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Research Article

Protective Effects of Adenovirus-Mediated Overexpression of Heat Shock Protein 70 (HSP70) in Rat Liver Cells Against Oxidative Stress

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Abstract

This study was aimed to study the protective effects of Adenovirus-mediated overexpression of heat shock protein 70 (HSP70) in rat liver cells against oxidative stress. Cultured Buffalo Rat Liver cells (BRL-3A) cells were divided into six groups. Groups SH and CH were transfected with Ad-CMV-HSP70, groups SN and CN were transfected with Ad-CMV-Null, and groups SC and CC were treated under the same conditions without adenovirus for 48 h. Groups SH, SN and SC were treated with 500 μM H_2O_2 for 3 h, and groups CH, CN and CC were treated under the same conditions without H_2O_2. The following parameters were determined for cells of each group: proliferation rate, lactic dehydrogenase (LDH) leakage rate, catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) enzyme activities, and glutathione (GSH) and protein carbonyl contents. In addition, the HSP70 mRNA abundance and HSP70 protein expression levels were examined using quantitative real-time PCR (qRT-PCR) and western blotting, respectively. The result shows that compared with cells of group SC, cells of group SH had a higher proliferation rate ($P<0.01$), CAT activity ($P<0.01$), GSH content ($P>0.05$), SOD activity ($P>0.05$), and protein carbonyl content ($P<0.01$), a lower LDH leakage rate ($P<0.01$) and GSH-Px activity ($P\leq0.05$), and higher HSP70 mRNA abundance ($P<0.01$) and HSP70 protein expression level ($P<0.01$). In summary, Adenovirus-mediated overexpression of HSP70 has certain protective effects on rat liver cells against H_2O_2-induced oxidative stress.

Keywords: *HSP70, BRL-3A, Oxidative stress, Adenovirus vector, Cold stress*

Sıçan Karaciğer Hücrelerinde Isı Şok Protein 70 (HSP70)'in Adenovirüs Aracılı Aşırı Ekspresyonunun Oksidatif Strese Karşı Koruyucu Etkileri

Öz

Bu çalışmada, sıçan karaciğer hücrelerinde ısı şok protein 70 (HSP70)'in Adenovirüs aracılı aşırı ekspresyonunun oksidatif strese karşı koruyucu etkilerinin araştırılması amaçlamıştır. Kültürlenmiş Buffalo Rat Karaciğer hücreleri (BRL-3A) altı gruba ayrıldı. SH ve CH grupları Ad-CMV-HSP70 ile, SN ve CN grupları Ad-CMV-Null ile transfekte edildi. SC ve CC grupları ise 48 saat boyunca aynı koşullar altında adenovirüs olmadan tedavi edildi. SH, SN ve SC gruplarına 3 saat boyunca 500 μM H_2O_2 uygulandı ve CH, CN ve CC grupları H_2O_2 olmadan aynı koşullara maruz bırakıldı. Her bir gruptaki hücreler için aşağıdaki parametreler belirlendi: proliferasyon hızı, laktik dehidrogenaz (LDH) sızıntı oranı, katalaz (CAT), glutatyon peroksidaz (GSH-Px) ve süperoksit dismutaz (SOD) enzim aktiviteleri, glutatyon (GSH) ve protein karbonil içeriği. Ek olarak, HSP70 mRNA yoğunluğu ve HSP70 protein ekspresyon seviyeleri, sırasıyla kantitatif gerçek zamanlı PCR (qRT-PCR) ve western blot yöntemi kullanılarak incelendi. Sonuçlar, SC grubundaki hücrelerle karşılaştırıldığında, SH grubundaki hücrelerin daha yüksek proliferasyon oranına ($P<0.01$), CAT aktivitesine ($P<0.01$), GSH içeriğine ($P>0.05$), SOD aktivitesine ($P>0.05$), ve protein karbonil içeriğine ($P<0.01$), daha düşük bir LDH salınım oranı ($P<0.01$) ve GSH-Px aktivitesine (P0.05) ve daha yüksek HSP70 mRNA varlığı ($P<0.01$) ve HSP70 protein ekspresyon seviyesine ($P<0.01$) sahip olduğunu gösterdi. Özetle, HSP70'in Adenovirüs aracılı aşırı ekspresyonu, sıçan karaciğer hücreleri üzerinde H_2O_2'nin neden olduğu oksidatif strese karşı belirgin koruyucu etkilere sahiptir.

Anahtar sözcükler: *HSP70, BRL-3A, Oksidatif stres, Adenovirüs vektörü, Soğuk stresi*

INTRODUCTION

Since Sies published Oxidative Stress in 1985, oxidative stress has increasingly been used as an indicator in research into biochemical systems for the influence of oxidative processes, particularly in the biomedical field [1]. Research over the last 30 years has shown that oxidative stress is closely involved in the majority of diseases and in aging of organisms. Many factors are known to cause oxidative stress, including electricity, cold, heat, and atmospheric

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pollution. Thus, many stresses such as cold stress, heat stress, and transport stress are closely related to oxidative stress. Oxidative stress provides a convenient entry point for solving the damage caused to animals by adverse stresses in livestock production.

