Liver Damage After Radiofrequency Ablation Combined with Transcatheter Therapy in Treating Rabbit VX2 Liver Tumors

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Research article

Keywords: Transcatheter arterial chemoembolization (TACE), Radiofrequency ablation (RFA), Transcatheter arterial embolization (TAE), HSP70, Apoptosis

DOI: https://doi.org/10.21203/rs.3.rs-36292/v1

Abstract

Background: To evaluate the effect of transcatheter therapies combining with radiofrequency ablation (RFA) treatment on hepatocellular necrosis, apoptosis and proliferation by using the rabbit VX2 tumor model.

Methods: Ninety six models were randomly divided into 4 groups: transcatheter arterial chemoembolization group (TACE), radiofrequency ablation group (RFA), TACE+RFA group and transcatheter arterial embolization (TAE) + RFA group. The above groups were further divided into two subgroups, A (15 rabbits) and B (9 rabbits). The subgroup B (control group) was followed up until animal death.

Results: The high expression of heat shock protein 70 (HSP70) was observed in the adjacent liver tissue in TACE and TACE+RFA. The highest increase of transaminase levels and serum HSP70 were detected in TACE+RFA group. TAE+RFA group had a low apoptotic rate and more hepatocyte proliferation as compared to TACE and TACE+RFA groups; and it had the longest end-point survival among these groups.

Conclusions: The TAE+RFA treatment had a better outcome than RFA, including a better liver tumor control, a less liver injury**,** and a longer survival than TACE+RFA**.** Compared to TACE and TACE+RFA procedures**,** TAE+RFA significantly decreased the liver injury, hepatocellular necrosis, apoptosis and systemic proinflammatory cytokine release caused by anticancer drugs application.

Background

Transcatheter therapy, including transcatheter arterial chemoembolization (TACE) and transcatheter arterial embolization (TAE), has been increasingly used as effective palliative treatments for unresectable hepatic tumors [1, 2]. TACE can promote tumor necrosis by directly kill tumor cells and block tumor's blood supply to achieve the therapeutic purposes. However, TACE and TAE induce ischemic or toxic injury in the tumors as well as in the hepatic tissues adjacent to the tumor, leading to hepatic failure occasionally [3]. Animal study revealed that the treatment of TACE induced prominent hepatocellular damage and hepatic fibrogenesis together with compromised liver function may be responsible for chronic liver decompensation [3, 4].

Radiofrequency ablation (RFA) considered to be one of the first-line treatments for early stage of hepatocellular cancer (HCC), particularly for patients with impaired liver function [5]. In patients with small HCC (<4 cm in size), RFA might provide therapeutic effects similar to those of surgical resection [6]. However, percutaneous RFA was more likely to be incomplete for the treatment of small HCC located at specific sites of the liver, which may be the better choice of open or laparoscopic surgery. Tumor recurrence due to incomplete ablation was a negative prognostic factor for survival [7]. To minimize recurrence rate and prolong survival, the TACE was used in combination with RFA (TACE+RFA) [5].

The decrease of blood flow caused by TACE reduced heat loss, thus permitting larger lesions to be ablated by RFA [8]. The combination of chemotherapy and hypoxia induced by TACE also reduced the temperature where coagulative necrosis occurred, which enables formation of larger thermal lesions [9]. Several studies have demonstrated the synergistic and cytotoxic effects of TACE + RFA on HCC [10, 11].

To determine the optimum combination strategy of transcatheter therapy and RFA, we carried out this experimental study that compared tumor growth and necrotic rate, the liver function, liver injury and end-point survival by using rabbit VX2 liver tumor model [12], we also investigated the cellular injury surrounding the necrotic zone produced by

TACE and the zone surrounding the RFA, TACE+RFA and TAE+RFA ablated tissue. This study aims to evaluate the efficacy of RFA combined with transcatheter therapies to aid in strategy selection practically.

Methods

Establishment of VX2 rabbit liver tumor model

All experiments were approved by the Animal Care Committee in China. The animals (purchased from Wuhan Wanqianjiaxing Biotechnology Co.,LTD.SCXK 2016-0011) were treated according to National Institutes of Health guide for the care and use of Laboratory animals and all efforts were made to minimize the number of rabbits used and their suffering. A total of 100 adult Japanese white rabbits weighing 3.0-3.5 kg were initially used in the study. The methods of VX2 carcinoma strain transplantation was followed as described by Virmani et al [12]. Two weeks after inoculation, the harvested VX2 tumor fragment implanted into the medial left liver lobe to generate liver tumors in carrier rabbits. On the 15 day after the implantation, each rabbit was undergoing magnetic resonance imaging (MRI, 1.5-T, Magnetom Avanto; Siemens Medical Solutions, Erlangen, Germany) and 96 of them detected the VX2 tumor. The rabbits' position and the T1WI and T2WI imaging parameters were described as previously [13]. The epigastria and backs of the rabbits were shaved before procedure.

