Origannal Article

The Effect of Sensitivity to Heat of Tween80 on Human Ovarian Cancer Cell Line SKOV–3 *

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ABSTRACT **Objective:** To prove the sensitizing effect of Tween80 to heat on the thermotolerance and expression of Heat shock protein(HSP) 70 in the human ovarian cancer cell line SKOV-3. **Methods:** The proliferation rate of cell was detected by methyltetrazolium (MTT) assay. The expression of Heat shock protein 70 was detected by immunocytochemical assay. **Results:** The cells were inhibited and killed after 42℃ hyperthermia firstly. But the cell proliferation rate of the groups reheated was quickened. The inhibition ratio of proliferation of groups treated by the combined treatment of Tween80 and 42℃ hyperthermia is higher than that of the cell of the control groups, the inhibition ratio of cell proliferation rise obviously after reheating compared to the control groups treated by 42℃ hyperthermia simply. After heating for 100mins, the expression of HSP70 of the experiment groups is weaker than groups treated by 42℃ hyperthermia simply after 8 hours. With increase of the concentration of Tween80, the inhibition effect of HSP70 expression is obvious.

Conclusions: Hyperthermia of 42℃ for 100 mins can induce thermotolerance of the human ovarian cancer cell line SKOV-3. Tween80 may inhibit thermotolerance of the cell and has a sensitizing effect on SKOV-3 treated by 42℃ hyperthermia.

Keywords: SKOV-3; Thermotolerance; Immunocytochemistry; MTT; Tween80

Tumor hyperthermia is a method which advances the temperature of body and/or tumor tissue by kinds of ways and treats malignant tumor by hyperthermia and secondary effect. Due to the needed therapy temperature is high, usually reached 40℃-43℃[1], it induces high mortality of tumor cell, and causes the damnification of human normal cell simultaneously. Otherwise, thermotolerance always accompanied by hyperthermia, which reduces its efficacy. Thermotolerance is a characteristic of cells, when they are heated beforehand or continually with nonfatal thermal dose their sensitivity descends. In order to resolve the above issues, we try to search for a kind of sensitizing agent of hyperthermia which can debase the temperature of hyperthermia and increase the effect. The experiment wants to let Tween80 combined with 42℃ hyperthermia to act on human ovarian cancer cell line SKOV-3 and investigate the effect of the thermotolerance and expression of HSP70.

MATERIALS AND METHODS

Cell culture

Human ovarian cancer cell line SKOV-3 was cultured in RPMI-1640 (USA sigma company) supplied with 10% fetal bovine serum (Shanghai Shangbao biological technology Ltd.) at pH 7.2. All cells were cultured in 5% CO2 cell incubator at 37 ℃ (Beijing Zhong xi yuan da Biotechnology Ltd.).

Method of hyperthermia

Cells were removed from the incubator. The used culture medium was exchanged by fresh culture medium or culture medium including Tween80（USA sigma company）. Then cells were heated in 42℃ constant temperature foster box (Jinan

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Yike instrument manufacturing Ltd. The temperature difference is less than 0.5℃) and put back into incubator immediately after hyperthermia.

Grouping

The experimental Groups were treated by different concentration of the Tween80(0.025%, 0.05%, 0.075%, 0.1%, 0.125%, 0.15%) combined with 42℃ hyperthermia. The control groups include:① Group treated by 42℃ hyperthermia simply; ② Groups treated by Tween80 of different concentration; ③Groups treated by 37℃ hyperthermia. We select 100 min as heating time considering the heating time is between 1 h to 2 h in clinic usually.

MTT assay

MTT assay was performed to assess the effect of different treatments on cell proliferation. Cells at logarithmic phase, concentration of 5×104/ml, were plated in 96-well plates for 24 hours(200μl/well), then the culture medium was exchanged. The experimental groups were added into 200μl culture medium which included Tween80 of different concentrations (0.025%, 0.05%, 0.075%, 0.1%, 0.125%, 0.15%). The control groups were added into 200μl pure culture medium. groups needed hyperthermia were put into 42℃ constant temperature foster box for 100mins immediately. Then they were put into cell incubator of 37℃, 5%CO2 and saturated humidity. Groups reheated were treated in the same way after incubation of 1h, 4h, 8h, 12h, 24h, 48h, 72h. 20μl MTT (5mg/ml, USA sigma company) were added into every well after incubation of needed time. The reaction was stopped after 4hrs of incubation by adding 150μl dimethylsulfoxide (DMSO). The optical density (OD) value was obtained by measuring absorbance at the wavelength of 492nm after oscillating for 5 mins, and the proliferation index calculated by the ratio of OD492 of experimental group to OD492 of control group. Each experiment was set up in triplicate, and repeated 3 times.