Several studies have shown that compensatory changes occur in the antioxidant defense system of mammals exposed to low temperatures [2]. Once an animal is exposed to a cold environment, the protein structure suffers more severe oxidative damage in the liver than in the kidney or heart [3]. Therefore, the injury mechanism of cold stress and anti-stress pathways in animals could be investigated through the study of oxidative stress in liver cells. The stress protein HSP70 is one of the major molecular chaperones, which helps to maintain cellular protein homeostasis and improve the tolerance of cells to stressors to maintain the normal physiological functions of cells. Our previous work showed that liver HSP70 rapidly increases in organisms under cold stress, helping the animal resist the external stimuli [4]. In the present study, we plan to transfect BRL-3A cultured *in vitro* with recombinant adenovirus carrying HSP70 to observe the proliferation of HSP70-expressing hepatocytes under H_2O_2-induced oxidative stress. Condition and degree of oxidative damage. The Results permitted the evaluation of the protective effect of recombinant adenovirus HSP70 transfection on rat liver cells against oxidative stress.

Newborn animals generally have weak ability to keep warm, wintering in northern alpine regions, and the impact of extreme weather on animals. The cold stress involved in these three aspects is gradually attracting scientific researchers' attention. Production practice and previous scientific research results have shown that cold stress can cause a decline in animal production performance, reduce animal disease resistance, and cause death in severe cases. Therefore, how to solve the problem of eagerly improving the ability of animals to resist cold stress in production practice has become an urgent issue in the field of animal husbandry research. In the process of cold stress in animals, the liver is damaged due to oxidative stress. This experiment studies animal cold stress from the perspective of liver cell oxidative stress, which will help to study the role of HSP70 in animal anti-cold stress, open up new ideas for cold stress research, reveal the mechanism of cold stress for the future and lay the foundation for anti-stress approach.

MATERIAL and METHODS

Materials

Competent *Escherichia coli* DH5α cells were obtained from the Animal Stress Biology Laboratory of Heilongjiang Bayi Agricultural University. The BRL-3A cell line was provided by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The adenovirus expression system (Adxsi Vector System) was purchased from SinoGenoMax (Beijing, China). Commercial kits for assaying WST-1 cell proliferation and cell toxicity, total GSH, total SOD activity, CAT, GSH-Px and BCA protein content (enhanced) were purchased from Beyotime Institute of Biotechnology (Haimei, China). Kits for protein carbonyl content assay and LDH assay were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The HSP70 monoclonal antibody was purchased from Abcam and the GAPDH monoclonal antibody and HRP-labeled goat anti-mouse IgG purchased from Santa Cruz (Santa Cruz, CA, USA). Other imported or domestic reagents were all analytical grade.

Cell Culture

BRL-3A cells frozen in liquid nitrogen were taken out, placed in a 37°C water bath environment and continuously shaken to allow rapid melting. The freezing tube was thoroughly sterilized with 75% ethanol, and the cell suspension was transferred aseptically to a 15 mL centrifuge tube. The cells were washed with 10 mL of cell culture broth (10% FBS) and centrifuged at 500x g for 5 min. The supernatant was removed and the cell pellet was washed again, re-suspended in 10 mL of cell recovery solution (20% FBS), transferred to a 75 cm^2 cell culture flask and incubated in an incubator (37°C, 5% CO_2). Thereafter, the cell culture broth (10% FBS) was replaced on a daily basis. When the cell density exceeded 1×10^6 mL^{-1}, the cell culture was digested with 0.25% trypsin (w/w) and then passaged into several 96-well plates, 6-well plates and 25 cm^2 cell culture flasks for routine incubation for 2-3 d. All experiments were performed on cells in the logarithmic growth phase and in good condition.

Experimental Grouping

The BRL-3A cells were randomly divided into six groups. These include three non-stressed groups: recombinant adenovirus Ad-CMV-HSP70 transfected (CH), empty vector Ad-CMV-Null transfected (CN) and no virus (CC), as well as three H_2O_2-stressed groups: recombinant adenovirus Ad-CMV-HSP70 transfected (SH), empty vector Ad-CMV-Null transfected (SN), and no virus (SC). Each group was set with 3-5 parallel replicates.

Recombinant Adenovirus Vector Construction and Target Cell Transfection

Recombinant adenovirus vector construction: The HSP70 gene sequence was amplified from Wistar rat spleen tissues using RT-PCR and cloned into vector pMD18-T to construct the recombinant cloning plasmid pMD18-T-HSP70. Nucleotide sequencing showed that the obtained sequence has a total length of 2269 bp and contains the complete CDS of HSP70. This is 100% homologous to the amino acid encoded by the same gene of rats (accession number: NP_114177) published in Genbank. The cloned HSP70 reading frame was sub-cloned into the pShuttle-CMV shuttle vector to obtain the recombinant plasmid

pShuttle-CMV-HSP70. The expression cassette of HSP70 was cut from the recombinant shuttle vector using I-Ceu I and I-Sce I, and ligated into to the pAdxsi vector carrying the adenovirus backbone, and identified via XhoI restriction enzyme analysis and sequencing. The recombinant adenovirus backbone plasmid was digested with PacI for linearization, and transfected into 293 (R) cell lines for virus packaging.

