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Original Article

The intracellular signaling pathway mediates cryoablation-induced functional changes in Kupffer cells

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Abstract: Objective: Cryoablation treatment for the unresectable local tumors has been considerably developed over the past 20 years. Cryoablation can destroy tumors and strengthen or induce either cellular immunity or an anti-tumor immune response. In this study, we aim to investigate the proliferation, phagocytosis, and secretion of Kupffer cells (KCs) following cryoablation and to explore their underlying molecular mechanisms. Method: The expression of scavenger receptors (SR) on KC surfaces were measured using western blot. The concentrations of TNF-α, IL-1β, and INF-γ were measured by ELISA in a supernatant of KCs culture. The expression of the P65 protein was measured by immunofluorescence staining. Result: 1. Low temperature inhibits the proliferation of KCs ($P < 0.01$), and this effect was significantly inhibited by pyrrolidine dithiocarbamate (PDTC) ($P < 0.01$). 2. The phagocytosis of KCs was significantly down-regulated ($P < 0.05$) by the necrotic products. 3. The concentration of the inflammatory factors TNF-α, IL-1β, and IFN-γ was increased when treated by low temperature or freeze-thawing necrotic products ($P < 0.01$). The effect was block by the PDTC ($P = 0.325$). 4. The necrotic products up-regulate the expression of the P65 protein ($P < 0.01$) and the NF-κB protein ($P < 0.05$), and this effect is blocked by PDTC ($P > 0.05$). Conclusion: Cryoablation changes the microenvironment around KCs, promotes the proliferation of KCs, activates the KCs, and enhances the function of KCs secreting cell factors, which induces the inflammatory reaction to clear relict tumor cells. The secretion function and proliferation of KCs is mediated by the NF-κB signaling pathway. The phagocytosis of the KCs did not dominate to clear away the freeze-thaw necrotic products.

Keywords: Cryoablation, Kupffler cells, low temperature, inflammatory factor, liver cancer

Introduction

In the mid-1850s, the freezing technique began to be used in tumor treatment, which could relieve pain and reduce the volume of tumors [1]. In 1961, since Cooper used liquid nitrogen as a cooling source and invented a freezer in which the range of frozen necrosis could be controlled, the freezing technique as a novel treatment technique has developed rapidly for the treatment of benign and malignant tumors [2]. Cryoablation treatment for unresectable local tumors has been considerably developed during the past 20 years, and has focused on treating kidney cancer [3], liver cancer [4], prostate cancer [5], metastatic cancer [6], and other solid tumors. At the same time, more and more reports showed that the apoptosis rate was significantly increased around the treatment areas, and the percentages of CD31 and CD41 in serum increased significantly after cryoablation, which suggests that cryoablation can destroy tumors and can strengthen or induce either the cellular immunity or the anti-tumor immune response [7-9]. It has been found that the labeled tumor cells in circulating blood could be swallowed by KCs within 24 hours, which shows that KCs could eliminate tumor cells by phagocytosis, after which the release of proteolytic enzymes directly leads to tumor cells being dissolved [10]. The destruction and dissolving of tumor cells could become important mediators of inflammation [11].

If the correlation between cryoablation and the change of the function of KCs can be confirmed, and the related mechanisms can be clarified, it will provide the theoretical evidence for the hypothesis that cryoablation for liver cancer can improve autoimmune function. For this, we

extracted KCs from rabbit livers and cultured them *in vitro*. Both the number and the function of the KCs were observed, and the expressions of NF-κB and related proteins were also observed after being double stimulated by low temperature and freezing-thaw necrotic products. The number and functions of the KCs were further compared after the NF-κB signaling pathway was blocked by PDTC. Thus, the mechanism of the functional change of KCs being induced by cryoablation was explored.

Methods and materials

Reagents

PDTC was purchased from the Bio Vision Company, USA. Fetal bovine serum was purchased from the Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China. Low sugar DMEM medium: ATCC Company, USA. Try pan Blue Solution: Sigma Company, USA. The GAPDH was from the Abcam Company. The protein marker (10-170 kDa) was from Fermentas. 0.45 μm PVDF membrane was from the Millipore Company. The SDS-PAGE gel kit, the protein extracting kits, the BCA protein concentration test kit, the ECL chemical lighting kit, the PMSF (100 mM), the phosphorylated protease inhibitor, the 5 × SDS-PAGE loading buffers, the 10 × Lichunhong dye, and the antibody elution kit were purchased from the Tuojie Biological Technology Company. The tria and glycine were purchased from the Guoyao Chemical Reagent Company. The anasthetic for the animals (Su-mian-xin II) was obtained from China Animal Health Products Co. Ltd. Other reagents were obtained from the Medical Experimental Department of Wuhan General Hospital of PLA.

