

Nrf2 inhibition affects cell cycle progression during early mouse embryo development

Ying LIN^{1, 2)*}, Liu-Cai SUI^{1)*}, Rong-Hua WU^{1)*}, Ru-Jun MA¹⁾, Hai-Yan FU¹⁾, Juan-Juan XU¹⁾, Xu-Hua QIU¹⁾ and Li CHEN¹⁾

¹⁾Reproductive Medical Center, Jinling Hospital, Clinical School of Medical College, Nanjing University, Jiangsu 210002, People's Republic of China

²⁾School of Life Sciences, Nanjing Normal University, Jiangsu, People's Republic of China

Abstract. Brusatol, a quassinoid isolated from the fruit of *Brucea javanica*, has recently been shown to inhibit nuclear factor erythroid 2-related factor 2 (Nrf2) via Keap1-dependent ubiquitination and proteasomal degradation or protein synthesis. Nrf2 is a transcription factor that regulates the cellular defense response. Most studies have focused on the effects of Nrf2 in tumor development. Here, the critical roles of Nrf2 in mouse early embryonic development were investigated. We found that brusatol treatment at the zygotic stage prevented the early embryo development. Most embryos stayed at the two-cell stage after 5 days of culture ($P < 0.05$). This effect was associated with the cell cycle arrest, as the mRNA level of *CDK1* and *cyclin B* decreased at the two-cell stage after brusatol treatment. The embryo development potency was partially rescued by the injection of Nrf2 CRISPR activation plasmid. Thus, brusatol inhibited early embryo development by affecting Nrf2-related cell cycle transition from G₂ to M phase that is dependent on cyclin B-CDK1 complex.

Key words: Brusatol, Cell cycle, Early embryo, Nrf2

(J. Reprod. Dev. 64: 49–55, 2018)

Mouse early embryo development is a complex process comprising a series of molecular and cellular events, including proper gene expression regulation [1, 2]. Following sperm fusion, the zygote undergoes its first cleavage into two cells and activates the subsequent process involving cleavage, compaction, blastulation and implantation [3]. The exact expression of specific genes is essential for embryonic development and cell commitment. The deficiency of several essential genes is shown to cause early embryonic development failure [4–6].

Cell cycle regulation is a vital process for cell survival during the embryonic development from zygote to blastocyst. Several genes, including those encoding the well-characterized cyclins and cyclin-dependent kinases, are responsible for regulating cell cycle progression [7]. The complex of *cyclinB* and *cyclin-dependent kinase1* (*CDK1*) regulates the G₂/M phase activity and is required for mitosis entry. It is well established that one of the most important factors affecting cell survival is transcriptional and translational genes, while genes related to cell cycle are essential in the mechanism of early embryonic development [5].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor involved in cellular defense mechanisms and elevates the

expression of target genes such as antioxidant defense enzymes and protein, including heme oxygenase-1 (HO-1), γ -glutamate cysteine ligase catalytic subunit (GCLC), superoxide dismutase (SOD), and catalase [8]. Numerous studies have shown that Nrf2 protects the cells from injuries caused by various microenvironment insults [9–11]. Moreover, recent studies indicated Nrf2-regulated cell cycle progression through the complex of cyclin B-CDK1 complex [12, 13].

Brusatol, extracted from a family of natural products known as quassinoids, has been considered as one of the most effective anti-neoplastic drugs and is known to regulate the expression of c-myc [14]. Recent studies have verified that brusatol may selectively and transiently inhibit Nrf2 signaling pathway and that the degradation of Nrf2 protein may serve as the major mechanism responsible for antineoplastic effects [15]. Ubiquitin C-terminal hydrolase L1 (UCHL1) is a deubiquitinating enzyme of the ubiquitin proteasome system and its depletion is associated with decreased cell proliferation [16].

The protein Nrf2 is known to play a major role in chemoresistance [17] by providing protection against oxidative stress and chemical toxicants in cancer cell lines. However, the role of Nrf2 in mouse early embryonic development remains unknown. In the present study, we investigated the role of Nrf2 in mouse embryonic development.