Preparation of cellslide

Logarithmic phase cells were digested by trypsinization and were made into cell suspension, the cell density was adjusted to 1 ×105/ml. The cover slips of 2×2cm2 were put into culture dishes; 150μl cell suspension was dropped on every cover slip. Proper quantity of culture medium was added after cell attachment, the experimental culture dishes were put into 42℃ constant temperature foster box for 100 mins after 24 h of culturing, then they were maintained in 37℃ cell incubator for different needed time. Cellslides were collected and washed two times with normal saline. Then they were put into the 4℃ refrigerator with 95% alcohol for 30 mins. Cellslides were fixed for reserving.

Immunocytochemical staining

Immunocytochemical staining was adopted to observe the expression level of HSP70 (Santa Cruz Biotechnology). The main steps are as follows: Cell slides were put into H2O2 of 3% concentration at room temperature for 10 mins, washed with PBS buffer solutions, added 10 % normal goat serum blocked antigen (Fuzhou Maixin Biotechnology development Ltd.) for 10mins, removed serum, and added mouse anti-human monoclonal antibody HSP70 diluted 1:50. After washed with PBS buffer solutions again, the second antibody was added. 10 minutes later, they were washed with PBS buffer solutions, added into reagent D (Horseradwash peroxidase-conjugated avidin). After washed with PBS buffer solutions, DAB (Fuzhou Maixin Biotechnology development Ltd.) was used as the chromogen for active HSP70 cytochemistry, resulting in a brown-yellow immunoreactive product. Cell slides stained with hematoxylin, dewatered conventionally, transpwerentize and sealed. Cell slides were observed and taken photographs. The location of expression in positive cells was cytoplasm and nucleus. Three cell slides in each group, immunocytochemical studies were performed in triplicate.

Statistical analysis

Statistical analysis was performed using statistical package SPSS10.0. to determine the significant difference (95% probability) of parameters between sample groups, variance analysis and t-test were utilized. The measuring data was expressed as mean number or mean number±standard deviation($\overline{X}$ ±s).

RESULTS

Inhibition ratio of cell proliferation was detected by methyltetrazolium (MTT) assay.

Detecting the inhibition ratio of cell proliferation at 1h, 4h, 8h, 12h, 24h after 42℃ hyperthermia for 100 mins (Fig 1), it can be seen that human ovarian cancer cell line SKOV-3 was inhibited and killed. The inhibition ratio of cell proliferation reached the top at 8h ($p<0.05$), the effect of hyperthermia was the best. As a result, we selected 8h after hyperthermia as the time of detection. Less than 0.1% concentration, the inhibition

effect of Tween80 for cells is low. A report said that Tween80 of 0.1% concentration had not obvious toxic and side effects in Vitro and in Vivo [2]. Considering the effect and normal cells without damnification, we selected 0.1% as concentration of Tween80 in the experiment. Detecting the inhibition ratio of cell proliferation of groups at different time after being treated by 0.1% Tween80, 42℃ hyperthermia and combined treatment of the two methods separately, it can be seen that the groups of combination has additive effect or synergistic effect on the SKOV-3 cell (Fig 1). Reheating at 4h, 8h, 12h, 24h, 48h after simply 42℃ hyperthermia can quicken the cell proliferation (Fig 2). The ratio of cell proliferation rose obviously in the groups of reheating at 8h ($p<0.05$). Later, the rising extent of proliferation ratio decreased gradually. At 72h the ratio of cell proliferation recover the state of heating firstly. After SKOV-3 cells were treated by different concentration of Tween80 (0.025%, 0.05%, 0.075%, 0.1%) for 100mins, the inhibition effect is weak at 8h. Detecting the inhibition ratio of cell proliferation after Tween80 of the above different concentrations combined with 42℃ hyperthermia for 100 mins, the inhibition ratio of proliferation of groups of combination was much higher than that of groups of hyperthermia simply and groups of medication simply (Fig 3). The result suggested that Tween80 combined with 42℃ hyperthermia had additive effect or synergistic effect on the SKOV-3 cell. Another 42℃ hyperthermia at 8h after Tween80 of 0.1% concentration combined with 42℃ hyperthermia, the ratio of cell proliferation was (81.1±5.43)%. compared with that of groups reheated after 42℃ hyperthermia simply (176.2±11.51)%, the difference was obvious ($p<0.05$).

