recapitulated in animal models of diabetic embryopathy before the safe dose of this supplement can be determined for use in humans.

Preconception care with insulin treatment and blood glucose-reducing agents decreases the incidence of birth defects in diabetic or obese pregnancies[41, 42]; however, even women with diabetes under modern preconception care have a higher chance of having babies with birth defects, compared with nondiabetic women.

Curcumin is a purified polyphenol from spice. Raw herb production use is still rare in pregnancies in Western cultures, but multivitamins and antioxidants have been tested to treat adverse pregnancy outcomes routinely[43]. Purified plant compounds are appealing for the treatment of maternal obesity-, diabetes-, inflammation-, hypertension- and tobacco intake-induced adverse pregnancy outcomes because they are natural products that may have lower

side-effects than traditional pharmaceuticals[41, 44-49]. Curcumin and other naturally occurring polyphenols[6] are highly attractive therapeutics because of their antioxidant properties, and because many poor pregnancy outcomes are the result of oxidative stress[50, 51].

Our previous work has demonstrated that oxidative stress and ER stress are the main causal events in diabetic embryopathy. Here, we show that curcumin can abrogate the damaging effects of high glucose-induced cellular stress. It is important for future studies to assess the effect of curcumin on the expression of endogenous antioxidant expression in relation with kinase signaling that is activated by maternal diabetes[13, 16]. By revealing the mechanism through which curcumin abolishes maternal diabetes-induced oxidative stress, this natural compound could become a potential and potent therapeutic to treat diabetic embryopathy.

The mechanisms underlying NTD formation are multifactorial and need to be further explored. Studies in human and animal models have demonstrated that the NTD incidences correlate with the severity of hyperglycemia in diabetic pregnancies[4, 52]. Hyperglycemia activates multiple metabolic pathways leading to excess production of reactive oxygen species and oxidative stress. Oxidative stress caused by mutations in SOD1 and SOD2 promoter regions, that potentially affect the expression of these two antioxidant enzymes, may be responsible for the formation of human myelomeningocele, a type of NTDs[53]. Oxidative stress mediates the teratogenic effect of hyperglycemia by altering developmental gene expression that is essential for neural tube closure[7, 17]. Recent studies have revealed several important oxidative stress markers in maternal circulation for the early diagnosis of birth defects and other adverse fetal outcomes[51, 54, 55]. Folic acid is effective in reducing human NTD formation but does not eliminate human NTDs[44]. Additionally, clinical studies suggest the failure of folic acid in preventing heart defects[56, 57]. Antioxidants such as curcumin may be good candidates for preventing human birth defects caused by maternal diabetes and obesity.

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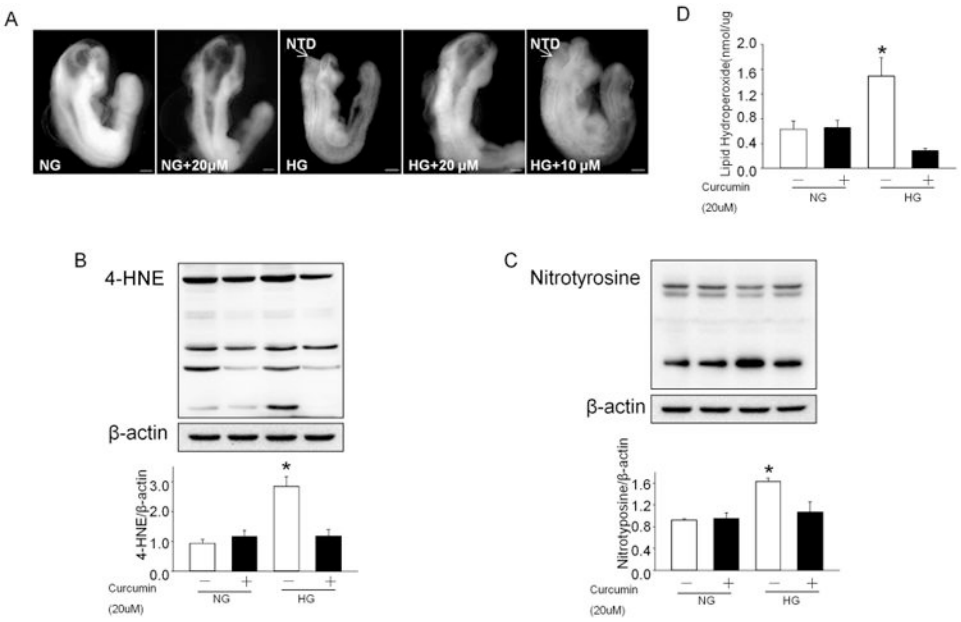