Target cell transfection: The BRL-3A cells were harvested at the exponential stage under good growth conditions and washed once with phosphate buffered saline (PBS). The cells of groups SH and CH were transfected with cell culture containing 1×10^7 pfu/mL Ad-CMV-HSP70 (MOI = 20); groups SN and CN were transfected with cell culture containing an equal amount of Ad-CMV-Null; and groups SC and CC were supplemented with an equal volume of virus-free cell culture. All six cell cultures were returned to the incubator (37°C, 5% CO_2). The plates were slightly shaken for 10 sec at 15 min intervals. Cultural broth was supplemented after 6 h. The total incubation period was 48 h.

Oxidative Stress Model

The oxidative treatment solution was prepared by diluting 30% H_2O_2 solution with cell culture broth to a final concentration of 500 µM. The culture broth was removed from all six groups of cell cultures, and the cell pellets washed once using PBS. The oxidative treatment solution was added to groups of SH, SN and SC, and an equal volume of H_2O_2-free culture medium was added to groups CH, CN and CC. All cell cultures were incubated for 3 h and then harvested for subsequent analyses.

WST-1 Cell Viability Assay

The WST-1 solution (10 µL) was added to cells from the six groups in 96-well plates and then incubated in an incubator for 1 h. The solution was thoroughly mixed before absorbance measurement using a microplate reader (at a detection wavelength of 450 nm; and a reference wavelength 630 nm). Each measurement was repeated three times.

Lactate Dehydrogenase Activity Assay

Six groups of BRL-3A cell cultures and the corresponding lysates were harvested from 25 cm^2 cell flasks for lactate dehydrogenase (LDH) activity assay using a commercial kit. The LDH leakage rate (%) = culture LDH activity/(culture LDH activity + cell LDH activity) × 100%.

Catalase Activity Assay

Six groups of BRL-3A cell culture lysates were harvested from 25 cm^2 cell culture flasks, and then centrifuged at 12.000x g (4°C) for 10 min. Catalase (CAT) activity was determined in the supernatant using a commercial kit, and the remaining material was used for protein content assay according to the BCA method.

Glutathione Peroxidase Activity Assay

Six groups of BRL-3A cell culture lysates were harvested from 25 cm^2 cell culture flasks, and then centrifuged at 12.000x g (4°C) for 10 min. Glutathione peroxidase (GSH-Px) activity was determined in the supernatant using a commercial kit, and the remaining material was used for protein content assay according to the BCA method.

Glutathione Content Assay

For the glutathione (GSH) content assay, the 25 cm^2 cell flasks of the six groups of BRL-3A cells were washed with 5 mL of PBS for 30 s. After the removal of PBS, cells were aseptically scratched and transferred to 1.5 mL micro-centrifuge tubes. The cell culture was centrifuged at 1.000x g for 3 min, and the supernatant removed. Thereafter, following the manufacturer's instructions, the GSH content of the deposit was assayed for each group of BRL-3A cell cultures.

Superoxide Dismutase Activity Assay

Six groups of BRL-3A cell culture lysates were harvested from the 25 cm^2 cell culture flasks, and then centrifuged at 12.000 g (4°C) for 10 min. The supernatant was used for the superoxide dismutase (SOD) activity assay using a commercial kit, and the remaining material was used for protein content assay according to the BCA method.

Protein Carbonyl Content Assay

The BRL-3A cell cultures in 25 cm^2 cell bottles were washed twice with pre-chilled PBS. After the removal of the PBS, the cells were scraped and transferred to 1.5 mL centrifuge tubes. The harvested cells were centrifuged and the supernatant discarded. The cells were resuspended in pre-chilled normal saline and subjected to ultrasonication (40% amplitude) in an ice bath for 5 s x 12, with 30 sec intervals. The protein carboxyl content was analyzed in 0.45 mL of homogenate using a commercial assay kit, and the remaining material was used for protein content assay following the BCA method.

qReal-time PCR

Total RNA was extracted using the Trizol method and cDNA was synthesized as a PCR template under standard conditions required by the reverse transcription kit. Specific primers were designed to amplify the HSP70-encoding gene using Primierprimer5.0. The upstream primer was 5'-GCTCGAGTCCTACGCCTTCAATA-3', and the downstream primer was 5'-TCCTGGCACTTGTCCAGCAC-3'. The amplification product was 105 bp in length. Specific primers for an internal reference gene β-actin were: upstream 5'-TCACCAACTGGGACG-3' and downstream 5'-GCATACAGGGACAACA-3'. The fluorescent quantitative real-time PCR (qRT-PCR) amplification was performed in three steps: 95°C denaturation for 2 min; 40 cycles of 94°C denaturation for 10 sec, 58°C annealing for 30 sec, and 72°C extension for 30 sec; and a final extension at 95°C for

30 min. Fluorescent signals were collected at the end of each cycle, and the melting solution curve analysis was performed after all cycles were finished. Results from qRT-PCR assays were used for relative quantitative calculation with the software package Line-Gene K provided with the qRT-PCR machine, following the maximum second derivative method.