Materials and Methods

Mice

Kunming mice were used in the present study. All experiments were approved by the Animal Care and Use Committee of Nanjing Jinling Hospital and performed in accordance with the institutional guidelines. The mice were housed in 12-h light/dark cycles, with free access to food and water.

Received: March 28, 2017

Accepted: October 30, 2017

Published online in J-STAGE: December 16, 2017

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Correspondence: Li Chen (e-mail: yepiaochen@126.com)

* Y Lin, L-C Sui and R-H Wu contributed equally to this work and were recognized as co-first authors.

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Table 1. Primer sequences and product sizes used for qRT-PCR

Genes	GeneBank accession no.	Primers	Primer sequences	Amplicon size (bp)
<i>GAPDH</i>	NM_008084.3	Forward	5'-CTTGTCAAGCTCATTTCCTGG-3'	133
		Reverse	3'-TCTTGCTCAGTGCCTTGC-5'	
<i>Cyclin B</i>	NM_172301.3	Forward	5'-CTGACCCAAACCTCTGTAGTG-3'	250
		Reverse	3'-CCTGTATTAGCCAGTCAATGAGG-5'	
<i>CDK1</i>	NM_007659.3	Forward	5'-TGCAGGACTACAAGAACC-3'	142
		Reverse	3'-GCCATTTTGCAGAGATTTCG-5'	
<i>HO-1</i>	NM_010442.2	Forward	5'-ACAGAGGAACACAAAGACCAG-3'	136
		Reverse	3'-GTGTCTGGGATGAGCTAGTG-5'	
<i>GCLC</i>	NM_010295.2	Forward	5'-ACCATCACTTCATTCCCAG-3'	148
		Reverse	3'-TTCTTGTTAGAGTACCGAAGCG-5'	
<i>SOD1</i>	NM_011434.1	Forward	5'-TGTGTCCATTGAAGATCGTGTG-3'	137
		Reverse	3'-TCCAGCATTTCAGTCTTTG-5'	
<i>EIF-1A</i>	NM_010120	Forward	5'-AAGAAGTCTGAAGGCCTATG-3'	170
		Reverse	3'-CAGAGAACTTGAAGGTAGC-5'	
<i>hsp70.1</i>	NM_010478	Forward	5'-TGCTGATCCAGGTGTACGAG-3'	204
		Reverse	3'-CGTTGGTGATGGTGATCTTG-5'	

Antibodies and chemicals

A rabbit polyclonal anti-Nrf2 antibody (ab137550) was purchased from Abcam (Cambridge, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was obtained from Thermo Fisher Scientific (Rockford, IL, USA). Brusatol (14907-98-3) was procured from Shanghai Tauto Biotech Co (Shanghai, China).

Zygote collection and culture

After 46–48 h of 10 IU pregnant mare's serum gonadotropin (PMSG) injection, mice (6–8-week old) were injected with human chorionic gonadotropin (HCG) and immediately mated with male mice. Eighteen hours later, the zygotes were harvested from oviduct ampulla. The enclosed cumulus cells were removed by repeated pipetting and the zygotes cultured in cleavage medium (CM, Quinn's 1026, SAGE, USA) supplemented with 10% serum protein substitute (SPS; Quinn's 3001, SAGE) and paraffin oil at 37°C under 5% CO₂. The embryos were transferred to blastocyst medium (BM, Quinn's 1029, SAGE) supplemented with 10% SPS (Quinn's 3001, SAGE) at the four-cell phase and collected at different time points for the subsequent assays.

Brusatol treatment

A solution of brusatol in dimethyl sulfoxide (DMSO, 1 mM) was diluted in CM to 20, 50, 100 or 200 nM concentration. The mouse embryos were cultured in this medium and used for immunofluorescence microscopy and western blot analysis. The controls were cultured in fresh CM with DMSO.