Experimental groups

Preparation of KCs cell suspension: After the rabbits were anesthetized via intramuscular injection with *Sumian-xin II* (0.2 ml/kg), they were sterilized, opened, exposed to the portal vein, and intubated, and the thoracic cavity was opened. Meanwhile, the inferior vena cava was ligated. A slow infusion of 100 ml PBS, at the rate of about 20 ml/min was administered, and the inferior vena cava underwent puncture bloodletting, repeated 3 times, in the color of liver sallow after the cessation of the perfusion of PBS. The collagenase perfusion of 50 ml was preheated at 37°C, then some liver tissue was quickly cut and broken after about 15 min and placed in the 37°C incubator to digest for 20 min. After filtering the 200-mesh filter, the collected liver suspension was centrifuged with PBS to 45 ml, 300 g × 5 min (4°C) for 2 times, and the supernatant was removed. The precipitated PBS was added to 45 ml. After being suspended sufficiently and then centrifugated at 50 g × 3 min (4°C), the supernatant was removed to a 50 ml centrifuge tube, and 550 g × 5 min (4°C) centrifugation was carried out to remove the supernatant. The above hepatocyte suspension was moved into a centrifuge tube containing 30% and 60% Percoll separation liquid, 800 g (20°C) slowly ascending and descending centrifugation 20 min, and the centrifugation can be seen in the milk white cell layer. We then collected the cell layer after cell suspension with 40% separate liquid again 600 g × 5 min (4°C) to centrifugation, supernatant, full percussion cell suspension with 20% fetal bovine serum in a low sugar DMEM medium. The washing of 1×10^6/ml was inoculated in 6-well plates, placed in the 5% CO_2 incubator cultivation under the condition of 37°C non-parenchymal cs, and then the cells were washed with PBS and continued to be cultured after 5-6 h.

The construction of a cell model for tumor cells attacking KCs at low temperature and freeze-thaw necrosis: The main attacking factors of KCs in the cryoablation were low temperature and necrotic substance, which we could construct in vitro. The KCs suspension with a concentration of 1×10^6/ml was divided into different culture bottles, and 5 ml of the suspension was added to each culture bottle. The experiment was divided into 8 groups, with 9 culture bottles in each group: ① The control group: Normal culture of KCs at 37°C; ② The 0°C group: Simulating the temperature of ice ball edge in cryoablation, the culture bottle was placed in an environment of 0°C for 20 min, and then removed and put back in the incubator at 37°C. After 5 min in the incubator, the culture bottle was placed in an environment of 0°C for 20 min again and then returned to the 37°C incubator. After 6 hours cultivation, the experiment was carried out; ③ The 5°C low temperature and the 10°C group: Simulating the temperature of the distance of 0.5 cm and

Table 1. KCs number in various groups

	No inhibitor	Inhibitor
Control (37°C)	14.50 ± 2.59	5.83 ± 2.31
0°C	11.00 ± 3.16	3.67 ± 2.07
5°C	12.67 ± 2.58	5.33 ± 1.37
Freeze thawing necrosis	25.83 ± 4.26	10.17 ± 1.83
Necrosis + 0°C	14.00 ± 1.79	5.50 ± 2.7
Necrosis + 5°C	15.17 ± 1.47	6.83 ± 2.85
Necrosis + 10°C	17.33 ± 1.97	7.17 ± 1.94

Note: 1. In the no-inhibitor groups, compared with the control group: the proliferation was inhibited in the 0°C and 5°C groups ($P < 0.01$ and $P < 0.05$) and was enhanced in the necrosis and necrosis + 10°C groups ($P < 0.01$ and $P < 0.05$); other groups, $P > 0,05$. 2. In the with-inhibitor groups, compared with the control group: the necrosis group, $P < 0.01$; 0°C group and necrosis + 10°C group, $P < 0.05$; other groups, $P > 0.05$. 3. Compared between with-inhibitor group and the without-inhibitor group, there were significant differences between the two groups of the same conditions, $P < 0.01$.

2 cm from the ice ball edge in cryoablation. The test method was the same as the 0°C group, but the temperature environment was different in terms of where the culture bottles were placed in the test. The 5°C group was placed in the 5°C environment twice for 20 min, and the 10°C group was placed in the 10°C environment; ④ The frozen necrotic substance group: The tumor tissue in the treatment center at the third day after cryoablation was made into a 1% cell suspension, and 2 ml was added to the KCs culture bottles, and the other culture conditions were the same as the control group; ⑤ The combined stimulation group: The KCs culture bottles were placed at different temperatures (0°C, 5°C and 10°C) and were divided into 3 groups. Frozen tumor necrosis cell suspension was added again to each group whose concentration was the same as the frozen necrotic substance group and the test methods were the same as the 0°C, 5°C, and 10°C groups respectively.

Intervention measures: After the 8 parallel groups were set up, PDTC was added to the culturing bottles with a final concentration of 100 umol/ml.

Cell count in the cultured KC in vitro

Following the methods described in previous studies [12, 13], the cells were counted with the blood cell count plate and the white cell count method. The methods were as follows. Wipe the counting plate with anhydrous ethanol or 95% ethanol solution, with a wipe, wiped on a coverslip, then the cover sheet was covered on a counting board. 0.4% *trypan* blue dye from the dropper was melted into cell suspension according to 1:1 ratio and dropped slowly from the edge of the counter to fill the gap between the counter and the cover. After a moment, the counter was placed under a low magnification (10 × 10 times) observation count. We randomly selected 6 fields of vision and counted the number of KCs and recorded the mean values. The data were expressed as the mean values ± the standard deviation.

ELISA detection of the secretion function of KCs

The concentrations of TNF-α, IL-1β, and INF-γ in the supernatant of the KCs were measured using the ELISA method (Bio-swamp Life Science Lab, Wuhan, China) following the method described in a previous study [14].

