
Rat Hepatoma BRL Cells Proliferation by Acrylamide and Alcohol Combination

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To cite this article:

Xiaoyun Shan, Pengqi Wang, Qing Feng. Rat Hepatoma BRL Cells Proliferation by Acrylamide and Alcohol Combination. *International Journal of Nutrition and Food Sciences*. Vol. 9, No. 3, 2020, pp. 78-85. doi: 10.11648/j.ijfnfs.20200903.12

Received: May 14, 2020; Accepted: June 9, 2020; Published: June 23, 2020

Abstract: Acrylamide, exists in carbohydrate-rich food heated at high temperatures, is a probable carcinogen to humans. Excessive alcohol intake can also lead to a variety of pathological changes in the body. Acrylamide in the foods and alcohol in the drinks are unavoidable. And studies have demonstrated that combination of the two substances which are taken into the body via diet may cause adverse effects in the cells, even induce impairments on testicular spermatogenesis in male offsprings. Both acrylamide and alcohol are mainly metabolized in the liver, where cytochrome P450 2E1 (CYP2E1) acts as the common metabolic enzyme of the two xenobiotics. This study aimed to explore the effects of acrylamide and alcohol combination on rat hepatoma BRL cells proliferation and the probable mechanisms. MTT, western blotting, EdU fluorescence staining, flow cytometry and PCR were used. Results showed that combination of acrylamide and alcohol at low doses promoted BRL cells proliferation through CYP2E1/Akt/ NF- κ B/cyclinB1/cyclinD1 activation and ROS production. The combined effects of acrylamide and alcohol largely depended on ROS level. Furthermore, acrylamide and alcohol could synergistically induce miR-21, which was related to the progress of liver regeneration. These data showed that combination of acrylamide and alcohol at low doses promoted BRL cells proliferation through inducing ROS production, which indicated that intake of fried starch food with small dose of alcohol might have positive effects on liver regeneration.

Keywords: Acrylamide, Alcohol, Proliferation, ROS, miR-21

1. Introduction

Acrylamide is a kind of water-soluble vinyl monomer formed from the hydration of acrylonitrile [1]. It has widespread application in many areas, such as drinking water purification, municipal sewage and industrial wastewater treatment, cosmetics and daily-use chemical additives, and laboratory reagents. Besides drinking water, people can also be exposed to a small amount of acrylamide from heating starchy foods (such as French fries, roasted coffee and bread, etc.) [2]. Due to its strong permeability, acrylamide can enter into the body through the digestive tract, respiratory tract, skin and mucous membrane quickly, with the fastest absorption in the gastrointestinal tract [3, 4]. Because of the various toxic

effects such as nerve, reproductive and genetic toxicity and carcinogenicity [5], acrylamide has caused extensive concern. Acrylamide metabolism in human and animal body is mainly through two competing ways: one is conjugating with reduced glutathione (GSH); another is generating epoxidation metabolites glycidamide by cytochrome P450 system, mainly CYP2E1, further conjugating with reduced GSH [6].

Moreover, in human daily activities, exposing to other environmental risk factors through respiratory tract, digestive tract and skin is inevitable as well. And, most of these factors act on the human body not by oneself. Thus, to study the toxicological effects of one poison, we should also consider its joint action with other materials. In recent years, with increasing consumption of alcohol, liver damage caused by alcohol is also on the rise year by year. Although

some studies indicate that light to moderate alcohol consumption is associated with a reduced risk of multiple cardiovascular outcomes [7, 8], the potential harm still cannot be ignored.

It is noteworthy that alcoholic beverages drinking could also accompany the intake of other foods, such as potato chips, popcorn and fried chicken, which contain acrylamide. Exposing to the two kinds of potentially toxic substances together may cause more severe toxic effects on the body, which has raised the attention of experts and society as well. One study by Sen E et al through evaluating sexual development in male mice after intake of 14 mg/kg acrylamide and 2 g/kg alcohol from gestation day 6 to postnatal day 21 suggests that consumption of acrylamide together with alcohol may induce impairments on testicular spermatogenesis in male offsprings, such as reduced gonadosomatic index of male offsprings and increased lipid peroxidation level and superoxide dismutase enzyme activity [9].