Figure 1. Curcumin treatment inhibited high glucose-induced oxidative stress and consequent NTD formation

A:Closed and open neural tube structures of 36 h cultured embryos in high glucose or normal glucose conditions, with or without curcumin; **B, C:** Protein levels of 4-HNE and nitrotyrosine in 24 h cultured embryos; **D:** Levels of lipid hydroperoxide (LPO) in 24 h cultured embryos. Experiments were repeated three times using three embryos from three different dams. NG: normal glucose; NG + Cur: normal glucose plus 20 μM curcumin; HG: high glucose; HG + Cur: high glucose plus 20 μM curcumin. *Indicates significant difference ($p < 0.05$) when compared to other groups.

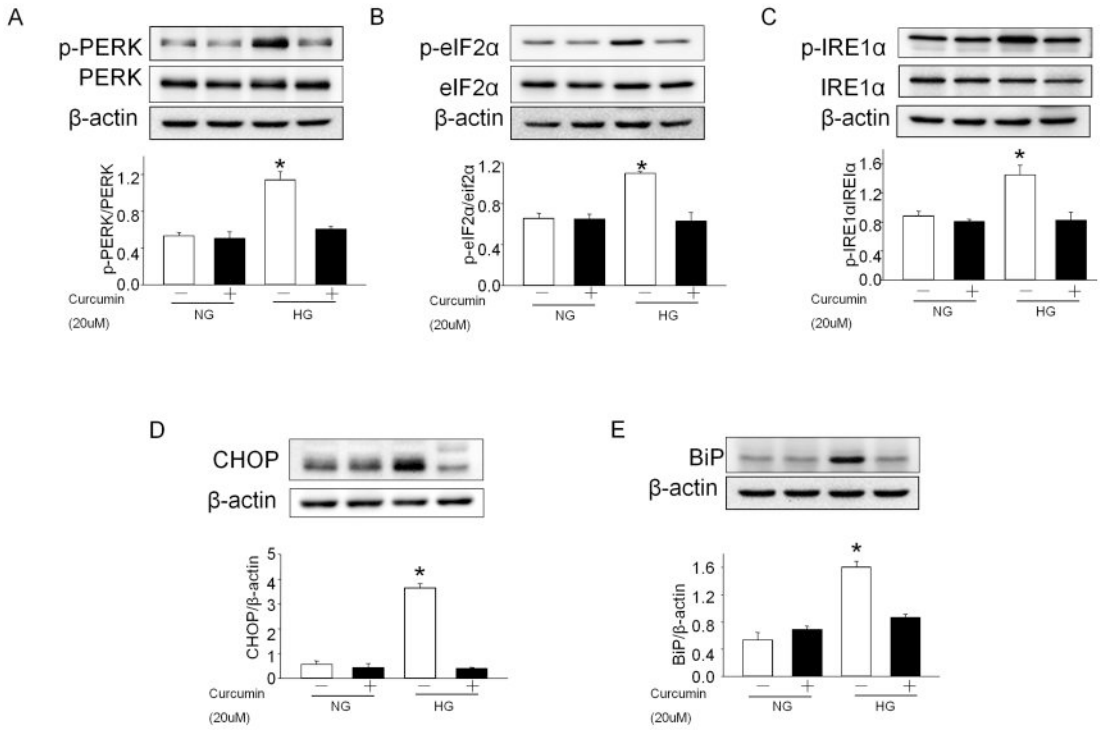

Figure 2. Curcumin treatment abrogated high glucose-induced ER stress

Protein levels of p-PERK (**A**), PERK (**A**), p-eIF2α (**B**), eIF2α (**B**), p-IRE1α (**C**), IRE1α (**C**), CHOP (**D**) and BiP (**E**) were determined in embryos cultured for 24 h under normal glucose and high glucose conditions, with or without 20 μM curcumin (cur) treatment. Experiments were repeated three times using three embryos from three different dams. NG: normal glucose; NG + Cur: normal glucose plus 20 μM curcumin; HG: high glucose; HG + Cur: high glucose plus 20 mM curcumin. * Indicates significant difference ($p < 0.05$) when compared to other groups.