Study Design

The 96 rabbits with VX2 liver tumor were randomly divided into 4 groups: 1) TACE group (consisting of 0.4 mg doxorubicin that was mixed into 0.4 ml iodized oil); 2) RFA group; 3) TACE+RFA group; 4) TAE+RFA group. RFA was performed in TACE+RFA and TAE+RFA groups 15 min after TACE or TAE [polyvinyl alcohol particles (PVA) 150-250 μm in size (Cook, Bloomington, IN, USA)] treatment [14]. Each above groups were further randomly divided into subgroup A (15 rabbits) and subgroup B (9 rabbits). Five rabbits from each subgroup A were sacrificed by sodium pentobarbital (100 mg/kg IV) on days 1, 3, 7 after procedures, respectively. The subgroup B was followed up until animal's death **(Fig. 1)**. All rabbits were treated humanely during the experiment as reported as previously [13].

The TACE and TAE procedures were performed according to digital subtraction angiography (DSA; Multistar, Siemens, Erlangen, Germany) [13]. The rabbits that suffered TACE, TAE or without treatment were placed on to a circuit pad in the supine position and RFA procedure was performed following an adequate anesthesia [13].

Hepatocellular necrosis score

The severity of injury in live tissue surrounding the zone of necrosis or coagulation was blindly graded according to modified Suzuki criteria [15]. The sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded on a scale of 0 to 4. Absence of necrosis, congestion, or centrilobular ballooning was scored as 0, whereas the presence of severe congestion/ballooning and 60% or greater lobular necrosis was scored as 4. Six fields of each sample were evaluated under light microscope with 100 magnification.

Serum and liver samples collection

Serum samples were collected 1 day before operation and on days 1, 3 and 7 after operation. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by standard enzymatic procedures. For TACE group, livers were incised along the long axis of tumor, its largest and the smallest diameters were measured on the largest slice. For RFA, TACE+RFA and TAE+RFA groups, the affected area of liver was incised

along the long axis of the RFA electrode tract and the samples were sectioned at 3-5 mm intervals. All samples were preserved in 4% paraformaldehyde for pathologic analysis.

Proinflammatory Cytokine Response

Serum TNF-α, the most important acute-phase response cytokine produced by hepatic macrophages, was assessed with ELISA by using polyclonal TNF-α goat anti-rabbit antibody (USCN Life Science, Wuhan, China) according to the manufacturer's instructions.

Hematoxylin - Eosin and heat shock protein 70 immunohistochemistry staining

The slides embedded in paraffin were sectioned at 4 μm. Hematoxylin - Eosin (HE) staining was performed in the standard fashion. The heat shock protein 70 (HSP70) levels of the samples were determined by immunohistochemistry staining [16]. All slides were evaluated with 200 magnification by two pathologists in a blinded manner. In TACE group, 5 photographs of HSP70 staining were taken from the liver tissue surrounding the zone adjacent to the necrosis. In RFA, TAEC+RFA and TAE+RFA groups, 5 photographs were taken from liver tissue surrounding the coagulation zone, respectively. The HSP70 expression was automatically calculated by integrated optical density (IOD) using Image-Pro Plus software (version 6.0, Media Cybernetics, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay of HSP70 in peripheral blood

One milliliter of peripheral blood taken from each rabbit's ear vein before treatment and on days 1, 3 and 7 after the treatment was placed into tubes containing no anticoagulants to separate serum (kept at -20°C). The serum HSP70 and tumor necrosis factor (TNF)-α were quantitated by Enzyme-linked immunosorbent assay (ELISA, Abcam PLC., Cambridge, UK).

Ninety-six well microtiter plates were coated with mouse anti-rabbit HSP70 capture antibodies in carbonate buffer overnight. 100 μL of the samples were added into the plates, incubated for 2 h. Plates were subsequently washed and PBS-gelatin buffer containing diluted biotin anti-HSP70 was added and incubated at room temperature for 1.5 h; the plates were washed again and incubated with streptavidin–HRP diluted in PBS gelatin for 20 min. Then the plate was washed to remove excess HRP conjugate and added Tetramethyl benzidine (TMB) substrate solution into each well. After the reaction stopped, the colorimetric reaction produced was read at a wavelength of 450 nm. The amount of signal was directly proportional to the HSP70 level in the sample and it was expressed as ng/ml.