Based on our previous study, which showed that low doses of acrylamide could accelerate the hepatocellular carcinoma cells malignant proliferation [10], this subject aimed to study the effects of low dose of acrylamide and low dose of alcohol, alone or in combination, on normal liver cell proliferation, and expected to get the based data about the effects of a small amount of alcohol and dietary acrylamide together on liver regeneration.

2. Materials and Methods

2.1. Cell Culture and Reagents

BRL cells were purchased from Cell Centre of Chinese Academy of Medical Sciences (Peking, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/mL, Gibco), and streptomycin (100 µg/mL, Gibco). These cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Acrylamide (purity > 99.5%, dissolved in ddH₂O in a concentration of 1000 mM/L) was purchased from Sigma-Aldrich (St Louis, MO, USA). Alcohol (purity > 99.7%) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. MTT Assay

Cell viability was assessed by the methyl thiazol tetrazolium bromide (MTT) assay. Cells were seeded in a 96-well plate at a density of 4×10³ cells per well in a final volume of 180 µL of medium. After indicated treatment, the cells were incubated with MTT solution (5 mg/mL) at 37 °C for 4 h. The formed formazan crystals were dissolved in DMSO at room temperature for 10 minutes. Then the absorbance was read at 490 nm with a microplate reader (Tecan /Infinite M200, Switzerland).

2.3. Western Blot Analysis

The experiments were prepared from seeding with 5×10⁵

cells. After indicated treatments, the cells were washed twice with PBS and suspended in a lysis buffer including 1 mM DTT, 0.1% protease inhibitor and 5 mM PMSF (KeyGEN BioTECH, China). Protein concentrations were measured by BCA Protein Assay Kit (Beyotime, China). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate 60 µg of protein, which was then transferred to PVDF membrane (Millipore Corporation, USA). The primary antibodies included rabbit anti-NF-κB/p65 monoclonal antibody (Cell Signaling technology, USA), rabbit anti-p-Akt monoclonal antibody (Cell Signaling technology, USA), rabbit anti-CYP2E1 monoclonal antibody (Epitomics, USA), mouse anti-Nrf2 monoclonal antibody (Cell Signaling technology, USA) and mouse anti-β-actin monoclonal antibody (BOSTER, China). Second antibodies included HRP-Conjugated AffiniPure Goat Anti-rabbit IgG (ZSGB-BIO, China) and HRP-Conjugated AffiniPure Goat Anti-mouse IgG (ZSGB-BIO, China). Immunoreactive proteins were visualized using ECL Western blotting detection reagents (GE Health-care, Buckinghamshire, UK). The bands were quantified with the Image J software.

2.4. EdU Fluorescence Staining

The 5-ethynyl-2'-deoxyuridine (EdU) fluorescence staining was used to detect the newly synthesized DNA in BRL cells (seeding with 4×10³ cells per well in a 96-well plate) after the indicated treatment. All steps performed following the manufacturer's instructions of Cell-Light™ EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China).

2.5. siRNA Transfection

BRL cells (2.5×10⁵) were transfected with predesigned human CYP2E1 siRNA or siRNA control (50 nM) (RiboBio, Guangzhou, China) in a six-well plate or a 96-well plate for 4 h with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif, USA) in a serum-free and antibiotic-free media. All steps performed following the manufacturer's instructions.

2.6. Flow Cytometry Assay

DCFH-DA fluorescent labeling was used to measure intracellular production of the reactive oxygen species (ROS) in BRL cells (seeded with 2.5×10⁵). After the indicated treatment, the cell supernatants were removed and 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) was added into each group. After incubation with DCFH-DA for 20 min at 37°C, the cells were washed twice with PBS and maintained in 600 µL PBS. The fluorescence images were captured by a fluorescence microscope (OLYMPUS U-RFLT50, Japan), under ×200 magnification with the filter which excitation at 470–490 nm and emission at 510–550 nm.

2.7. Real-Time Polymerase Chain Reaction

Total RNA was extracted by the RNAiso Plus (TaKaRa

BioTechnology, Dalian, China) following the manufacturer's protocol. For miRNA quantitative analysis, total RNA was reversely transcribed using the PrimeScript™ RT Master Mix (TaKaRa BioTechnology, Dalian, China), and qPCR was performed using SYBR Premix Ex Taq™ II (TaKaRa BioTechnology, Dalian, China). The miRNA primers were purchased from RiboBio Co., Ltd. (Guangzhou, Guangdong, China). All qPCR was performed with the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Expression of miRNA was defined from the threshold cycle, and relative expression levels were calculated using the 2^{-Ct} method after normalization with reference to the expression of U6 small nuclear RNA.