Immunostaining of the NF-κB P65 protein

Immunofluorescence was used to measure the expression of the NF-κB P65 protein, following the method described in a previous study [15]. ① PBS was added from the waiting detected specimen to the attenuating specimen. ② Using a dropper, an antibody was added to the attenuated specimen. ③ The slides were rinsed with PBS 1-2 times and immersed in the three cylinders in turn. ④ We slowly added fluorescent, labeled, anti-human globulin antibodies. ⑤ We then observed the fluorescence intensities of the specimens using a laser scanning confocal microscope.

Western blot for SR and NF-κB protein

After being cultured for 6 hours, the expressions of SR and the expressions of the NF-κB protein in each group were measured using western blot, using the Quantity One software processing system to analyze the optical density of the target band.

Western blotting was carried out as described previously [16]. Blots were probed with rat anti-SR antibody (Invitrogen Corporation, USA.) or rat anti- NF-κB antibody (SC-109, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The optical density of the target band was calculated.

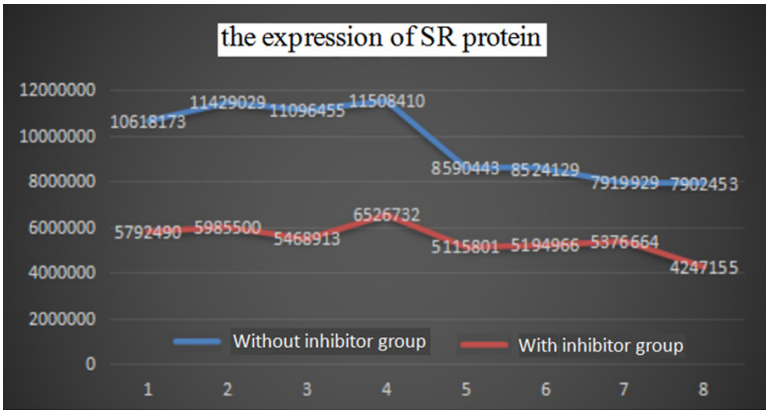

Figure 1. Numerical comparison of scavenger receptor expression.

Figure 2. In each group without inhibitor. Note: 1. In without inhibitor groups, Compared with control group: 0°C, 5°C and 10°C group, P > 0.05; necrosis group and combined groups, P < 0.05. 2. In with inhibitor groups, Compared with control group: all groups, P > 0.05. 3. Compared between with inhibitor group and without inhibitor group, these were significant difference between two grous of the same conditions, P < 0.01.

Figure 3. SR protein expression in each group with inhibitors. Note: 1. In without inhibitor groups, Compared with control group: 0°C, 5°C and 10°C group, P > 0.05; necrosis group and combined groups, P < 0.05. 2. In with inhibitor groups, Compared with control group: all groups, P > 0.05. 3. Compared between with inhibitor group and without inhibitor group, these were significant difference between two grous of the same conditions, P < 0.01.

Data analysis

SPSS 19.0 software was used. The count data was analyzed using Student's *t*test and variance analysis. The measurement data was analyzed using an χ^2 test. A single factor variance analysis was used for comparison among the groups. A Friedman test was used for the intragroup comparisons. The test level was P < 0.05.

Results

*Low temperature inhibits KC proliferation and frozen necrotic substances promote KC proliferation (**Table 1**)*

Compared with the control group, low temperature inhibited the proliferation of KCs in vitro in a temperature dependent manner: When the temperature was 0°C and 5°C, the difference was statistically significant (P < 0.05). On the other hand, frozen necrotic substances promoted the proliferation of KCs, and the difference was significant (P < 0.01).

The effect on the proliferation of KCs induced by low temperature was significantly inhibited (P < 0.01) in the presence of the NF-κB signaling inhibitor PDTC: Frozen necrotic substances could still promote the proliferation of KCs. However, this effect was significantly decreased (P < 0.01) in the presence of the NF-κB signaling inhibitor PDTC.

*The KCs phagocytosis function test (**Figures 1-3** and Table S1)*

The phagocytosis function of KCs can be tested indirectly by testing SR with western blot. The results suggested that the SR expression was down-regulated by the necrotic products significantly in the absence of the inhibitor. There were significant differences among the necrosis group, the combined stimulation group, and the control group (P < 0.05). For the low temperature group, the change had no statistical difference (P > 0.05). With the presence of the inhibitor, there was no significant difference among the various groups (P > 0.05).

*KCs secretion function test (**Figures 4-6** and Tables S2-S4)*

The secretion function of KCs showed that the concentration of TNF-α, IL-1β and IFN-γ were different under the various stimulation conditions, but the bar diagrams show that the changing trends were very similar in the non-inhibitor group. After the KCs were stimulated by low temperature or freeze-thawing necrotic

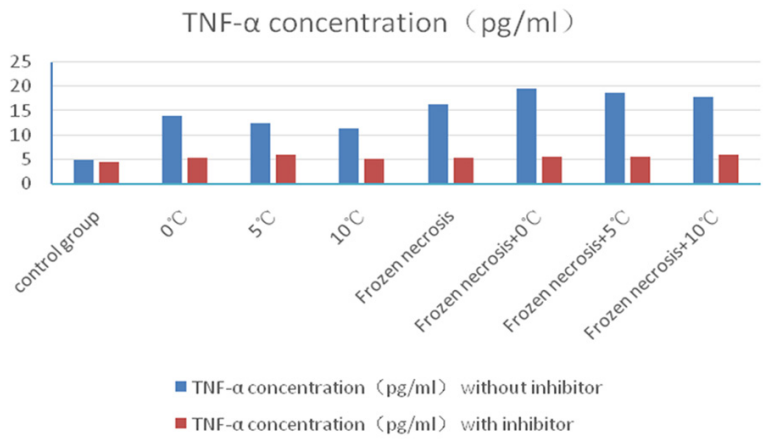

Figure 4. Detection of KCs to secrete TNF-α concentration.