Liver apoptosis

The apoptosis index (AI) of hepatocyte apoptosis in liver and tumor cells were assessed with the method of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL). The results were semi quantitatively scored by averaging the number of apoptotic cells per field at 200 × magnification. Five microscopic fields of each sample were evaluated.

Liver Regeneration

Liver tissues surrounding the ablation or embolized zone were immunostained with anti-Ki-67 antibodies (monoclonal mouse anti-rat Ki-67 antigen; MIB-5; Dako, Carpinteria, California), which allowed to detect all active parts of the cell cycle and was a strong positive correlation with proliferating antigen expression and bromode-oxyuridine / thymidine incorporation [12]. The immunostained sections were counterstained with HE. The

proliferation index was defined as the percentage of Ki-67 positive hepatocytes per total hepatocyte by counting 6 fields for each section at 200× magnification.

Statistical Analysis

Data analysis was carried out with SPSS software (Chicago, IL, U.S.A.). Quantitative data were expressed as means ± SD and analyzed by ANOVA. The survival curves were constructed by the Kaplan-Meier method and compared by log-rank test and χ^2 tests for survival analysis. Differences in counts between each groups and different time points were analyzed by the Kruskal-Wallis H test and Mann-Whitney U test. *P* value <0.05 was considered to indicate statistical significance.

Results

On the day 15 after tumor implantation, 96 rabbits were confirmed the VX2 liver tumor formation by MRI. The tumor sizes were 11.5 to 16.9 mm (mean, 12.8±3.5 mm). Ten rabbits in the TACE group and all rabbits in the TACE+RFA group were loss of appetite up to 3 days after the procedure. The rest of the rabbits returned to normal diet and physical activity, and no animals died within 3 days after surgery.

The animal survival in subgroup B after treatment was shown in **Fig. 2**. Mean survival in RFA group (35.9 days ±11.2) was higher than that in TACE (26.5 days ±7.9; P<0.05) and TACE+RFA groups (16.9 days ±4.8; P<0.05). TAE+RFA group had the longest mean survival (50.2 days ±16.5).

Hepatic necrosis, tumor growth and necrotic rate on day 7 postoperative

On postoperative day 7, the Suzuki score of hepatic necrosis in TACE+RFA group was significantly higher than the others **(Table 1)**, but there were no significant differences among the TACE, RFA and TAE+RFA groups. The differences of tumor growth rate and necrotic rate between the TACE+RFA and TAE+RFA were not significant. The decrease of tumor growth rate and the increase of necrotic rate in the TACE+RFA and TAE+RFA groups were significantly differed from TACE and RFA groups, that were the same as we previous reported [13].

Changes in liver function

There were not significant changes in ALT and AST levels among the four groups one day before the operation. The statistical differences of ALT and AST levels in the four groups at a different time point were the same as we reported previously [13]. ALT and AST reached the highest levels on day 3 in groups TACE, TACE+RFA and TAE+RFA , and on day 7 in RFA , P<0.05. The TACE group's levels were higher than that of the RFA and TAE+RFA on days 3 and 7; and the TACE+RFA's levels were the highest among the groups on days 3 and 7 after operation; The ALT and AST levels in TAE+RFA were significantly higher than RFA on day 3, but were not on day 7 **(Table 2)**.

Serum TNF-α level

The peaked TNF-α level on day 1 were gradually dropped on days 3 and 7 in four groups. The TNF-α levels in TACE, RFA, TACE+RFA and TAE+RFA groups on day 1 were significantly elevated as compared to their concentration on days 3 and 7 **(Table 3)**.

HSP70 expression in liver tissue

IOD of HSP70 expression in the transitional zone of ablation in liver increased significantly on days 1, 3 and 7 in all 4 groups. HSP70 expression on days 1 and 3 in TACE group was significant increased as compared with RFA group. HSP70 expression in TACE+RFA group on days 1, 3 and 7 was significantly higher than others. The HSP70 expression in TAE+RFA group on days 1 and 3 was significant higher than that in TACE and RFA groups, respectively. There was no significant difference for HSP70 expression in TACE, RFA and TAE+RFA groups on the day 7 **(Figure 3)**.