2.8. Statistical Analyses

The results were presented as the mean ± standard deviation (SD) compared with the controls. A one-way analysis of variance (ANOVA), which was followed by a Tukey HSD test for the multiple comparisons, was used to detect the differential effects of acrylamide and alcohol on BRL cells. A *P* value < 0.01 was considered statistically significant.

3. Results

3.1. Synergistic Collaborative Effect of Low Doses of Acrylamide and Alcohol on BRL Cells Proliferation

After treating BRL cells for 24 hours with different concentrations of acrylamide or alcohol, MTT assay was performed to detect cell viability. As the results shown in Figure 1a, acrylamide at less than 1000 μM had no significant effect on cell activity. In the same way, only the alcohol reaching at 100 mM or higher produced cytotoxic effect (Figure 1b). Then, we chose the acrylamide (50, 80, 100 μM) and alcohol (1, 5, 10 mM) doses, which acted no significant effects, for their combined experiment, finding that each group of the combination was able to promote the BRL cells survival. In the group co-treated with 100 μM acrylamide and 10 mM alcohol, the cell activity increase was most pronounced (Figure 1c).

In order to further validate the combined effects on cell proliferation of acrylamide and alcohol, EdU fluorescence staining assay was performed to detect the newly synthesized DNA in BRL cells. The results showed that EdU positive cells in the acrylamide and alcohol treated separately group after 24 h had no significant difference compared with the controls, while the combined treating group increased markedly (Figure 1d). These results suggested that acrylamide and alcohol combination could activate the DNA replication activity.

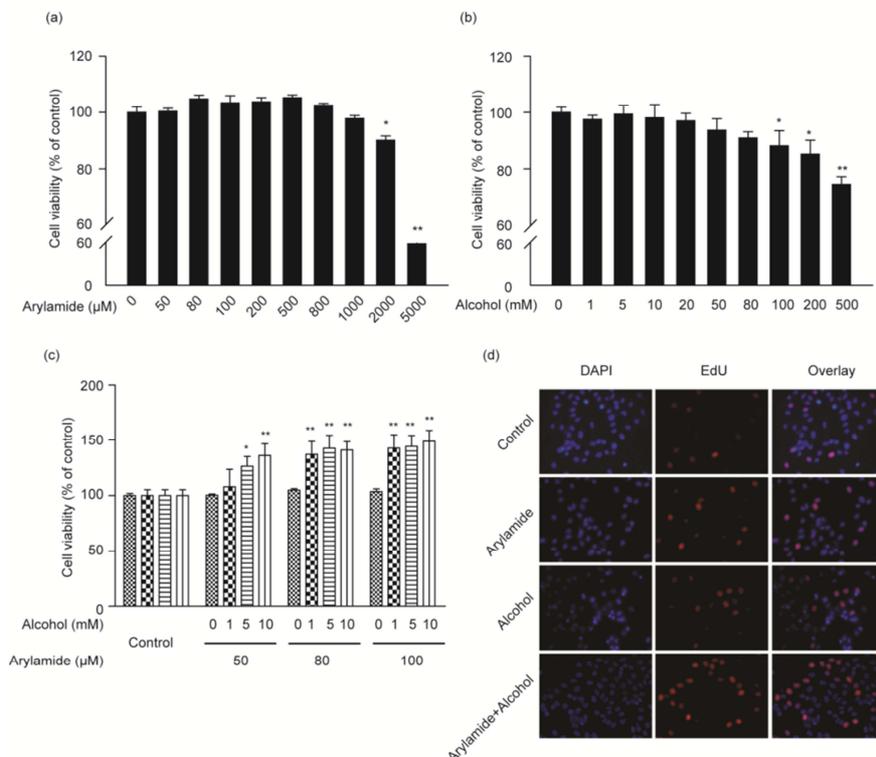


Figure 1. Effect of acrylamide and alcohol on proliferation of BRL cells.