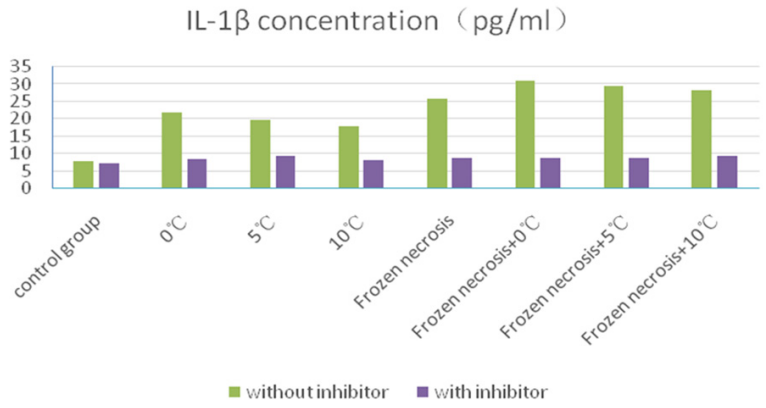

Figure 5. Detection of KCs to secrete IL-1β concentration.

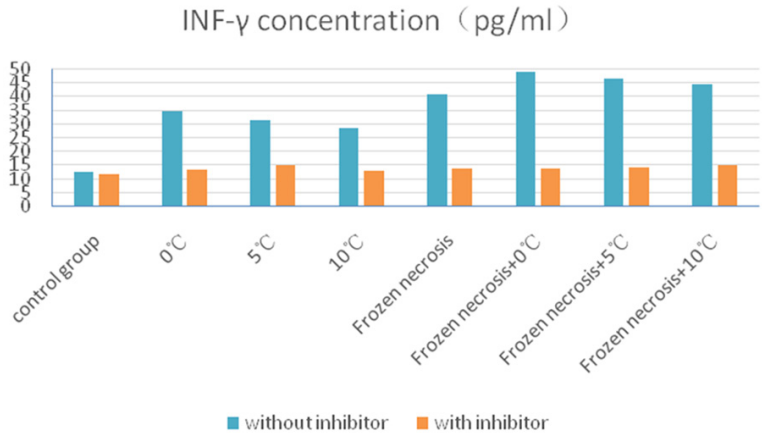

Figure 6. Detection of KCs to secrete IFN-γ concentration. Note: Three figures were similar except for numbers. 1. In without inhibitor groups, Compared with control group: all groups, $P < 0.01$; 2. In with inhibitor groups, Compared with control group: all groups, $P > 0.05$; among 8 groups, Friedman test: $\chi^2 = 2.25$. $P = 0.325$. 3. Compared between with inhibitor group and without inhibitor group, these were significant difference between two groups of the same conditions (except control groups), $P < 0.01$.

products or combined stimulation, the secretion of the inflammatory factor was increased ($P < 0.01$). The superimposed effect was shown by the combined stimulation of low temperature and freeze-thawing necrotic products. In the presence of PDTC, the secretion level of the inflammatory factors showed no significant difference among the 8 groups ($P = 0.325$).

Expression and immunofluorescence intensity of P65 protein in the NF-κB signaling pathway

The immunofluorescence results showed that low temperature inhibited the expression of the P65 protein. There was a significant difference between the 0°C group and the control group ($P < 0.05$). There was no significant difference between the 5°C or the 10°C group and the control group ($P > 0.05$). Compared with the control group, the necrotic products up-regulates the expression of the P65 protein ($P < 0.01$). There were no significant differences between the control group and the combined groups ($P > 0.05$). After the PDTC was added, the inhibiting effect of the low temperature was more apparent. Compared with the control group, the expression of the P65 protein was significantly down-regulated in the 0°C group ($P < 0.01$), the 5°C group ($P < 0.05$), and the 0°C + frozen necrosis group ($P < 0.05$). There was no significant difference between the control group and the 10°C group ($P > 0.05$). There was no significant difference in P65 protein expression between the necrotic products group