The levels of serum HSP70 (ng/ml) in different groups

The levels of serum HSP70 in all groups postoperative were peaked on day 1, decreased on day 3 and decreased to the lowest on day 7. Its levels in TACE group on days 1, 3 and 7 were significantly higher than that in RFA groups. The HSP70 day 1 levels in TACE, TACE+RFA and TAE+RFA groups were significant higher than theirs day 3 and day 7 levels. The serum HSP70 levels in TACE+RFA group on days 1, 3 and 7 were much higher than that the others, respectively. There were no significant differences between TACE and TAE + RFA group and between TAE + RFA and RFA group on the days 1, 3 and 7 HSP70 levels **(Figure 4)**.

Hepatocyte apoptosis

A representative TUNEL staining showed the day 1 images in the 4 groups **(Figure 5)**. The percentage of apoptotic cells (i.e., TUNEL-positive) in TACE group on days 1 and 3 were greater than TAE+RFA and RFA group, respectively. The percentage of hepatocyte apoptosis in TACE+RFA group on days 1, 3, and 7 post-operation were the highest when compared with others. There was no significant difference in percentage of apoptotic cells among TACE, RFA and TAE+RFA groups on day 7. As well, there was no significant difference in percentage of apoptotic cells between RFA and TAE+RFA on days 3 and 7.

Hepatocyte proliferation

Hepatocyte proliferation in the liver tissue surrounding the zone of necrosis or coagulation was evaluated by anti-Ki-67 staining **(Figure 6)**. The percentage of Ki-67-positive hepatocytes was gradually increased on day 1, peaked on day 3 and decreased on day 7 in these groups **(Table 5)**. The percentage of Ki-67-positive hepatocytes in TAE+RFA group on days 1, 3 and 7 had significant increases compared to TACE+RFA and RFA groups ($P<0.05$). The frequency of proliferation cells of the TAE+RFA group showed the highest levels on days 1, 3 and 7 ($P<0.05$). There were no significant differences for hepatocyte proliferation frequencies in TACE, TACE+RFA and RFA groups on day 7.

Discussion

The so-called heat-sink effect (the cooling effect produced by adjacent blood flow when tumors situated near large vessels) represents one of the limitations for liver RFA [9]. Reduction of the necrotic zone by heat-sink effect may increase the risk of the local recurrence [18]. To overcome the heat-sink effect, many strategies to improve completeness of RFA have been to target these residual viable cells with adjuvant chemotherapy, vascular occlusion such as TAE, and adjuvant chemotherapy followed by vascular occlusion such as TACE [8, 14, 19].

With the synergistic cytotoxic effects for TACE+RFA treating HCC, TACE decreases blood flow to the tumor, making subsequent RFA more effective, as there was less heat loss by convection [8, 10, 11]. In our previous study, we found TACE+RFA was more effective for achieving tumor destruction than TACE, RFA or TAE+RFA, on treating rabbit

VX2 liver tumors [13]. However, TACE+RFA with the most serious liver dysfunction showed no difference in achieving tumor control compared with TAE+RFA [13].

Li et al. [20] found reducing chemotherapy doses and times in the course of TACE treating HCC can play a positive effect to patients' long-term survival ratio, which not only have no influence to its short-term curative effect but also could protect liver function. Some researchers even gave up TACE and took advantage of the synergistic cytotoxic effects of the chemotherapeutic drugs by RFA combined with intravenous liposomal doxorubicin to achieve more complete tumor treatment and greater survival [21]. By inducing tumor cells' apoptosis and inhibiting HSP70 expression, they found combining RFA with adjuvant liposomal drug therapies can achieve more complete tumor treatment and greater survival [21]. Shibata et al. [22] suggested the combination treatment may not be necessary for TACE+RFA; RFA has the equivalent effectiveness on including local tumor progression rates, over-all survival rates, and local progression-free survival rates during the treatment of human small (≤3 cm) HCCs.

To determine the optimum combination strategy of transcatheter therapy and RFA, we carried out this experimental study to not only compare tumor control by tumor growth and necrosis, but also compare the liver function, liver injury and end-point survival. We measured ALT and AST to compare change of liver function, HSP70 expressed and liver proliferation in liver surrounding the ablation area or infarct area to compare the ability of hepatic cells recovered from stimulations, TNF-α and HSP70 in serum and hepatocyte apoptosis to compare liver tissue injury in the 4 treatments in a rabbit VX2 liver tumor model.