Western Blot Analysis

Cells were harvested and total cellular protein was extracted from the lysate. Ten micrograms of protein sample were loaded onto SDS-PAGE containing 10% polyacrylamide, and then transferred to nitrocellulose membranes using the wet transfer method. The membrane was rinsed in PBST for 5 min three times, and then incubated in 5% non-fat milk solution for 1 h. The membrane was rinsed with PBST for 10 min three times and then cut into two pieces, which contained the HSP70 band and internal reference GAPDH band, respectively. These two membranes were incubated in 1:2000 and 1:5000 primary antibody solutions, respectively, at 37°C for 1 h. Thereafter, the membranes were rinsed with PBST for 10 min three times, and then incubated in 1:5000 secondary antibody solution at room temperature for 1 h. The membranes were then rinsed with PBST for 10 min three times. The membranes were then developed, fixed and exposed to X-ray film, which were photographed. The photographs were analyzed using Image J and statistical data were processed with Excel.

Statistical Analysis

Analysis of variance (ANOVA) was performed using SPSS 17.0. All measurements are presented as mean values ± standard deviation (X±Sx).

RESULTS

Ad-CMV-HSP70 Construction

According to XhoI restriction enzyme analysis and sequencing analysis, the recombinant adenoviral vector Ad-CMV-HSP70 was successfully constructed. The viral particles were purified through CsCl density-gradient centrifugation and the titer of recombinant adenoviral was calculated based on the absorbance (A260 and A260/A280) and TCID50. The titer of recombinant adenoviral reached 1.0×10^{11}pfu/mL, which was suitable for further transfection experiments.

Cell Viability

Of the three non-stressed groups, the average OD values followed the order of CC>CN>CH, with significant differences among the groups (P<0.01) *(Fig. 1)*. For the three stressed groups, the average OD values were highest in the SN group followed by SH and SC, with significant differences among the groups (P<0.01). The average OD values were lower in the stressed group than in the non-stressed group (P<0.01), suggesting that the cell proliferation rate was decreased. The largest decrease was found in SC (0.491), followed by SN (0.107) and SH (0.007) *(Fig. 1)*.

LDH Leakage Rate Variation

The LDH leakage rate and cellular content were both higher in cells of the groups with oxidative stress compared with those of the groups without stress *(Table 1)*. Of these, the LDH leakage rates of groups SN and SC were significantly higher than those of the corresponding groups without stress (P<0.01). The LDH leakage rate was higher in the group SH compared with the group CH (P<0.01), but the LDH cellular contents have no significant difference (P>0.05) between the two groups. The LDH leakage rates of groups CH and CN were significantly higher than that of group CC

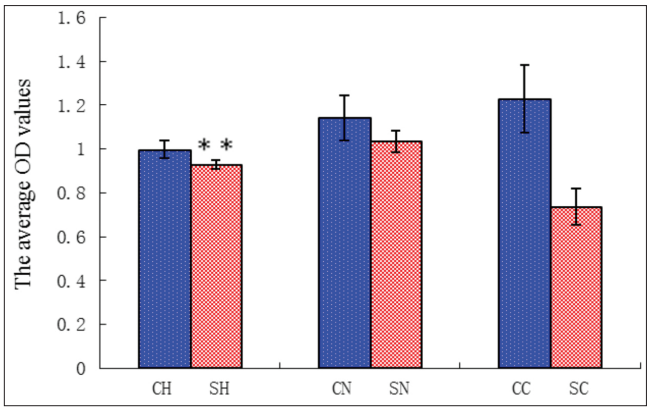

Fig 1. BRL-3A cell proliferation rate indicated by the average OD values. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

Table 1. *Lactate dehydrogenase activity of BRL-3A cells*

Group	Leakage Rate U/L	Cellular Content U/gprot	Total U
CH	896.13±58.69 [a]	1.84±0.05 [a]	48.71±1.42 [a]
SH	941.58±14.36 [b]	1.86±0.01 [a]	49.23±0.15 [a]
CN	784.53±46.26 [c]	1.97±0.11 [ab]	51.70±2.77 [a]
SN	1049.46±68.86 [d]	2.04±0.02 [b]	54.19±0.74 [b]
CC	756.54±45.63 [c]	2.00±0.22 [ab]	52.28±5.59 [ab]
SC	1413.94±23.20 [e]	2.27±0.01 [c]	60.76±0.21 [c]

Different superscript letters in the same column represent statistically significant differences (P<0.01)