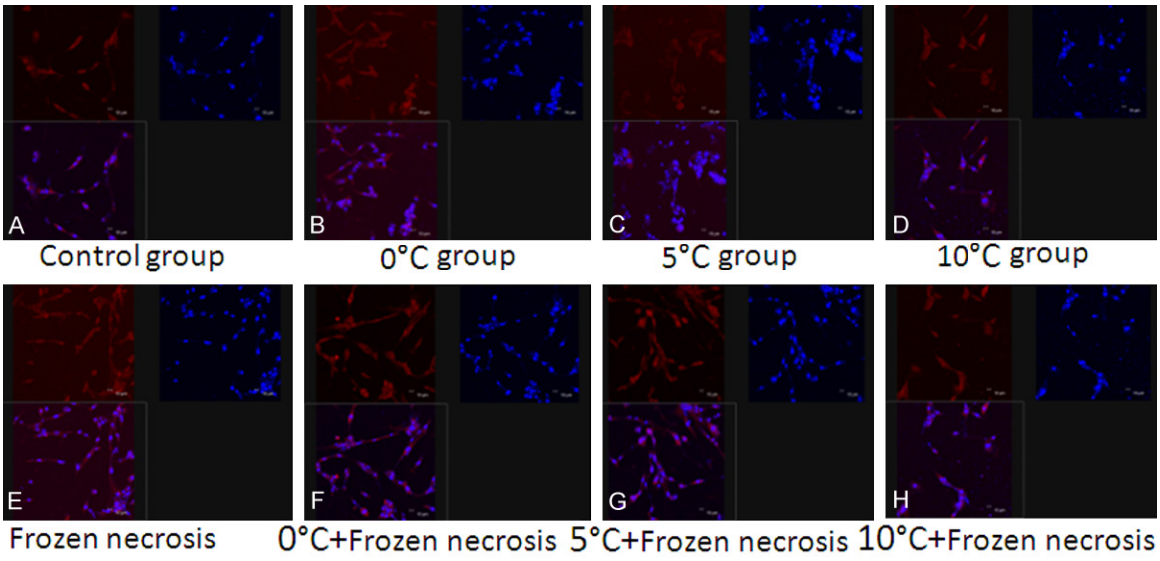

Figure 7. Immunofluorescence results of NF-κB P65 protein without inhibitor.

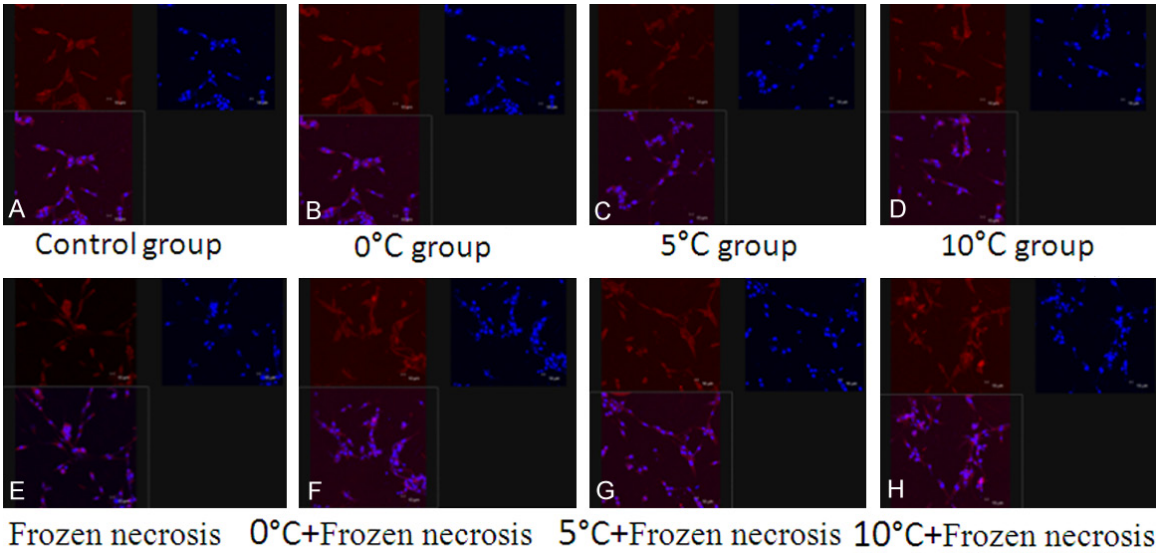

Figure 8. Immunofluorescence results of NF-κB P65 protein in the presence of inhibitor.

and the control group (P > 0.05). (**Figures 7**, **8** and **Table 2**).

*Protein expression of NF-κB (**Figures 9-11**)*

These results showed that low temperature stimulation had no significant effect on the expression of the NF-κB protein, and there was no significant difference between the control and low temperature groups (P > 0.05). The expression of the NF-κB protein in the compound stimulation groups and the necrotic group was significantly up-regulated (P < 0.05). However, there was no change in the expression of the NF-κB protein in all the groups with an inhibitor, whose line charts were almost in a straight line.

Discussion

The KCs participate in a variety of important physiological and pathological processes in the liver, such as clearing necrotic cells and tissue fragments, inducing inflammatory reactions, and inhibiting carcinogenesis via its phagocytosis and secretion functions [17-19]. The KCs function is affected by various factors such as endotoxin, lipopolysaccharide, necrotic tissue

Table 2. Fluorescence intensity of the NF-κB P65 protein in each group

Group	Without inhibitors	With inhibitors
Control group	224	209
0°C	158	98
5°C	186	116
10°C	179	129
Frozen necrosis	394	205
Frozen necrosis + 0°C	199	109
Frozen necrosis + 5°C	264	154
Frozen necrosis + 10°C	282*	196

Note: 1. In the without-inhibitor groups, a low temperature inhibited the expression of the P65 protein. Compared with the control group, the 0°C group, $t = 4.107$, $P < 0.05$; 5°C or 10°C group, $P > 0.05$. The necrotic products could up-regulate the P65 protein expression. Compared with the control group, necrotic group, $t = 6.732$, $P < 0.01$. There was a superimposed effect in low temperature simulation and necrotic products. Compared with the control group, the necrosis+0°C group, $t = 2.643$, $P > 0.05$; necrosis + 5°C group, $t = 3.371$, $P > 0.05$; necrosis + 10°C group, $t = 4.783$, $P < 0.05$. 2. In the with-inhibitor groups, compared with the control group: 0°C group, $t = 7.564$, $P < 0.01$; 5°C group and necrosis + 0°C group, $P < 0.05$; other groups, $P > 0.05$. 3. Compared between the with-inhibitor group and the without-inhibitor group, there were significant differences between the two groups of the same conditions (except the control groups), $P < 0.05$.