Up to 2000 μM, acrylamide distinctly inhibited the viability of BRL cells after 24 h of treatment, as analyzed by MTT assay; Likely, alcohol showed no significant cytotoxicity on BRL below 100 mM (a and b). Low doses of acrylamide and alcohol combination largely increased BRL cells viability, especially at 100 μM and 10 mM respectively (c). The EdU-positive cells (red fluorescence staining) increased distinctly after co-treatment acrylamide (100 μM) and alcohol (10 mM) for 24 h measured by EdU fluorescence staining assay (d). Results are presented as the mean ± SD from three independent experiments (in triplicate for each experiment). Statistical differences to the controls are shown as **P* < 0.01, and ***P* < 0.001.

3.2. Acrylamide and Alcohol Induced Cell Cycle Protein Increasing

In this study, after 24 hours of treatment with 100 μ M of acrylamide and 10 mM of alcohol, cyclinD1 and cyclinB1 raised in BRL cells (Figure 2a). Then, the cell cycle proteins

were measured at different time points and increased in a time-dependent way. But when reaching to 36 h, they returned to normal (Figure 2b). These results suggested that low doses of acrylamide and alcohol can increase the expression of cyclinD1 and cyclinB1.

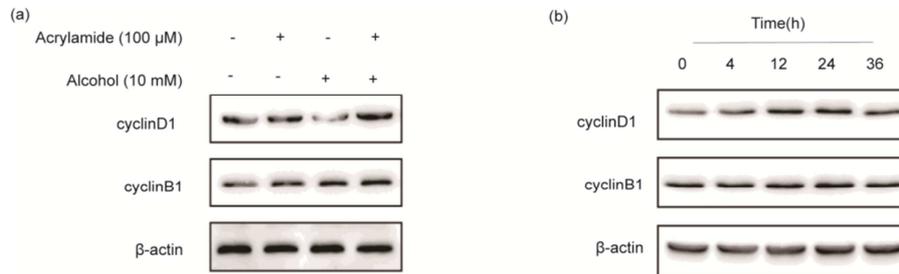


Figure 2. Effect of acrylamide and alcohol combination on cyclinD1 and cyclinB1 expressions.

Acrylamide and alcohol combination largely increased cyclinD1 and cyclinB1 expressions in BRL cells (a). Acrylamide and alcohol combination showed obvious effects on upregulation of cyclinD1 and cyclinB1 for 12 and 24 h treatment, but not for 4 and 36 h (b).