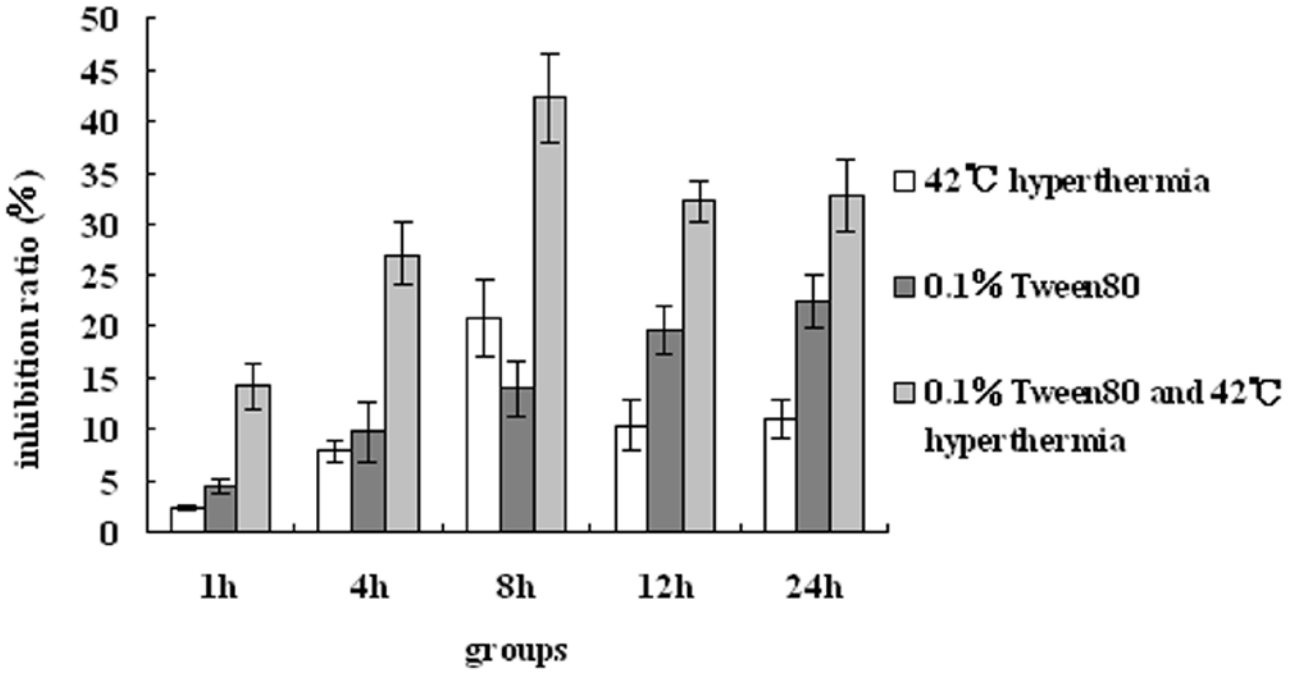

Fig.1 The inhibition ratio of cell proliferation at different time after being treated by 0.1%Tween80, 42 hyperthermia and combined treatment of the two methods.

The fig showed that the inhibition ratio of cell proliferation after 42 hyperthermia firstly for 100 mins at 1h、4h、8h、12h、24h, it can be seen that human ovarian cancer cell line SKOV-3 was inhibited and killed. The inhibition ratio of cell proliferation reached the top at 8h ($p<0.05$)