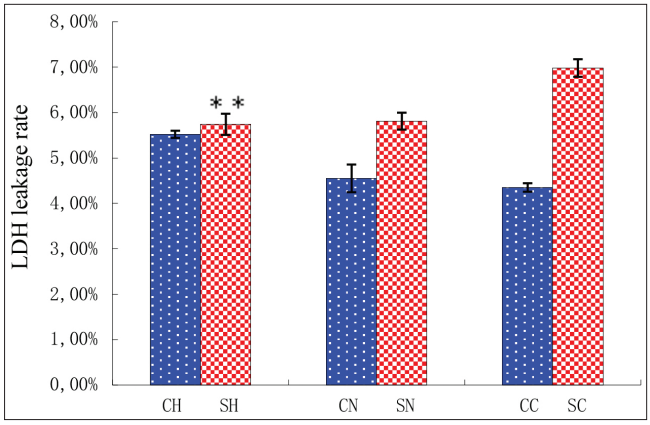

Fig 2. LDH leakage rate in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

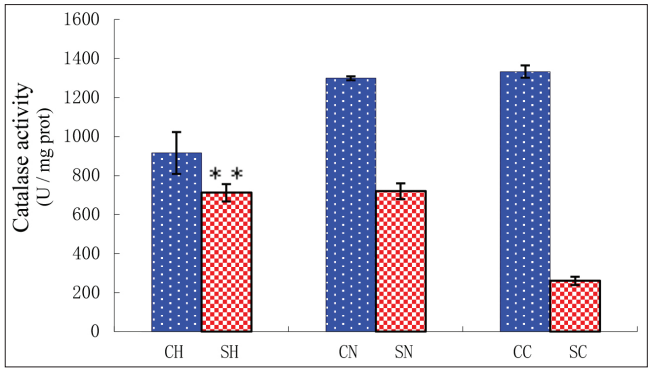

Fig 3. Catalase activity in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

(P<0.01), but significantly lower in groups SH and SN compared with group SC (P<0.01). There were no significant differences in LDH leakage rate between groups SH and SN (P>0.05) *(Fig. 2)*.

Catalase Activity

The CAT activity was significantly lower in cells of the stressed groups compared with those of the non-stressed groups (P<0.01) *(Fig. 3)*. Of these, the CH group had lower CAT activity than groups CN (P≤0.05) and CC (P<0.01), whereas the SH group had higher CAT activity than the SC group (P<0.01), with no significant difference from that of the SN group (P>0.05) *(Fig. 3)*.

Glutathione Peroxidase Activity

The GSH-Px activity was lower in cells of the groups subjected to oxidative stress compared with those of the non-stressed groups. However, such difference was only significant between the corresponding virus-free groups (P≤0.05) *(Fig. 4)*. For the non-stressed groups, the CH group had significantly lower GSH-Px activity than groups CN (P<0.01) and CC (P<0.01). Of the three stressed groups, SH had a lower GSH-Px activity than SN (P<0.01) and SC (P≤005) *(Fig. 4)*.

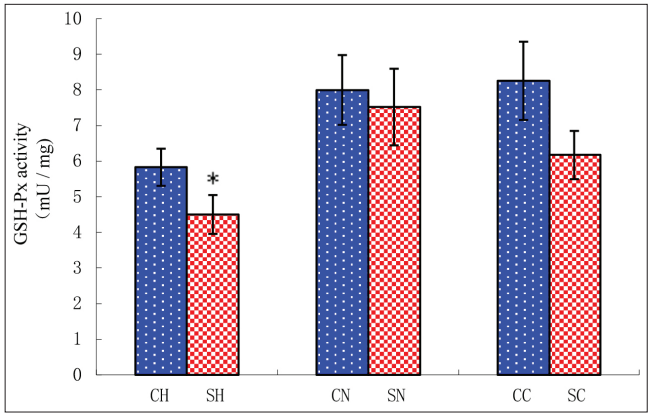

Fig 4. GSH-Px activity in BRL-3A cells of different groups. Results are expressed as mean ± SD. *P≤0.05 for comparison with group SC

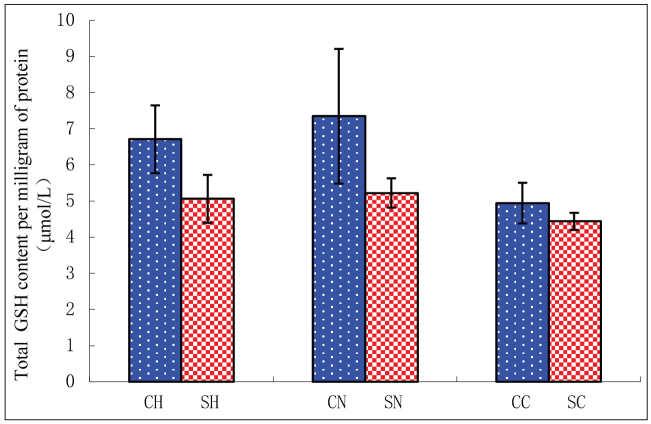

Fig 5. GSH content in BRL-3A cells of different groups. Results are expressed as mean ± SD. P>0.05 for group SH versus group SC

Glutathione Content

The GSH content was significantly higher in the CH group compared with the CC group (P≤0.05), whereas there were no significant differences between the GSH contents of groups SH and SC (P>0.05) *(Fig. 5)*. The GSH content was significantly reduced in the two groups with adenovirus transfection upon oxidative stress (P≤0.05), but no significant decline was found in the virus-free groups after oxidative stress (P>0.05) *(Fig. 5)*.

Superoxide Dismutase Activity

The SOD activity was significantly lower in cells of the CH group compared with those of the CN group (P≤0.05), but significantly higher than the CC group (P<0.01) *(Fig. 6)*. The SOD activity was significantly lower in cells of the SH group than that of the SN group (P<0.01), with no significant difference with the SC group (P>0.05) *(Fig. 6)*.