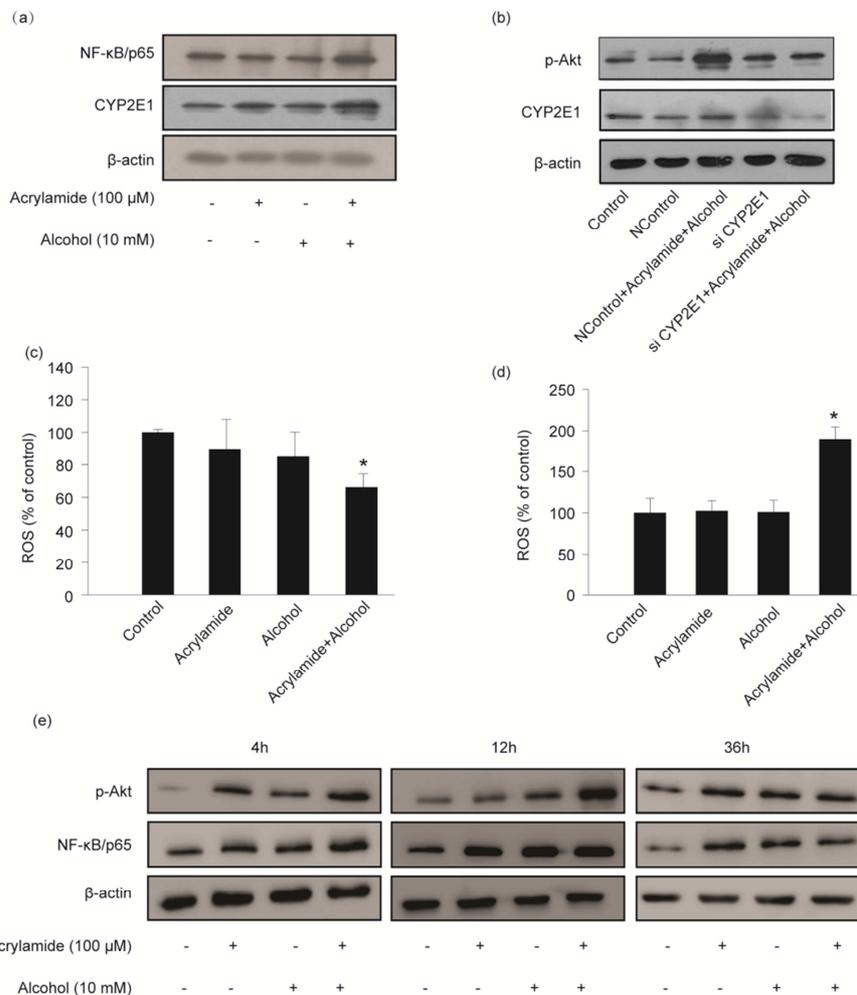


Figure 3. Effect of acrylamide and alcohol combination on ROS.

Acrylamide and alcohol combination significantly induced NF- κ B/p65, p-Akt and CYP2E1 expressions in BRL cells. And knockdown of CYP2E1 could prevent acrylamide and alcohol combination to induce p-Akt (a and b). Co-treatment for 24 h was able to induce production of ROS, but not for 4 h (c). Though co-treatments for different time periods all increased NF- κ B/p65 and p-Akt expressions, once reaching to 36 h, the effect weakened (d). Results are presented as the mean \pm SD from three independent experiments (in triplicate for each experiment). Statistical differences to the controls are shown as * P < 0.01, and ** P < 0.001.

3.3. Acrylamide and Alcohol Combination Could Induce Cellular ROS

CYP2E1 is one of the main metabolic enzymes in the liver. Previous studies indicated that acrylamide or high doses of alcohol can induce CYP2E1 expression [10]. In this study, we treated BRL cells alone or in combination with acrylamide and alcohol for 24 h. Though individual treatment did not induce CYP2E1 expression, the combined group significantly raised CYP2E1, which suggested that acrylamide and alcohol had an important synergy on CYP2E1 (Figure 3a). In addition, the proteins related to oxidative stress, such as Akt and NF- κ B, were both phosphorylated in the combined treating group. As shown in Figure 3a and Figure 3b, NF- κ B/p65 and p - Akt expression were much higher than the controls. However, once CYP2E1 was knocked down, the co-treatment was not able to phosphate Akt anymore. As CYP2E1 can control the NF- κ B/p65 expression [10] and cell proliferation is able to be mediated by the activation of NF- κ B via PI3K/Akt pathway [11], which means that acrylamide and alcohol might adjust cell proliferation through the molecular way as CYP2E1/Akt/NF- κ B/cyclinB1/cyclinD1. Studies have confirmed that moderate ROS is necessary for liver regeneration, as ROS at too low or too high levels can destroy the oxidation/antioxidant balance and inhibit the liver

proliferation and regeneration [12]. We tested the level of ROS in cells after treatment for 24 h, finding that the joint group had a marked increase in the level of ROS (Figure 3d). In addition, several REDOX proteins expression at other various time points such as 4, 12 and 36 h was detected. After 4 and 12 h, both p - Akt and NF- κ B/p65 was raised obviously, and the antioxidant Nrf2 was significantly lower after 12h (delete this sentence). To 36 h, various protein levels turned back to normal level again (Figure 3e).

3.4. BRL Cells Activity Enhancement by Combination of the Acrylamide and Alcohol Depended on REDOX Level

In this study, acrylamide and alcohol combination obviously increased the number of BRL cells and cells were highly stretched after 24 h. Once the antioxidant N-acetyl-L-cysteine (NAC) was pretreated to cells, the expression of p - Akt and Nrf2 (antioxidant) changed. As collaboration effect was antagonized by NAC, acrylamide and alcohol could not increase p - Akt and decrease Nrf2 any more (Figure 4a and 4b). The cell proliferation was also inhibited (Figure 4c). These indicated that BRL cells activity enhancement by combination of the acrylamide and alcohol largely depended on cell oxidation level.