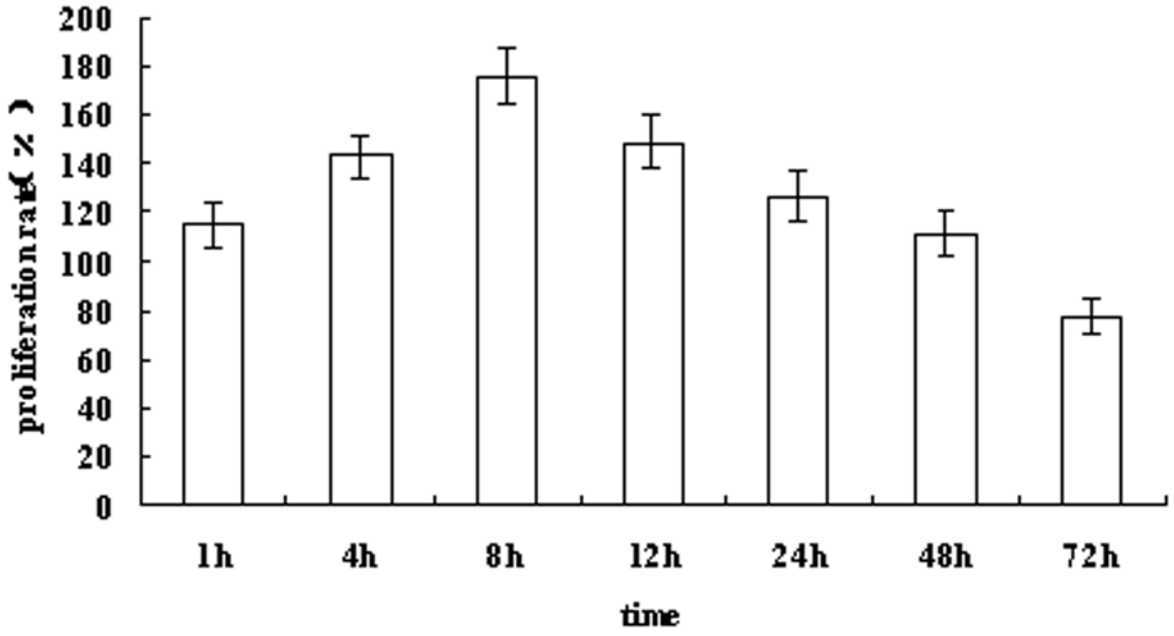

Fig. 2 The ratio of cell proliferation at different time after reheating

Fig.2 showed that at 4h,8h,12h,24h and 48h after simply 42℃ hyperthermia can quicken the cell proliferation. The ratio of cell proliferation rose obviously in the groups of reheating at 8h ($p<0.05$). Later, the rising extent of proliferation ratio decreased gradually. At 72 h the ratio of cell proliferation recover the state of heating firstly.

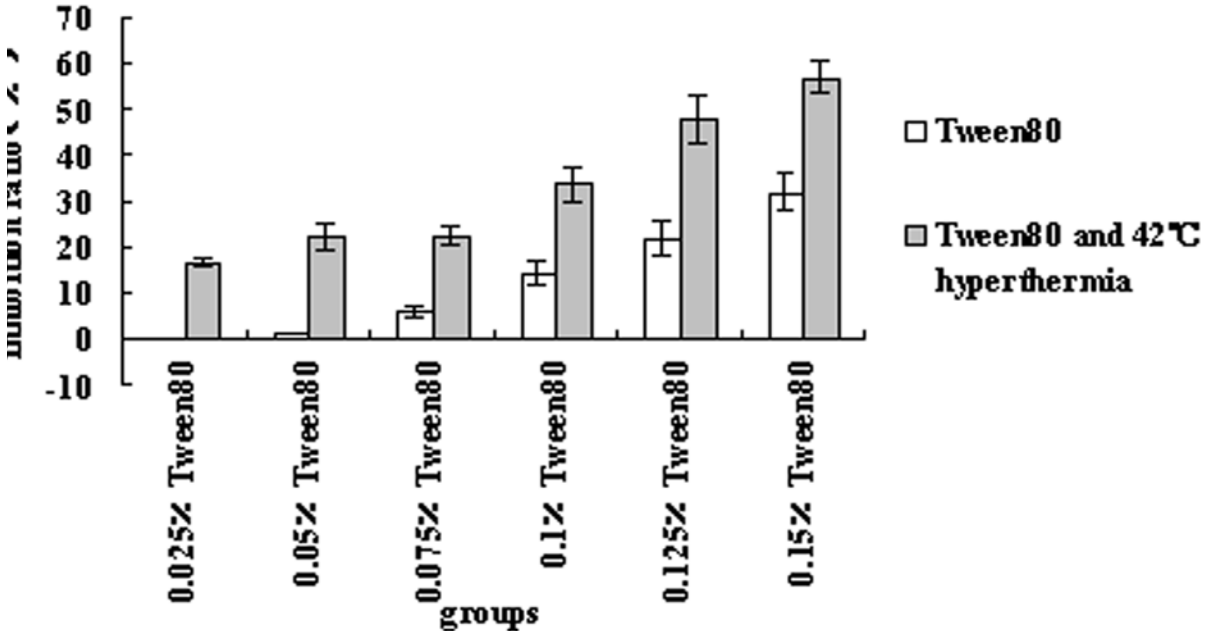

Fig.3 The inhibition ratio of proliferation of cell treated by Tween80 of different concentration and combined treatment of Tween80 and 42 hyperthermia after 8 hours