Activation of Nrf2

The cytoplasm of pronucleus-stage zygotes was microinjected with Nrf2 CRISPR activation plasmid (sc-421869-ACT; Santa Cruz Biotechnology, San Jose, CA, USA) to induce Nrf2 protein overexpression. The plasmid was diluted with nuclease-free water to obtain a stock concentration of 0.04 µg/µl, and 2.5 pl plasmid was

injected into the zygotes with a Narishige microinjector. The control embryos were microinjected with phosphate-buffered saline (PBS). After injection, all the embryos were cultured in brusatol-treated CM.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Around 50 embryos from each group were used to extract total RNA using an RNA queous-Micro kit (Ambion, TX, USA) and cDNA was quantified by qRT-PCR using a Roche Light Cycler 96 Real-time PCR system (F. Hoffmann-La Roche, Basel, Switzerland). The fold change in the gene expression was estimated using $\Delta\Delta C_t$ method with the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as the internal control. Primer sequences are listed in Table 1.

Western blotting analysis

A total of 100 embryos were lysed in Laemmli sample buffer containing protease inhibitors. The samples were heat denatured for 5 min at 100°C, separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked in TBST (Tris-buffered saline containing 0.1% Tween 20) and 5% nonfat milk for 1 h. Following incubation, the membrane was overnight treated at 4°C with primary antibodies as follows: rabbit anti-Nrf2 antibody (1:1000), rabbit anti-UCHL1 antibody (1:1000), and anti-actin antibody (1:2000). After three TBST washes, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at 20°C and processed using an enhanced chemiluminescence (ECL) Plus western blotting detection system.

Immunofluorescence and fluorescence microscopy

For Nrf2 staining, embryos were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 for 20 min. After 1-h blocking in 1% bovine serum albumin (BSA)-supplemented PBS, the samples were incubated overnight at 4°C with anti-Nrf2

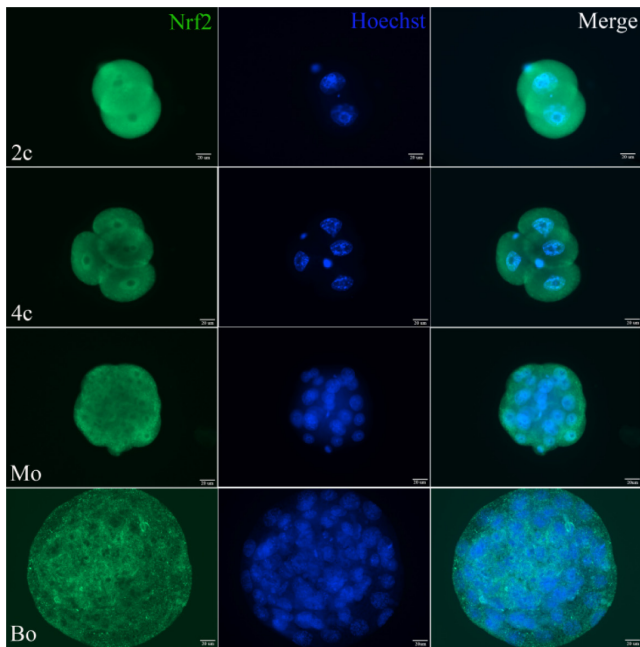


Fig. 1. The subcellular localization of Nrf2 during mouse embryo development. Immunofluorescence staining indicated that Nrf2 primarily concentrated in the cytoplasm and near the nucleus. Green, Nrf2; blue, DNA. Scale bar, 20 μ m.

antibody. The embryos were washed thrice in PBS and labeled with a goat-anti rabbit IgG at room temperature for 1 h. Hoechst 33342 (blue) was used for chromosome staining. The embryo samples were mounted on an anti-fade medium (Vectashield, Burlingame, CA, USA) and examined under a fluorescence microscope (IX3-SVR, Olympus, Japan).

Labeling with EdU for DNA replication

Two-cell embryos were labeled *in vitro* with EdU to confirm their arrest phase. EdU is a thymidine analogue and may substitute thymine (T) into the cells during DNA synthesis. EdU is assessed based on the direct and accurate detection of DNA replication activity by Apollo[®] fluorescent dye. The EdU-specific reaction is widely used in cell proliferation [18, 19]. Two-cell embryos were treated with 25 μ M EdU 18 h after insemination and cultured until 2.5 days after the embryo development [20]. About 50 arrested two-cell embryos were collected and EdU-labeled embryos were stained using an EdU detection kit (RiboBio, Guangzhou, China).