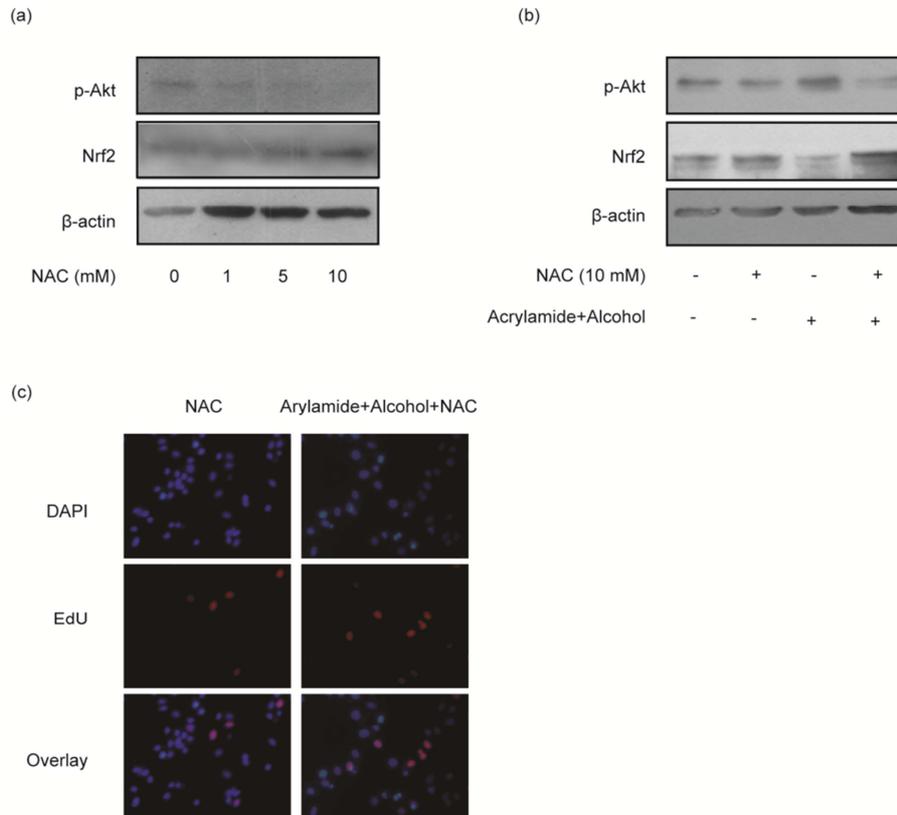


Figure 4. Antagonistic effects of NAC on acrylamide and alcohol combination in BRL cells.

NAC from 1 to 10 mM could regulated the levels of NF- κ B/p65 and p-Akt effectively in BRL cells (a). Pretreatment of NAC for 2 h, acrylamide and alcohol combination lost the ability to induce NF- κ B/p65, p-Akt and proliferation in BRL cells (b and c).

3.5. Combination Treatment of Low Doses of Acrylamide and Alcohol Induced miR - 21 Expression in BRL Cells

As one of the earliest discovered tiny RNA, miR-21 has been linked to regulation of cell proliferation, migration, and apoptosis of tumor process including liver cancer, lung cancer, breast cancer and prostate cancer and other tumors [13]. Several studies have documented the significant induction of miR-21 in the mouse liver during the proliferative phase of liver regeneration after partial hepatectomy [14, 15]. A study from Li J J showed that miR-21 could obviously upregulated and promoted BRL-3A cell proliferation, which involved in rat liver regeneration induced by partial hepatectomy [16]. Mentioned above, combination of alcohol and acrylamide could promote rat liver BRL cells proliferation, we examined the expression of miR-21 in BRL cells at different treating times. As shown in Figure 5, combined treatment raised miR-21 expression independently.

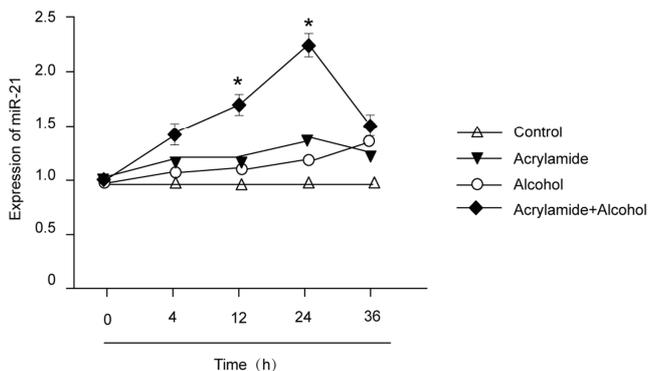


Figure 5. Effect of acrylamide and alcohol combination on miR-21 in BRL cells.