Fig.3 showed that after SKOV-3 cells were treated by different concentration of Tween80 (0.025%, 0.05%, 0.075%, 0.1%) for 100mins, the inhibition effect is weak at 8h. The inhibition ratio of cell proliferation of groups of combination after Tween80 of the above different concentrations combined with 42℃hyperthermia for 100 mins was much higher than that of groups with hyperthermia simply and groups with medication simply.

Immunocytochemical staining of HSP70 in cells

The expression of HSP70 was not obvious when SKOV-3 cells were cultured at 37℃ (fig 4A), and the nucleus staining was brown-yellow in 70% cells and all cells have cytoplasm staining at 8h after cells treated by 42℃ hyperthermia for 100mins (fig 4B). The cytoplasm and nucleus staining became weaker at 12h after Hyperthermia, but the number of staining cells did not decrease. After 24h hyperthermia, the number of cells with nucleus staining fell obviously, reaching 9%, the staining degree lightened. But the number of staining cells was 100% still (fig 4C). The nucleus staining disappeared entirely almost after 48h, the cytoplasm staining was light yellow (fig 4D). After 72h, the staining of the cells became weaker obviously, the number fell obviously also, the staining nearly reaches the state before Hyperthermia. Having been treated by different concentration of Tween80 (0.025%, 0.05%, 0.075%, 0.1%) combined with 42℃ hyperthermia for 100 mins, it can be seen that the degree of staining of cells fell gradually with the increase of the concentration of medication in the above mentioned concentrations after 8 hours. (The cell number of nucleus staining falls, and the cytoplasm staining becomes weaker). The staining effect of cells in the groups treated by 0.025% Tween80 combined with 42℃ hyperthermia (fig 4E) near that of the control group treated by 42℃ hyperthermia simply. Only a few of cells had light yellow staining in the group of 0.1%Tween80 combined with 42℃ hyperthermia (fig 4H), the staining effect near that of the control group treated by 37℃ hyperthermia simply. In the groups treated by 0.05% Tween80 combined with 42℃ hyperthermia (fig 4F) and 0.075% Tween80 combined with 42℃ hyperthermia (fig 4G), the staining degree of cells was between that of the group treated by 0.025% Tween80 combined with 42℃ hyperthermia and that of the group treated by 0.15% Tween80 combined with 42℃ hyperthermia.

DISCUSSION

The experimental results suggested that the proliferation rate of the cells reheated increased obviously at 8 hours after 42℃ hyperthermia for 100mins, the cells recovered the effect of growth inhibition of hyperthermia firstly when the cell was reheated after 72 hours, which suggested that the tolerance of cells was strongest and the toxicity of hyperthermia was lowest at 8 hour after hyperthermia. The time of thermotolerance lasted for 3 days, the killing effect of hyperthermia for 100mins was mild for the cells. The inhibitory effect was weak for cells when the concentration of Tween80 was less than 0.1%. 0.1% Tween80 combined with 42℃ hyperthermia had significant synergistic effect. In the experiment, the inhibition ratio of proliferation of cell near the effect of simply first hyperthermia when the cell treated by 0.1%Tween80 and 42℃ hyperthermia was reheated after 8 hours. The cells died in a certain degree. It completely changed the experimental results of cells increased obviously after simply reheated in 42℃.

Many experiments have similar results[3]. The thermotolerance appeared and regressed in a time-dependent manner. The cells easily appear thermotolerance with the lasting of heating time. But the reported results suggested the peak of thermotolerance is in the period of 2 hours to 18 hours. The time of complete regress is from 2 days to 7 days. The biggest degree of thermotolerance was different. The time of the peak of thermotolerance in this experiment was same with the mentioned above. In our experiment, the proliferation rate of the cells reheated in 48 hours after hyperthermia increased obviously. The result was different with the previous reports which suggested the cell reheated was inhibited still, only the inhibition ratio of proliferation of cell decreased in a certain degree comparing to the ratio of first hyperthermia. The difference may be caused by different heating temperature, time and different kinds of cells.