Protein Carbonyl Content

The protein carbonyl content was significantly higher in cells of the stressed group compared with the non-stressed group (P<0.01) *(Fig. 7)*. There were no substantial

differences in protein carbonyl content amongst the non-stressed groups (P>0.05), but the protein carbonyl content was significantly higher in cells of the SH group compared with groups SN and SC (P<0.01) *(Fig. 7)*.

HSP70 mRNA Abundance

The stressed groups had significantly higher HSP70 mRNA expression levels than non-stressed groups (P<0.01), and the SH group had significantly higher HSP70 mRNA expression levels than groups SN and SC (P < 0.01) *(Fig. 8)*.

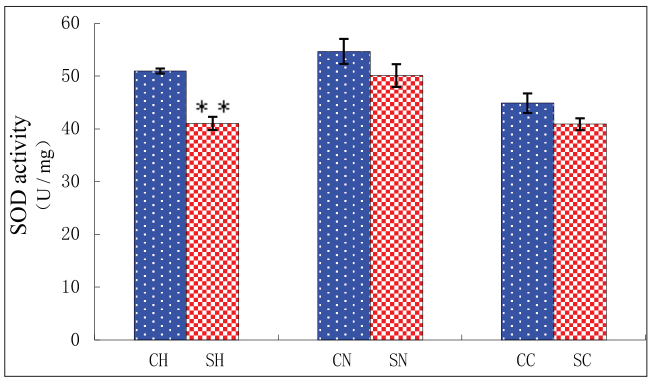

Fig 6. SOD activity in BRL-3A cells of different groups. Results are expressed as mean ± SD. *P≤0.05 for comparison with group SN

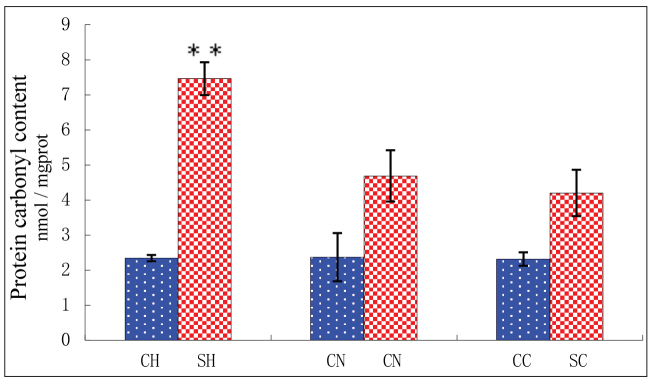

Fig 7. Protein carbonyl content in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC

Hsp 70 Protein Expression Level

As shown in *Fig. 9* and *Fig. 10*, cells of the stressed groups had significantly higher HSP70 protein expression levels than the non-stressed groups (P<0.01), and the SH group had significantly higher HSP70 protein expression level than groups SN and SC (P<0.01)

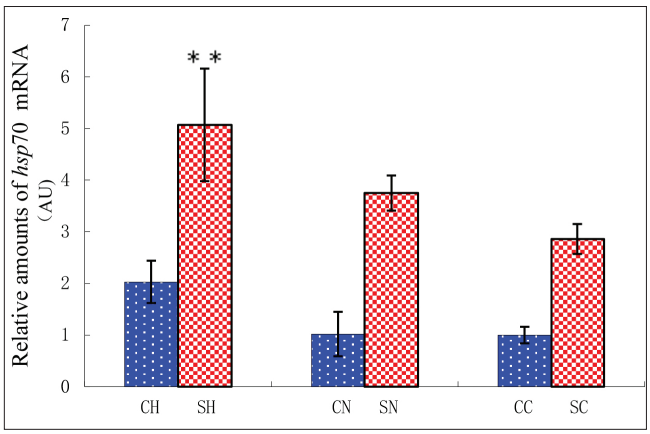

Fig 8. HSP70 mRNA abundance in BRL-3A cells of different groups. Results are expressed as mean ± SD. **P<0.01 for comparison with group SC group SC

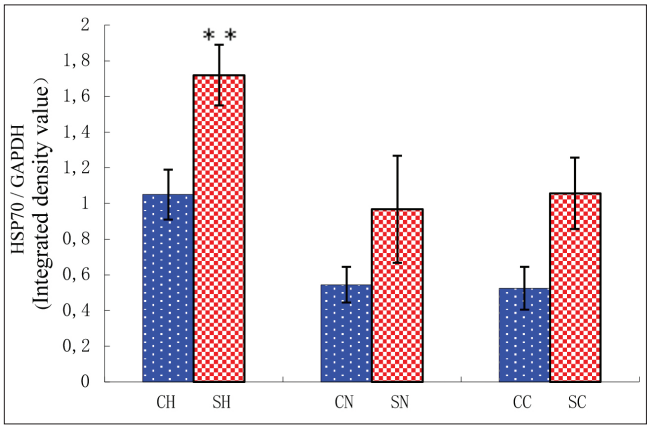

Fig 10. Hsp 70 protein expression levels in BRL-3A cells of different groups. Results are expressed as mean ± SD. ** P<0.01 for comparison with group SC