Statistical analysis

Statistical comparisons were made with Student's *t*-test and analysis of variance (ANOVA) when appropriate. Quantitative data were analyzed with Prism 5 software (GraphPad, San Diego, CA, USA). Data are presented as means \pm standard deviation (SD), unless otherwise indicated. Values of $P < 0.05$ were considered as statistically significant.

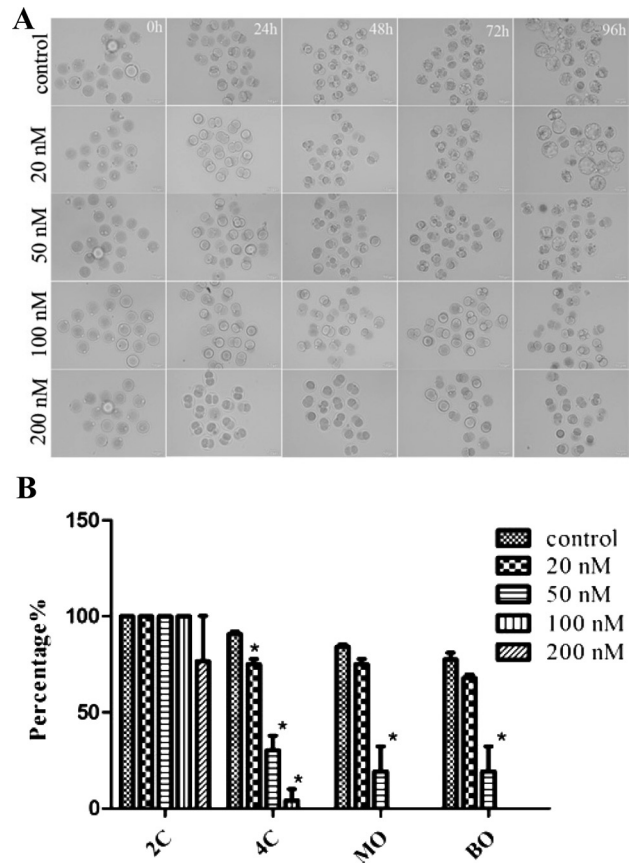


Fig. 2. Effects of brusatol treatment on mouse early embryonic development. A) Embryos were treated with different concentrations of brusatol to select optimal conditions; the control group was treated with DMSO. Embryos failed to develop into the four-cell stage, severely affecting the blastocyst formation in response to brusatol treatment. Original magnification $\times 200$. B) Rates of embryo development after 24, 48, 72 and 96 h culture in different treatment groups (control and 20, 50, 100, and 200 nM). We determined 50 nM brusatol as the optimal concentration for subsequent experiments. * Significantly different ($P < 0.05$).

Results

Expression pattern of Nrf2 during mouse early embryonic development

The zygotes harvested from the oviduct were cultured for 24, 48, 72 and 96 h to obtain two-cell, four-cell, morula, and blastocyst stage embryos, respectively. We explored the localization of Nrf2 at different stages in early embryos by immunofluorescence staining and found that Nrf2 was expressed in the cytoplasm and accumulated in the nucleus from the two-cell stage up to the blastocyst stage (Fig. 1). Moreover, immunofluorescence staining verified the specificity of Nrf2 antibody (data not shown).

Brusatol treatment prevented early embryonic development

To evaluate the impact of Nrf2 on embryonic development, the embryos at the zygote phase were treated with various concentrations

of brusatol (20, 50, 100 and 200 nM) for 0, 24, 48, 72 and 96 h. The control group was treated with equivalent DMSO. Images were recorded by a camera on a stereomicroscope. As shown in Figs. 2A and 2B, most embryos underwent cleavage and developed into two-cell stage after 24 h. After 48 h, the percentage of embryos developing into four-cell stage was significantly decreased in groups treated with 20, 50, 100, and 200 nM brusatol as compared with the control group. None of the embryos developed into four-cell stage in 200 nM brusatol treatment group. After 72 h, no significant difference was observed between control and 20 nM brusatol treatment group, but a significant difference was observed with 50 nM brusatol treatment group. However, no embryo had entered this stage in 100 nM brusatol treatment group. After 96 h, approximately $19.25 \pm 13.1\%$ blastocysts were observed in 50 nM treatment group as compared with $77.5 \pm 3.5\%$ in the control group ($P < 0.05$), whereas $68 \pm 1.8\%$ blastocysts were observed in 20 nM treatment group ($P > 0.05$). These results suggest that brusatol treatment resulted in the failure of early embryonic development in a dose-dependent manner. The concentration of 50 nM brusatol was selected for subsequent experiments.