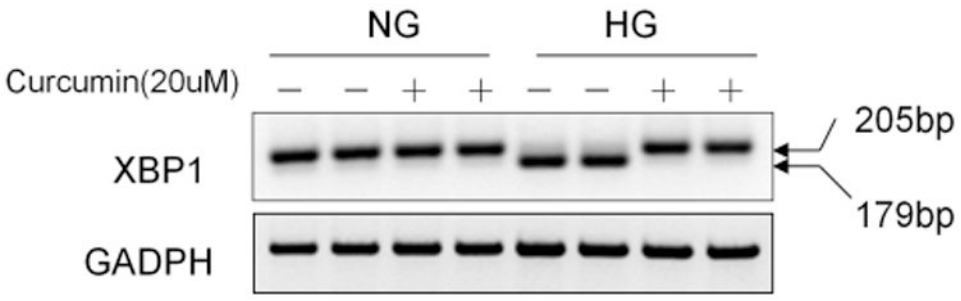

Figure 3. Curcumin treatment abrogated high glucose-induced XBP1 mRNA splicing

XBP1 mRNA splicing in embryos cultured for 24 h under normal glucose and high glucose conditions, with or without 20 μM curcumin treatment. Arrows point to the actual size of the bands. NG: normal glucose; NG + Cur: normal glucose plus 20 μM curcumin; HG: high glucose; HG + Cur: high glucose plus 20 μM curcumin; XBP1, X-box binding protein.

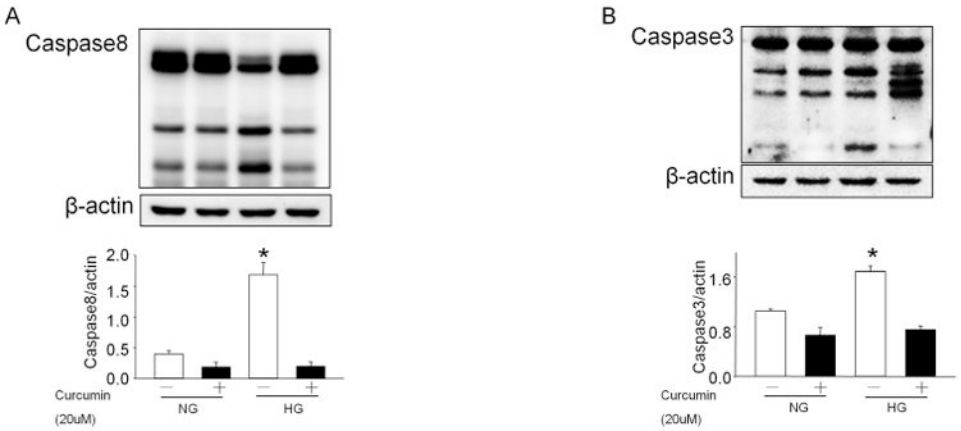

Figure 4. Curcumin treatment reduced high glucose-induced caspase activation

Protein levels of caspase 8 (**A**) and caspase 3 (**B**) in embryos cultured for 24 h under normal glucose and high glucose conditions, with or without 20 μM curcumin treatment. Experiments were repeated three times using three embryos from three different dams. NG: normal glucose; NG + Cur: normal glucose plus 20 μM curcumin; HG: high glucose; HG + Cur: high glucose plus 20 μM curcumin. * Indicates significant difference ($p < 0.05$) when compared to other groups.

Table 1
Curcumin treatment ameliorates high glucose-induced NTD formation

Group	Total number of embryos	Number of NTD embryos	NTD rate(%)
NG	24	2	8.3
NG + 20μM cur	24	3	12.5
HG	24	13*	54.2
HG + 20μM cur	23	3	13.0
HG + 10μM cur	25	10**	40

NG: normal glucose (100 mg/dl glucose); NG + 20 μM cur: normal glucose plus 20 μM curcumin (cur); HG: high glucose (300 mg/dl glucose); HG + 20 μM cur: high glucose plus 20 μM cur; HG + 10 μM cur: high glucose plus 10 μM cur.

* Indicates the HG group are significant different when compared with the NG, the NG + 20 μM and the HG + 20 μM groups.

** Indicates that the HG + 10 μM group and the HG group are not significantly different, and the HG +10 μM group are significantly different when compared with the NG group, the NG + 20 μM group and the HG + 10 μM group. Statistical difference was analyzed by the *Chi square* test.