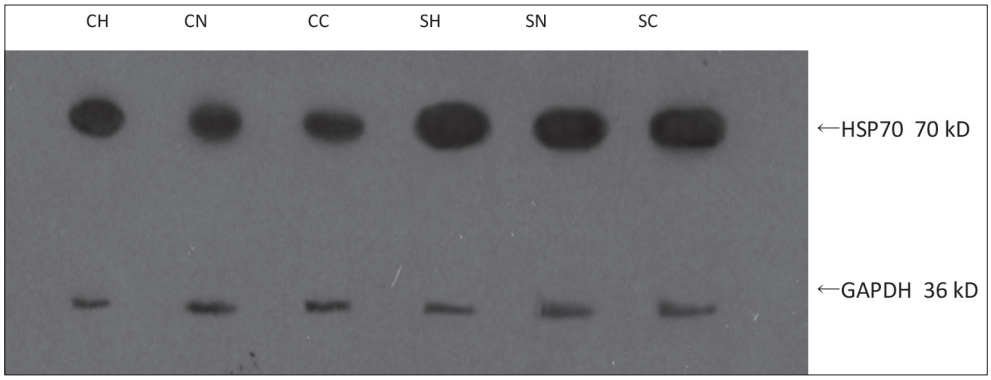

Fig 9. Detection of HSP70 by western blotting. The position of the molecular weight markers is indicated

DISCUSSION

The cell viability results from this study showed that average OD values in cells of the three stressed groups were significantly lower than those of non-stressed groups (P<0.01). This indicated that the 3 h oxidative stress induced by 500 μM H_2O_2 on BRL-3A cells caused decreases in cell proliferation rates or damage to mitochondria. Of the three non-stressed groups, the average OD value was highest for the cells of the CC group, followed by CN and CH (P<0.01). We speculated that both Ad-CMV-HSP70 and Ad-CMV-Null (MOI = 20) cause cell damage or death to a certain degree. The WST-1 assay also showed that the average OD values of Ad-CMV-HSP70 were lower than those of Ad-CMV-Null; therefore, the HSP70-containing adenovirus vector likely caused greater damage to cells under non-stressed conditions. This was confirmed by the subsequent LDH assay. However, the changes in the average OD value of the non-stressed group and the stressed group showed that, compared with cells transfected with empty vector or virus-free cells, transfection of BRL-3A cells carrying HSP70 is advantageous in resisting H_2O_2-induced damage, as indicated by smallest decreases in their average OD values (P<0.01).

Lactic dehydrogenase release is an important signal of apoptosis. When the cytomembrane is damaged, LDH will be released from *in vitro* cell cultures into the culture medium, and the release amount reflects the degree of damage to cells caused by physical factors or drug treatment [5]. In the present study, all stressed groups had substantially higher LDH leakage rates than non-stressed groups, suggesting that the 3 h 500 μM H_2O_2-stress caused damage to BRL-3A cell membranes, leading to the increased LDH leakage rate. In addition, our measurements of LDH leakage were probably less than the actual LDH leakage from cells, because the acidity of the H_2O_2 solution could cause partial inactivation of the LDH leakage. In 2018, Wu et al. found that HSP70 reduced the leakage of LDH in rats pretreated with hyperbaric oxygen [6]. Our results showed that overexpression of HSP70 reduced LDH leakage. Our results showed that the LDH leakage rate was significantly lower in the SH group than in SC (P<0.01), despite a lack of significant difference between groups SH and SN (P>0.05). These results indicated that the adenovirus-mediated HSP70 transfection of BRL-3A cells had a protective function, but it remains uncertain whether such a function was the result of exogenous introduction of HSP70.

Catalase is an important component of the cellular ROS scavenging system. It has an important role in maintaining the balance of intracellular ROS metabolism, and catalyzes the production of H_2O and O_2 from H_2O_2 [7]. Hence, the H_2O_2 stimulation to BRL-3A cells could cause intracellular CAT consumption, accounting for the substantial reduction of CAT activity in cells of the stressed groups compared with the non-stressed groups (P<0.01). In addition, CAT activity was substantially lower in cells of the group CH compared with other non-stressed groups (P≤0.05), possibly due to the overexpression of HSP70. In Cheng and Dimitrovskas' experiments, a similar negative correlation was also observed between HSP70 and CAT [8,9]. We speculate that CAT and HSP70 are both transiently expressed proteins, thus the rapid expression of HSP70 potentially imposed a competitive inhibition effect on the expression of CAT during the stress process.