Brusatol down-regulated Nrf2 expression

We examined the expression of Nrf2 in mouse early embryo by immunofluorescence staining and found that the fluorescence intensity of Nrf2 was significantly decreased in brusatol-treated group as compared with the control group (Figs. 3A and 3B). This observation was further confirmed by western blot analysis (Figs. 3C and 3D). In addition, qRT-PCR analysis indicated the decreased expression of *Nrf2* (Fig. 3E). The intensity of Nrf2 protein was significantly decreased in brusatol-treated group ($0.785 \pm 0.13\%$) as compared with the control group ($P < 0.05$). To test the correlation between brusatol-induced Nrf2 reduction and ubiquitin-proteasome system, UCHL1 levels were determined by western blot analysis (Figs. 3D and 3E). Brusatol treatment led to a significant reduction in UCHL1.

Brusatol treatment decreased mRNA level of Nrf2-mediated antioxidant genes

To determine the mechanism contributing to brusatol-mediated down-regulation of Nrf2, the mRNA expression of Nrf2 and Nrf2 target genes, *HO-1*, *GCLC*, and *SOD1*, was determined by qRT-PCR in control and brusatol-treated groups. Brusatol significantly decreased the level of Nrf2 (Fig. 3C) and relevant antioxidant genes, including *HO-1*, *GCLC*, and *SOD1* (Fig. 4).

Brusatol treatment decreased the mRNA level of cyclin B, CDK1, eif-1a, and hsp70.1

To investigate the cause of mouse early embryonic cleavage failure, we examined the mRNA expression of *cyclin B* and *CDK1* by qRT-PCR after brusatol treatment of two-cell embryos. As shown in Fig. 5A, the relative expression of *cyclin B* and *CDK1* was markedly decreased upon brusatol treatment as compared with the control group ($P < 0.05$). After 48 h, the embryos contained integrated nuclear membranes and incorporated EdU, suggestive of their arrest in G₂/M phase (Fig. 5B) [21]. We detected the mRNA expression of zygotic gene activation (ZGA)-related genes, *eif-1a* and *hsp70.1* (Fig. 5C) and found that brusatol treatment significantly decreased the expression of *eif-1a* and *hsp70.1* genes ($P < 0.05$).

Overexpression of Nrf2 by microinjection of Nrf2 CRISPR activation plasmid into the cytoplasm of the zygote partially rescued the embryo development potency

To evaluate if brusatol treatment prevented the mouse embryonic development through Nrf2, we injected the zygote with Nrf2 CRISPR activation plasmid during brusatol treatment and observed the embryonic development (Fig. 6A). The overexpression efficiency of the plasmid was verified by western blot analysis (Figs. 3D and 3E). The relative expression of Nrf2 protein was markedly increased in the Nrf2 activation group as compared with the brusatol-treated group ($P < 0.05$), similar to the expression of UCHL1. The percentage of embryos at four-cell stage was markedly higher in Nrf2 activation group ($42.7 \pm 8.3\%$) as compared with brusatol-treated group ($23.3 \pm 2.9\%$; $P < 0.05$; Fig. 5B), which was similar to that of morula and blastocyst ($P < 0.05$). These results showed that the overexpression of Nrf2 partially rescued the embryo development potency, suggestive of the effect of brusatol on the mouse embryonic development through Nrf2.

Discussion

Here, we used brusatol to investigate the effects of Nrf2 on mouse early embryonic development. Our results suggest that brusatol exhibited adverse effects on early embryonic development through Nrf2 down-regulation.