Acrylamide and alcohol combination induced miR-21 in BRL cells after 12 and 24 h treatment. Results are presented as the mean \pm SD from three independent experiments (in triplicate for each experiment). Statistical differences to the controls are shown as * $P < 0.01$, and ** $P < 0.001$.

4. Discussion

Cell proliferation acts a very important role in organism growth, development, reproduction and genetic basis. However, cell proliferation is not always a good thing, especially for these people with cancer. On the one hand, unlimited proliferation makes the patient's body consuming large nutrients. On the other hand, a variety of toxins release and cancer cells transfer throughout the body, inducing a series of symptoms, such as weight loss, weakness, anemia, fever, impaired viscera function and even death. Initiation, proliferation and termination compose the liver regeneration process. Some signaling pathways/proteins like NF- κ B and miR-21 have been found involved in the process [14]. The induction of NF- κ B and reduction of P450 activity can also be initiated by augments of liver regeneration (ALR) treatment [17].

Simultaneous consumption of acrylamide-containing foods

and alcoholic beverages is inevitable. Studies have shown a higher genotoxic potential of alcohol/acrylamide combination treatment compared to acrylamide treatment alone in HepG2 cells. But the treating concentration is much higher (alcohol (15–240 mM) and acrylamide (5 mM)) [18]. To understand the effect of low dose of alcohol/acrylamide combination, we examined the change of BRL cell proliferation and the possible mechanism. In this study, the results conformed the promoting effects on BRL cells proliferation of low dose of acrylamide and alcohol combination from different respects, such as cell cycle, ROS level and miR-21 expression.

Cell cycle regulation plays a key role in cell growth process. CyclinD1 and cyclinB1 are two common cell cycle proteins which drive cells proliferation at cycle G1/S and G2/M, respectively [19]. When cells were treated with acrylamide and alcohol for different time periods, both cyclinD1 and cyclinB1 over expressed except for 36h. These indicated that acrylamide and alcohol function had strong timeliness.

The regulation of cell proliferation by ROS exists a lot of controversy. On one hand, ROS act as a proliferation promoting factor and increase both normal and cancer cells proliferation [20]. On the other hand, ROS level has a negative correlation to cell activity [21]. NAC, as a common antioxidant, can increase GSH content, remove oxygen free radicals and inhibit NF- κ B activity and are widely used in clinical and experimental research in the field of respiratory, cardiovascular and nervous system as well as in AIDS [22]. In this study, low dose of acrylamide and alcohol increased the proliferation of the cells. Once the antioxidant NAC was pretreated, cell proliferation was inhibited. And we found that the cell proliferation induced by this combination depended on the ROS levels, indicating that acrylamide and alcohol could promote liver regeneration similar to liver resection and H₂O₂ treatment.

Liver regeneration is an important repair response to liver injury. But chronic ethanol consumption inhibits and delays liver regeneration in experimental animals. Moreover, upregulation of miR-21 following partial hepatectomy (PHx) is more robust in livers from ethanol-fed rats than in control rats, despite the inhibited cell proliferation, in apparent conflict with a pro-proliferative role of miR-21 in liver regeneration [23], which needs a comparative analysis of potential miR-21-affected functions during regeneration between naive and ethanol-adapted livers. However, in our study, low concentration of acrylamide and alcohol combination largely also facilitated the overexpression of miR-21 and cell proliferation.

In conclusion, high dose of acrylamide and alcohol together or alone was found to have genotoxicity, cytotoxicity and even carcinogenicity, but we showed that combination of low dose of acrylamide and alcohol could induce BRL cell proliferation through regulating cell cycle, ROS level and miR-21 expression. The data also proved that these functions dropped off after a certain time threshold. The results in this study applied in vitro model gave first hint of a possible benefit in liver regeneration entailed by simultaneous consumption of

acrylamide-containing foods and alcoholic beverages. However, further studies are needed to act in rat models.

5. Conclusion

The present study showed that the combination of low doses of acrylamide and alcohol was able to promote BRL Cells proliferation through the molecular way as CYP2E1/Akt/ NF- κ B/cyclinB1/cyclinD1. And the enhancement of BRL cells activity by combination of acrylamide and alcohol largely depended on cell oxidation level and raised miR-21 expression.

Acknowledgements

The study was funded by Scientific Research Project of Hunan Education Department (16C1389) and Science and Technology Project of Hengyang City (2015KS10).

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