Glutathione peroxidase is an important enzyme that occurs widely in organisms and catalyzes the decomposition of H_2O_2. It specifically catalyzes H_2O_2 reduction to protect the structural and functional integrity of the cell membrane. In this study, the H_2O_2-stressed group had substantially lower GSH-Px activity than the corresponding non-stressed group. This was probably because the oxidation of oxygen free radicals led to carbonyl production from amino acid residues of the GSH-Px protein, further changing the secondary structure of GSH-Px and reducing its α-helix percentage [10]. Our results showed that regardless of the stress, the lowest GSH-Px activity occurred in cells transfected with Ad-CMV-HSP70. These results suggested that the expression of HSP70 reduced GSH-Px activity, consistent with previous findings. In 2019, Liu et al.[11] found that GSH-Px activity is negatively correlated with HSP70 expression. The low-molecular-antioxidant GSH is the substrate of GSH-Px. GSH can eliminate oxygen free radicals and H_2O_2, stabilize sulfhydryl enzymes, prevent oxidative damage, and combine with toxic substances to accelerate their catabolism [12]. In the present study, the two adenovirus transfection groups had substantial decreases in GSH content upon H_2O_2 stress (P≤0.05), indicating that H_2O_2 caused a certain degree of oxidative damage to cells. However, no significant differences were observed between GSH contents of the SH group and other stressed groups in the present study. It was likely that HSP70 first reduced the GSH-Px activity, and subsequently inhibited GSH production.

Superoxide dismutase can eliminate superoxide anion radicals to protect cells from damage. It plays a vital role in maintaining the balance of oxidation and antioxidation in organisms [13]. Our results showed that all BRL-3A cells of the stressed groups had lower SOD activity than the non-stressed groups (P≤0.05), consistent with previous findings. For example, Guesmi found that H_2O_2 treatment of rats can significantly reduce the SOD activity in the testis of rats [14]. It was likely that H_2O_2 or OH-• enhanced the inactivation effect of H_2O_2 on SOD and increased the protein carbonyl content of the enzyme, further reducing the enzymatic activity. In addition, the relationship between HSP70 and SOD activity is inconclusive. In the present study, we found that SOD activity was substantially lower in cells of the SH group compared with the SN group (P<0.01). This could be related to the exogenous introduction of HSP70, but the detailed mechanism(s) remain unclear.

The protein carbonyl content is a sensitive indicator of protein oxidative damage. A high protein carbonyl content indicates that the protein suffers larger oxidative damage and that the cell and living organism has suffered a greater degree of oxidative stress [15]. In the cold-exposure animal test, the protein carbonyl contents of different varieties of rat livers were significantly increased compared with the control after short- or long-term cold exposure [16]. In our study, the protein carbonyl content was significantly higher in cells of the stressed groups compared with the non-stressed groups, suggesting that H_2O_2 caused oxidative damage to BRL-3A cells. In addition, the protein carbonyl content was higher in the SH group compared with the other stressed groups. According to the theory that protein carbonyl content correlates with the extent of oxidative damage, the damage to cells of the SH group should be the largest, which is consistent with results from other assays of cell proliferation, LDH leakage rate and antioxidant enzyme activities. It was likely that the cells of group SH contained more HSP70 than other groups, and that the overexpressed HSP70 functioned as the 'death squads' that were first attacked by oxidative stress. The sacrifice of overexpressed HSP70 protected other proteins against the attack of oxidative stress. In addition, some researchers consider that HSP70 becomes the target of carbonylation through 4-hydroxy azelaic acid [17]. Presently, the protective mechanisms of HSP70 against oxidative damage to cellular proteins remain unclear. Further study is needed to produce sufficient experimental evidence to confirm our speculation.

Comprehensive comparisons of relevant indicators between the cells of groups SH and SC showed that adenovirus-mediated HSP70 transfection imposed a certain protective effect on rat liver cells against oxidative stress. However, it remains unclear whether this protective effect is entirely caused by the exogenous introduction of inducible HSP70, as various indicators showed no significant differences between groups SH and SN. There are several potential reasons. Firstly, the adenovirus vector itself imposed a certain degree of stress to BRL-3A cells, and this stress can initiate the self-protection of cells, thus allowing transfected cells more advantages than virus-free cells in response to subsequent oxidative stress. Secondly, although inducible HSP70 can protect cells from stress damage, it needs to synergize with other members of the Hsp family for better performance. In this study, exogenous introduction of inducible HSP70 increased the expression level of inducible HSP70. However, the contents of synergized proteins would show no corresponding increases. Hence, the protective function inducible HSP70 could not come into play completely. Third, the BRL-3A cells can produce endogenous inducible HSP70 under stress. Thus, a large amount of endogenous HSP70 can be expressed initially, even in the absence of exogenous introduction of HSP70 under stress. Consequently, the HSP70 level of stressed BRL-3A cells transfected with Ad-CMV-HSP70 was not higher than that of cells free of the virus. Fourth, the adenoviral vector is associated with transient transfection. The exogenously introduced HSP70 is only available for transcription but not replication. In addition, HSP70 mRNA has a short half-life in normal cells and is easily degraded; thus, high levels of translation and expression of exogenous HSP70 cannot be guaranteed.

In summary, HSP70 is likely to be the most important intermediary substance in cells response to oxidative stress. An increasing number of reports have shown that HSP70 is negatively correlated with oxidative stress [18]. However, our results suggest that the adenovirus-mediated HSP70 transfection only has a certain protective effect on rat liver cells against oxidative stress. Further study is needed to explore the anti-oxidant mechanism in liver cells associated with HSP70.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

All authors read and approved the final version of the article. Designed the study: Designed the study: Guo Jingru. Conducted the analyses and wrote the first draft of the article: Hu Huijie, Liu Hongrui, Yuan Jianbin. Tables and results: Ji Hong, Zhang Xu.

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