We evaluated the localization of Nrf2 during embryonic development. Immunofluorescence staining of Nrf2 in embryos at various developmental stages (from two-cell to morula stage) revealed that Nrf2 primarily accumulated in the cytoplasm, consistent with the reports of a previous study showing Nrf2 accumulation in the cytoplasm of endometrial glandular cells [22]. The expression pattern encouraged us to further investigate the possible roles of Nrf2. The knockdown of Nrf2 expression was shown to inhibit proliferation by inducing apoptosis in various cancer cells, and Nrf2 overexpression resulted in converse phenotypes [23, 24]. However, little is known about the role of Nrf2 in early mouse embryo. To evaluate the effects of Nrf2 on mouse embryonic development, zygotes were pretreated with brusatol. Brusatol down-regulated Nrf2 expression in mouse early embryo, as evident from western blot results. In addition, brusatol prevented the early embryo cleavage at concentrations from 50 nM, resulting in cell cycle arrest. Studies have shown that Nrf2-knockout mice are viable and may mature to give birth; however, these mice show accelerated ovarian failure and develop autoimmune dysfunction [25]. This observation indicates that Nrf2 is a transcriptional factor controlling wide spectra of substrates and plays an important role in reproduction.

The transcription factor Nrf2 is involved in redox-sensitive signaling and antioxidant response by activating cytoprotective protein or antioxidant enzymes [26, 27]. Therefore, we detected Nrf2 target genes, including *HO-1*, *GCLC*, and *SOD1* by qRT-PCR. The decrease in the expression of these genes is indicative of the oxidative stress, suggesting that these genes are likely to be the targets of Nrf2. In addition, Nrf2 degradation is Keap1-dependent; Keap1-Nrf2 pathway is a dynamic balance [28, 29]. The mechanism underlying brusatol-induced Nrf2 inhibition via ubiquitination pathway is partially confirmed by the decreased expression of UCHL1 protein.

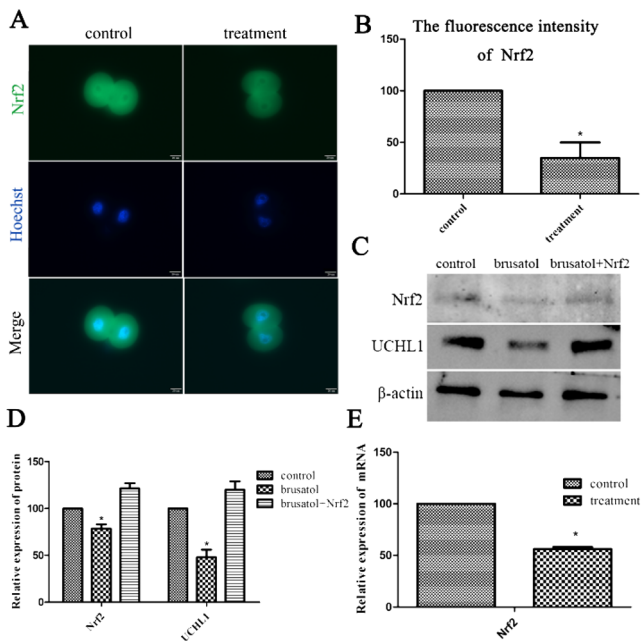


Fig. 3. Effects of brusatol treatment on the expression of Nrf2. A) Images of control and brusatol treatment group by immunofluorescent staining. B) Nrf2 fluorescence intensity analysis of the control and brusatol treatment group. The fluorescence intensity of Nrf2 was significantly decreased in brusatol treatment group as compared with the control group. Green, Nrf2; blue, DNA. Scale bar, 20 μ m. C) Nrf2 and UCHL1 protein expression after brusatol treatment by western blotting. D) Quantitative analysis of the relative expression of Nrf2 protein. Western blot analysis showing the decline in the expression of Nrf2 and UCHL1 in brusatol-treated group. * Significantly different as compared with the control and Nrf2 activation groups ($P < 0.05$). E) Nrf2 mRNA expression in brusatol treatment group as compared with the control group.