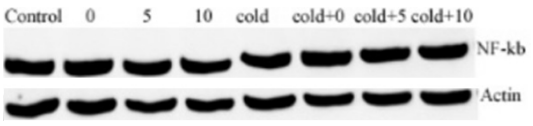

Figure 9. Protein expression of NF-κB in no-inhibitor group.

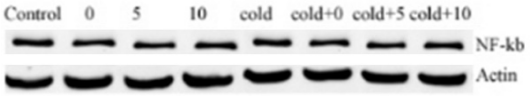

Figure 10. Protein expression of NF-κB in inhibitor.

or cells, and stress [20]. Activated KCs can produce many cytokines including TNF, IL, and IFN, which can kill target cells, so as to play its biological roles [21]. Through rapid temperature changes, cryoablation can destroy tumor cells and produce low temperature stimulation to the tissues around the treatment area so as to induce a stress reaction. The stress reaction and cellular harmful substances can cause changes to the KCs [22].

Our results have demonstrated that the change of the inflammatory factors was very obvious. Although the concentrations of TNF-α, IL-1β and INF-γ were different, their bar graphs were very similar, with no significant statistical changes. The KCs enhanced the secretion of inflammatory factors after the stimulation of the low temperature or freeze-thaw necrosis, and there was a superimposed effect. However, there were no significant differences among the temperature subgroups. These results demonstrated that KCs were activated by cryoablation and secreted plenty of inflammatory factors, such as TNF-α, IL-1β, and INF-γ. These factors could guide inflammatory cell aggregation, mediate the inflammatory response, and clear away necrosis cells or tissue fragments [23]. This reaction process might be the main mechanism for killing any remaining tumors after cryoablation. In addition, this study showed that the cryoablation-induced secretion of inflammatory factors could be prevented by PDTC, a specific inhibitor in NF-κB signaling pathway. So, it was fully confirmed that the secretion function of KCs could be regulated by the NF-κB signaling pathway after cryoablation.

This study has demonstrated that the low temperature stimulation without freeze-thawing necrotic products or the presence of body stress reaction could not promote the KCs proliferation *in vitro*. It has been reported that a low temperature reduced cell metabolism and cytokine activity [24]. The lower the temperature, the much more obvious the inhibition effect was. In this study, at either 0°C or 5°C, the KCs decreased in quantity. There was a significant different in the quantity of KCs between the low temperature and control groups. However, the freeze-thawing necrotic substance promoted the proliferation of the KCs. The KCs increased in quantity. There was a significant difference compared with the control group. So, it could be concluded that the freeze-thawing necrosis products stimulate the proliferation of KCs and further enhance the cellular immune response. After PDTC was added, the necrotic products could still stimulate the KCs proliferation. This suggests that the NF-κB signaling pathway is closely related to the KCs proliferation, and there might be other pathways in the KCs proliferation after cryoablation.

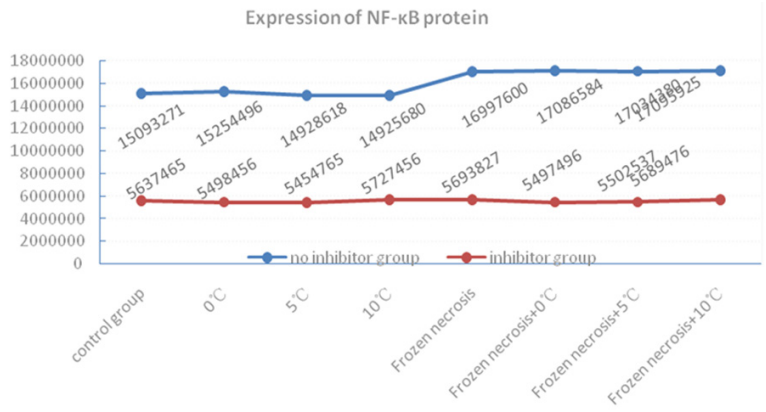

Figure 11. Expression of NF-κB protein. Note: 1. In without inhibitor groups, Compared with control group: 0°C, 5°C and 10°C group, $P > 0.05$; necrosis group and combined groups, $P < 0.05$. 2. In with inhibitor groups, Compared with control group: all groups, $P > 0.05$. 3. Compared between with inhibitor group and without inhibitor group, these were significant difference between two groups of the same conditions, $P < 0.01$.

KCs have another important function-phagocytosis, which is closely associated with SR. In 1979, Goldstein discovered firstly that there was a binding site in macrophages which is used to uptake and degrade the acetylated LDL and was named as SR1 [25]. Other SRs were discovered later [26]. All the SRs composed an SR family. SR, which is expressed on the surface of KCs inside the liver, is a transmembrane protein and also a group of heterogeneous molecules on the surface of phagocytes which present at least in 6 different molecular forms. Their ligands include chemical modified proteins, polynucleotides, polysaccharides and phospholipids, which can promote KCs endocytosis to swallow damaged proteins, cells and cell debris of inflammation or tissue injury, and participate in the body's defense response [27]. They also participate in the identification and elimination of pathogens that reflect the phagocytic ability of KCs [28]. This study showed that SR, which indicates the phagocytic function of the KCs, increased with a rise in temperature in a low-temperature environment. However, SR was shown to be down-regulated significantly after being stimulated by the tumor necrotic products. We thought that the reason for the down-regulation of the SR proteins might be either because plenty of SR proteins were consumed by the phagocytosis of KCs or an inhibited expression of the SR proteins because of increased inflammatory factors [29]. It was certain that the phagocytosis of the KCs did not dominate to clear away the tumor necrotic products after cryoablation.