Many studies suggested thermotolerance was related to HSP family. HSP nearly exists in all eukaryotic cells. The best-characterized aspect of acquired thermotolerance is the production of heat shock proteins [4]. In the heat shock response, a range of stressful stimuli activate the transient induction of a set of genes encoding HSPs, while ongoing gene expression is down-regulated [5,6]. These induced proteins play a role in cellular repair mechanisms by functioning as molecular chaperones, mediating the refolding or degradation of stress-damaged proteins, and thus promoting cell recovery [7-9]. The accumulated HSPs also play a role in protective mechanisms by increasing cellular resistance to a subsequent stress, a phenomenon known as thermotolerance[10-14] . HSP70 subfamily was one of the most conservative and important kind in the HSP family, which was the best predictor of thermotolerance also[15,16]. The HSP70 combines with structure of cells, the combination promotes stability of cells, prevent damage of cells caused by the definition of oxygen or decomposition of ATP, and achieve the goal of protection of cells[17]. Brian RB[18] analyzed the relationship between HSP70 and other stress proteins and proved that they have reaction each other. Other stress proteins can not make up the result of Drosophila virilis′s death after heat stress caused by the lack of HSP70. HSP70 plays an important role in thermotolerance, which can not be replaced by other stress proteins. The staining result of immunocytochemistry in the experiment verified our conjecture mentioned above. The expression of HSP70 reached the peak at 8 hour after cells were heated for 100mins. Its staining degree was strongest. The number of cell with nucleus staining was most. All these returned to normal level after 3 days. After