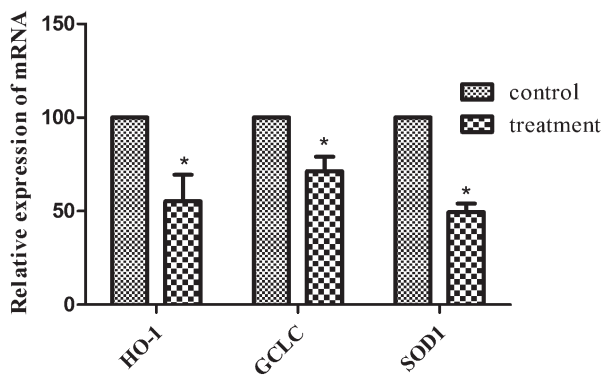


Fig. 4. Effects of brusatol treatment on Nrf2-mediated oxidative stress. Brusatol reduced the mRNA level of Nrf2 target genes, *HO-1*, *GCLC*, and *SOD1*, as analyzed by qRT-PCR.

However, several recent studies suggest that Nrf2 knockdown may lead to cell cycle arrest in MHCC-97H cells. In addition, Nrf2 knockout promoted cell cycle arrest in Hep3B cells, while Nrf2

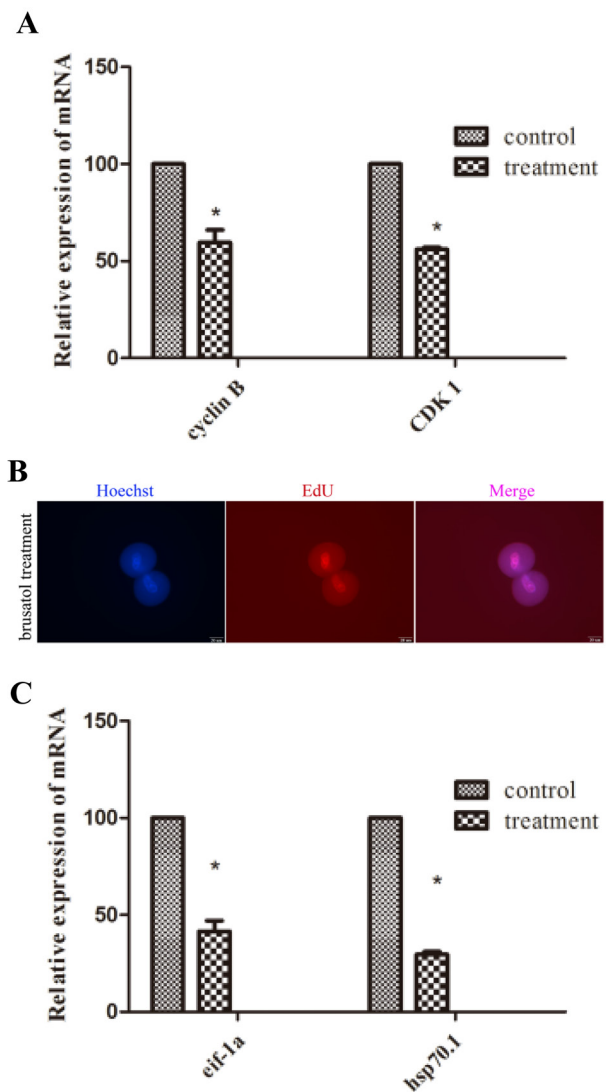


Fig. 5. Effects of brusatol treatment on *cyclinB* and *CDK1* mRNA expression. A) Two-cell embryos were collected to examine *cyclin B* and *CDK1* mRNA expression in brusatol-treated group by qRT-PCR. The mRNA expression of *cyclin B* and *CDK1* was significantly decreased in brusatol treatment group as compared with the control group. B) Embryos were arrested in G_2/M phase, as analyzed by EdU immunofluorescence staining. Red, EdU; blue, DNA. Scale bar, 20 μ m. C) Brusatol reduced the mRNA level of ZGA-related genes, *eif-1a*, and *hsp70.1*, as analyzed by qRT-PCR.

overexpression resulted in antagonistic effects [30]. Together these results indicate that Nrf2 not only protects the cell from oxidative stress but also maintains the order of cell cycle. Therefore, we examined *cyclin B* and *CDK1* mRNA expressions and found that *cyclin B*, the G_2/M cyclin associated with *CDK1* activation, promotes the M-phase entry during embryo cell cycle [31, 32]. We hypothesize that Nrf2 may also be involved in the transcriptional regulation of cyclins in early mouse embryos. Our EdU assay results showed that brusatol induced cell cycle arrest mainly in G_2/M phase. *Cyclin B* and *CDK1*