NF-κB is a transcription factor family that regulates a large number of genes that are involved in important physiological processes, including cell survival, inflammation, and immune responses. More recently, the constitutive expression of NF-κB has been associated with several types of cancer [30]. NF-κB signaling is an important factor in the development of inflammation-associated cancers, too [31]. Through the detection of related proteins in the NF-κB signaling pathway, this study explored the mechanism by which KCs induced inflammatory reaction. NF-κB is an important nuclear transcription factor in cells and an important component of cellular immune and inflammatory reactions [32]. It participates in the expression and the regulation of multiple genes [33]. It was thought that NF-κB was the key factor in regulating the KCs activation [34]. NF-κB played a key role in the regulation of some cellular information transcription [35]. And it was a marker of cell activation and an important factor for the activation of the inflammatory response [36]. It was showed that the NF-κB signaling pathway was a potential target for tumor therapy [37]. In resting cells, P65 and NK-κB consisting of two aggregates did not show any transcriptional activity. Under the stimulation of viruses, LPS, or an active oxygen intermediate product, P65 identified specific DNA sequences and further induced the production of mRNA, and finally transcribed, produced, and released various cytokines [38]. This process was known as the classical activation pathway. If this signaling pathway plays a similar role in the changes of the immune function caused by cryoablation, it would be helpful to explore the mechanisms of immune function changes after cryoablation. The most direct way to examine them would be to block the NF-κB signaling pathway. It was already confirmed that PDTC can inhibit the expressions of NF-κB protein at different levels of the NF-κB activating channel and can further block the NF-κB signaling pathway. Whether the blockade

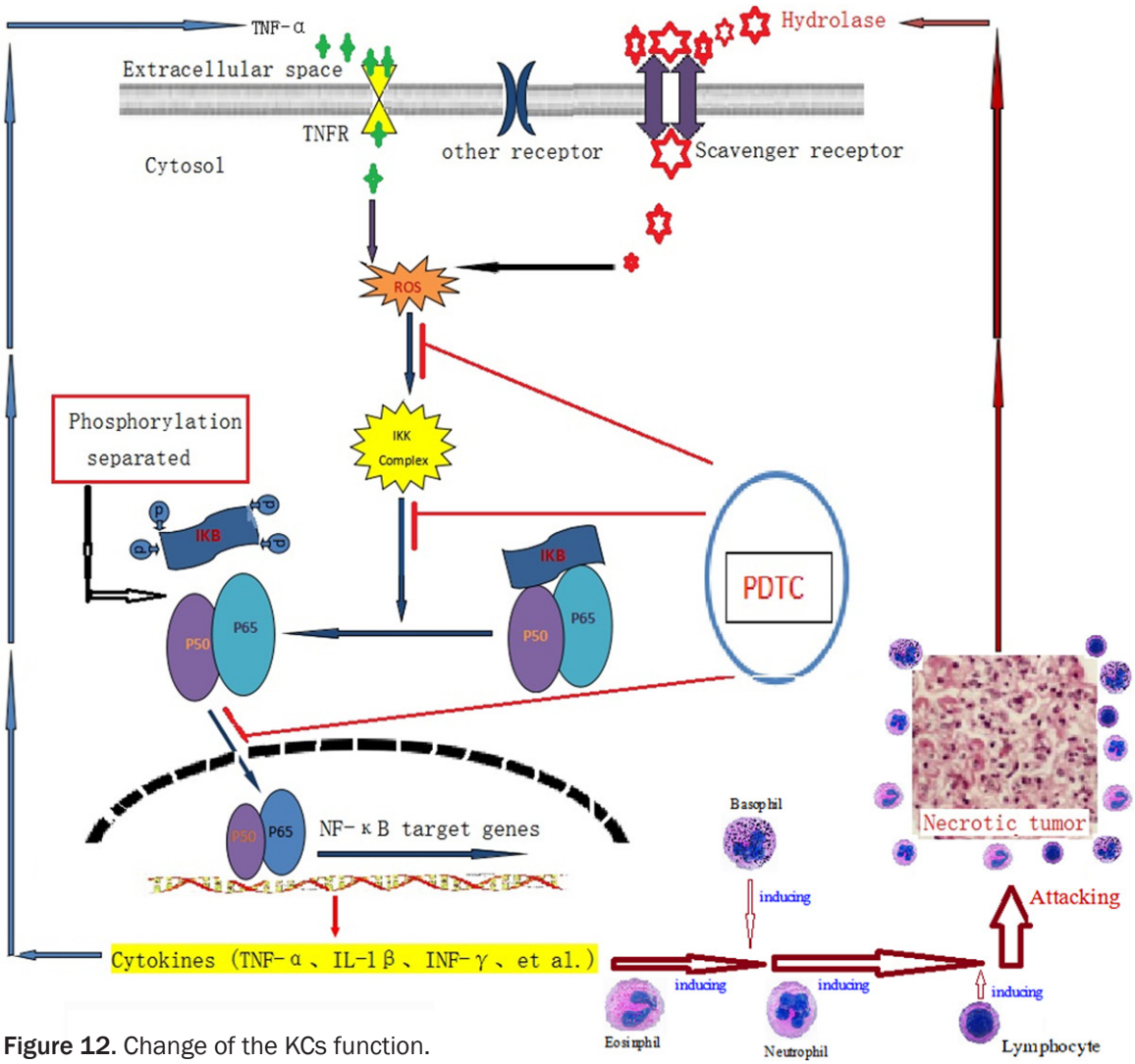

Figure 12. Change of the KCs function.