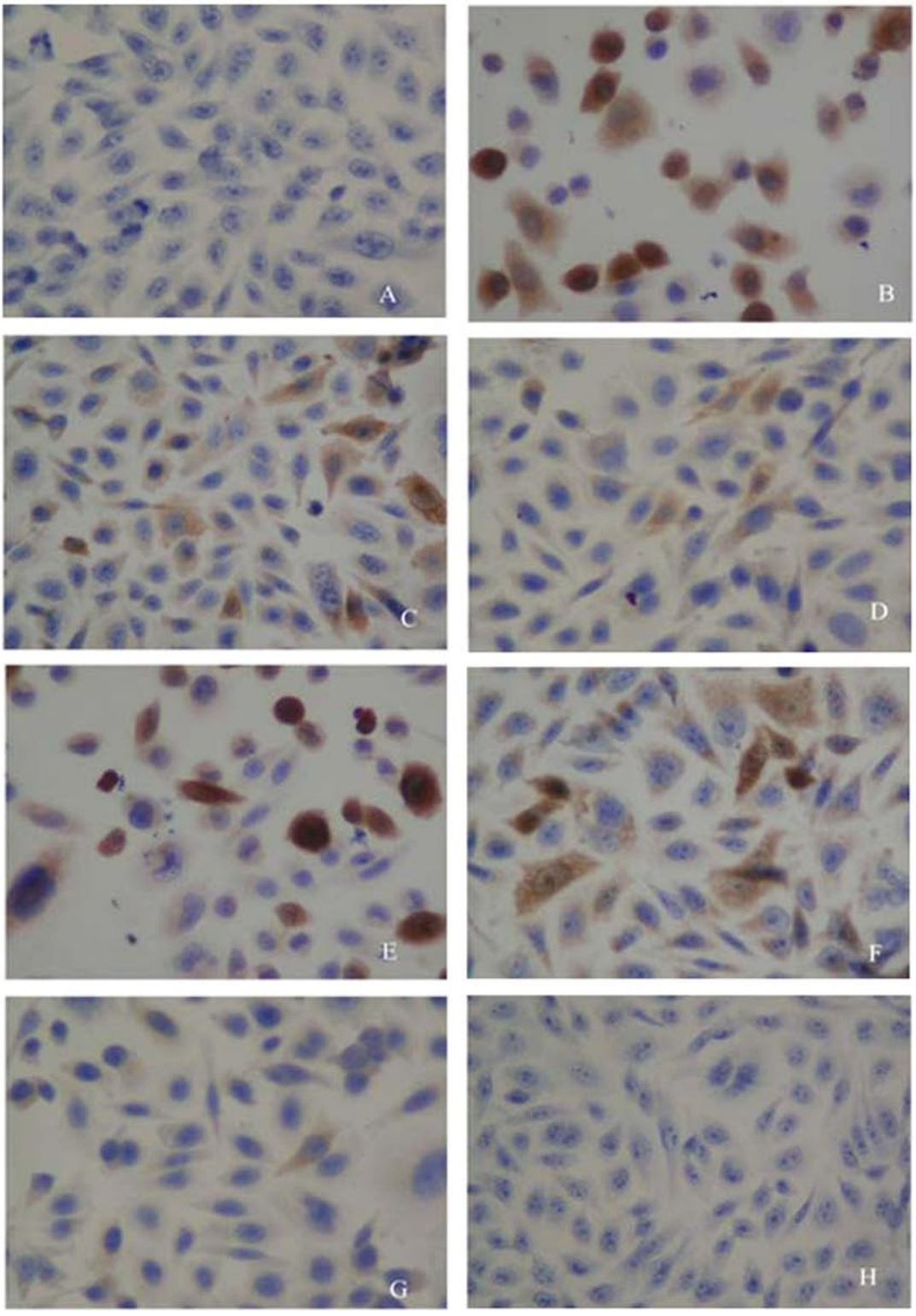

Fig.4 Immunocytochemical staining of HSP70 in cells treated by different methods

A: the control group of 37℃ B: 8h after 42℃ hyperthermia C: 24h after 42℃ hyperthermia D: 48h after 42℃ hyperthermia
E: 8h after 0.025% Tween80 combined with 42℃ hyperthermia F: 8h after 0.05% Tween80 combined with 42℃ hyperthermia
G: 8h after 0.075% Tween80 combined with 42℃ hyperthermia H: 8h after 0.1%Tween80 combined with 42℃ hyperthermia

heating for 100mins, the expression of the HSP70 of the cell groups treated by the combined treatment of different concentration of the Tween80 (0.025%, 0.05%, 0.075%, 0.1%) combined with 42℃ hyperthermia was weaker than that of the groups treated by simply 42℃ hyperthermia after 8 hours. With the increase of the concentration of Tween80, the inhibited effect of the HSP70 expression was obvious. The staining effect of the groups treated by the combined treatment of 0.1% Tween80 and 42℃ hyperthermia was the same as the control groups treated simply by 37℃ hyperthermia, which showed that Tween80 can inhibit the expression of HSP70 and the appearance of thermotolerance. In a certain scope, the inhibition effect was more obvious with the increase of concentration, which was the same as the previous results[19].

Studies of cytology show that Tween80 was a kind of safe and low toxic cosolvent, which can be regarded as drug excipient and food additive. Tween80 was reported firstly in 1990 as a hyperthermia sensitizer[20]. The combination Tween80 with 42℃ hyperthermia which acting on human gastric cancer cell line BGC-823 can decrease the critical temperature of treatment 2℃[21,22], and inhibit the activity of succinate dehydrogenase. It can combine with lipid membrane in cell membrane system. This can quicken the fluidity of membrane, change varieties of characteristics of lipid membrane and decrease the critical temperature of activation of enzyme[20]. The point is same with the effect of hyperthermia for membrane of cells, which is the foundation of strengthening the effect of hyperthermia. The experimental result of Mahua TC et al showed also that the cell lipid membrane was related to thermotolerance [23]. The expression of HSP70 was the manifestations of cell stress response, which can inhibit the happening of cell apoptosis. Signal thransfer of cell heat stress response was closely related to activation of membrane protein, it can lead to a series of changes of enzyme, protein and charged groups integrated cell membrane when fluidity of cell membrane increase, reaching a certain level[24]. It perhaps was part reason of the effect of Tween80 on thermotolerance and the expression of HSP70. Besides, Tween80 can enhance hyperthermia′s effect of changing the permeability of cell membrane and affect the stability of cell internal environment. The low pH environment in cells inhibited the expression of HSP70 in a certain degree [25].

In conclusion, 42℃ hyperthermia for 100 mins can induce high expression of HSP70 and high thermotolerance in SKOV-3 cell line. The thermotolerance was most obvious at 8h after hyperthermia and disappeared almost after 3 days. Tween80 and 42℃ hyperthermia have additive action or synergwastic effect on SKOV-3 cell line. Tween80 can inhibit the expression of HSP70 and formation of thermotolerance. It is expected that Tween80 will become a sensitizing agent of hyperthermia in clinical.

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