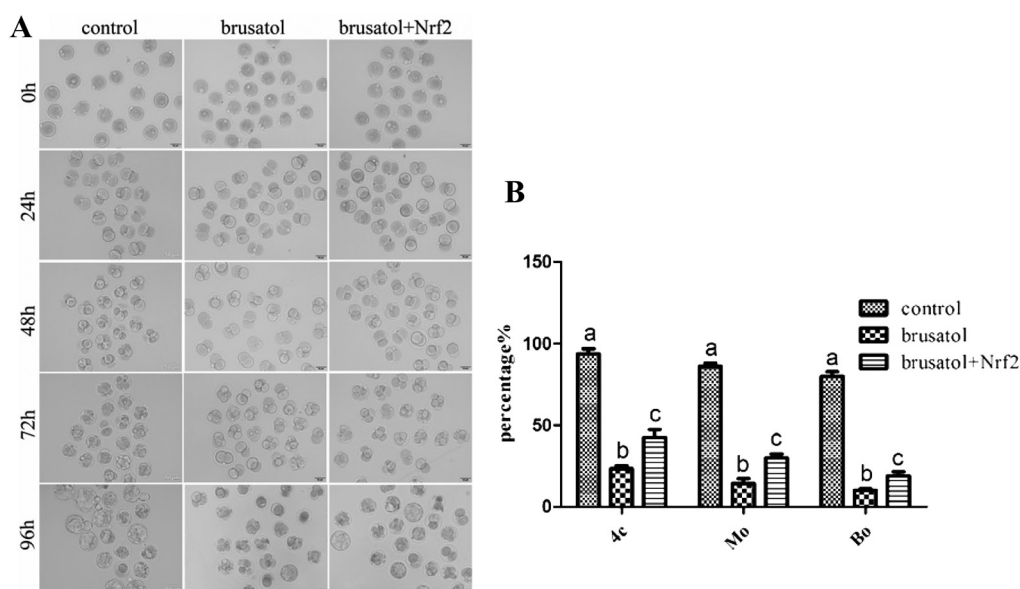


Fig. 6. Effects of microinjection of Nrf2 CRISPR activation plasmid in the cytoplasm of the zygote on the embryo development potency. A) Embryos were microinjected with Nrf2 CRISPR activation plasmid in a medium supplemented with brusatol for 24, 48, 72 and 96 h. Nrf2 activation group showed a significant increase in the four-cell stage embryos as compared with brusatol treatment group. B) Microinjection with Nrf2 CRISPR activation plasmid partially rescued the embryo development potency. The embryo development rate from four-cell to blastocyst stage was significantly increased as compared with the embryos treated with brusatol. As shown in Fig. 3C, the expression of Nrf2 protein was increased in Nrf2 activation group as compared with the brusatol treatment group. a, b, and c represent the significant differences in three groups. Original magnification $\times 200$.

mRNA expression were significantly decreased following brusatol treatment as compared with the control group. Furthermore, the onset of ZGA is a critical event for embryonic development [20] and the decreased expression of *eif-1a* and *hsp70.1* genes indicates that Nrf2 inhibition affects ZGA of early mouse embryo. Based on these results, we explored the role of Nrf2 in embryonic development. However, the ultimate function and mechanisms of Nrf2 in mouse early embryonic development require further exploration.

We overexpressed Nrf2 by the microinjection of the zygote with Nrf2 CRISPR activation plasmid and found that the embryo development rates were partially rescued as compared with the brusatol treatment group. These results indicate that Nrf2 promotes the early embryo development and brusatol affects Nrf2 expression.

Taken together, our results show that brusatol prevents early embryo development through the down-regulation of Nrf2 and this effect may be mainly dependent on *cyclin B-CDK1*-associated cell cycle transformation from G_2 to M phase. Nrf2 may serve as a new factor affecting embryo cleavage and blastocyst formation and Nrf2 detection may be used as a novel diagnostic and therapeutic approach for assisted reproduction technology.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant number 81503655) and Foundation of Jinling Hospital, Clinical School of Medical College, Nanjing University (2014027&2014030).

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