was successful or not could be determined by detecting the expression of the key protein-the P65 protein in the NF-κB signaling pathway and the expression of the terminal protein-the NF-κB protein.

Compared with the classical activating pathway of the NF-κB signaling pathway at a normal temperature, the KCs activity is lower, the combination of p65 and IKK is closer, and the expression of p65 is lower at a lower temperature, as this experiment confirmed. There was cytotoxin inside the freeze-thaw necrotic substance. After the necrotic substance was added to the KCs culture bottle, the stimulating signal was combined to the receptor on the KCs membrane, such as SR, and was phagocytosed by KCs firstly. And then, the IKK, the upstream kinase of IKB, was phosphorylated and further activated through a multistage cascade reaction, resulting in the degradation of IKB. P50 had a nuclear location signal. After losing the limitation of IKB, P50 would take the RelA (P65) to move to the intranuclear. P65 combined with the homeopathic element residing on the gene promotor or enhancer inside the cells, and it further regulated and controlled the transcription of the target gene and induced both the expression and the transcription of NF-κB mRNA and released various cytokines (such as TNF-α and IL-1β, etc.) [39]. From our results, it appears that the expressions of P65 and NF-κB are up-regulated, and the secretion of inflammatory factors was increased in the groups treated with the freeze-thawing necrotic substance.

PDTC plays the role of inhibiting the protein expression of NF-κB in the different levels of the NF-κB activating pathway, such as prevent-

ing the upstream IKK phosphorylation and reducing the nuclear translocation of P65, so as to prevent the NF-κB signaling pathway, inhibit NF-κB protein expression and cause a decrease in inflammatory cytokines. Our results provided strong evidence that the activation of the KCs by freeze-thawing necrotic substance was regulated and controlled by NF-κB signaling pathway. This mechanism could be displayed by the diagrammatic sketch (**Figure 12**).

In summary, cryoablation could change the microenvironment around KCs, promote the proliferation of KCs, activate the KCs, and increase the release of the secreting cell factors from the KCs, which could induce the inflammatory reaction to clear relict tumor cells. The effect on the secretion function and the proliferation of KCs by cryoablation was mediated through the NF-κB signaling pathway. In addition, we thought that the phagocytosis of KCs did not dominate in clearing away the freeze-thaw necrotic products.

Disclosure of conflict of interest

None.

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Table S1. The KCs phagocytosis function test

	No inhibitor	Inhibitor	T
Control	10520544.0 ± 90749.2	5764754.3 ± 57497.1	T = 76.7 (P < 0.001)
0°C	11401372.0 ± 25555.8	5896203.0 ± 164535.9	T = 57.3 (P < 0.001)
5°C	11091848.7 ± 116977.5	5456900.7 ± 56973.3	T = 75.0 (P < 0.001)
10°C	11436500.3 ± 114354.2	6543143.7 ± 54499.4	T = 66.9 (P < 0.001)
Cold	8563434.3 ± 57206.4	5127795.3 ± 62049.1	T = 70.5 (P < 0.001)
Cold + 0°C	8541676 ± 53874.1	5177190.0 ± 70248.5	T = 65.8 (P < 0.001)
Cold + 5°C	7982509.0 ± 61156.5	5398161 ± 21739.9	T = 68.9 (P < 0.001)
Cold + 10°C	7949273 ± 83542.6	4294848.3 ± 45917.7	T = 66.4 (P < 0.001)

KCs cell secretion function test (**Figures 4-6**)

Table S2. TNF-α concentration

	No inhibitor	Inhibitor
Control group	4.963	4.630
0°C	13.930	5.355
5°C	12.598	6.016
10°C	11.431	5.121
Freezing necrosis	16.341	5.492
Freezing necrosis + 0°C	19.672	5.501
Freezing necrosis + 5°C	18.663	5.605
Freezing necrosis + 10°C	17.896	6.001

Table S3. IL-1β concentration

	No inhibitor	Inhibitor
Control group	7.841	7.362
0°C	22.010	8.515
5°C	19.905	9.565
10°C	18.062	8.142
Freezing necrosis	25.819	8.733
Freezing necrosis + 0°C	31.082	8.747
Freezing necrosis + 5°C	29.487	8.912
Freezing necrosis + 10°C	28.276	9.542

Table S4. IFN-γ concentration

	No inhibitor	Inhibitor
Control group	12.389	11.631
0°C	34.776	13.454
5°C	31.450	15.112
10°C	28.538	12.865
Freezing necrosis	40.796	13.798
Freezing necrosis + 0°C	49.110	13.821
Freezing necrosis + 5°C	46.590	14.082
Freezing necrosis + 10°C	44.677	15.076