In our present study, less HSP70 expressions and apoptotic hepatocytesis, compared with TACE+RFA group, were observed on the liver tissue in the TACE, RFA and TAE+RFA groups, suggesting that there were less hepatocytes suffered hyperthermia, hypoxia and cytotoxicity and they were reversible cellular injury or apoptotic state. HSP70 expression on day 7 postoperative in TACE, RFA and TAE+RFA groups was not significantly different may indirectly indicate that their hepatocytes were more easily to be recovered than TACE+RFA. The synergistic cytotoxic effects of TACE in TACE+RFA which induced transient impairment in liver function or potential chronic liver injury is probably a consequence of TACE+RFA has less effect on liver dysfunction and end-point survival compared with TAE+RFA.

Tissue injury also resulted in the release of HSP70 from damaged cells into the blood [23]. Elevated serum levels of HSP70 had been demonstrated during trauma, myocardial infarction, hepatic ischemia-reperfusion and liver resection [24, 25]. Higher HSP70 levels of the patients could be a reflection of their inflammatory status. In this study, serum HSP70 levels on day 1, day 3, and day 7 of the TACE + RFA group were significantly higher than those of the other three groups, indicating that the most severe tissue damage and inflammatory status occurred on days 1, 3 and 7 of the TACE + RFA group.

Cell death in the liver occurs mainly by apoptosis or necrosis. In this study, the main treatment measures included TACE and RFA. Doxorubicin used in TACE group can cause apoptosis and cell death through mitotic catastrophe [26]. RFA employs is a high-frequency causing a coagulative necrosis of the targeted tissue [27]. The combination of these two methods for the treatment of HCC, not only can cause cell apoptosis, but also lead to necrosis. However, the ALT and AST levels in the TACE + RFA group on days 3 and 7 after operation, the HSP70 expression and the percentage of hepatocyte apoptosis in TACE + RFA group on days 1, 3, and 7 post-operation were significant higher than that in TACE, RFA and TAE + RFA, suggesting that this combination of treatment at the same time also significantly damage the liver function. TACE+RFA led to severe hepatocellular necrosis in the adjacent liver tissues, serum TNF-α level significantly increased. In TAE+RFA group, liver function was not markedly affected,

and liver parenchymal necrosis was mild based on its histologic analysis, which showed that hepatic arteries embolized by using PVA did not develop prominent hepatocellular damage as compared to RFA group. Additionally, There was no significant difference of hepatocellular necrosis in the adjacent liver tissues between RFA and TAE+RFA groups, based on their serum levels of TNF-α. Therefore, we believe that TAE and RFA joint use is worthy of clinical consideration of the program. In the absence of anticancer drugs, TAE + RFA is safe and effective for patients with unresectable liver malignancies like TACE + RFA. The advantage of this combination is that it can reduce the blood supply in the tumor area and reduce the loss of heat generated by RFA, so the lesion can be effectively eliminated.

The limitations of this study is: First, more rabbit VX2 liver tumor models should be built in TACE+RFA group since that had a high mortality on the 7 days after procedures; and the observation time points of 12 hour and 14 day should have been set to detect real-timely changes of various detection indexes in the 4 groups. Second, more animals should be observed for the survival in the 4 groups. Finally, because treating human HCC need multiple courses of TACE, RFA or TACE+RFA, their hepatocellular injury or liver regeneration cannot be really mimicked. These results from present study should be confirmed in a clinical setting with a careful prospective study in patients with HCC.

Conclusions

In conclusion, TAE+RFA has less effect on liver injury, hepatocellular necrosis and apoptosis as compared to TACE and TACE+RFA in the rabbit VX2 liver tumor model. It is likely to have potential for future standard treatment of small liver tumors, especially considering many other liver problems that need to be addressed by interventional therapy.

Abbreviations

RFA: radiofrequency ablation; TACE: transcatheter arterial chemoembolization group; TAE: transcatheter arterial embolization; HSP70: heat shock protein 70; HCC: hepatocellular cancer; PVA: polyvinyl alcohol particles; DSA: digital subtraction angiography; AST: aspartate aminotransferase; ALT: alanine aminotransferase; HE: Hematoxylin - Eosin; IOD: integrated optical density; TNF: tumor necrosis factor; ELISA: Enzyme-linked immunosorbent assay; TMB: Tetramethyl benzidine; AI: apoptosis index; TUNEL: triphosphate nick-end labeling.

Declarations

Ethics and consent to participate

All experiments were approved by the Animal Care Committee in China. The animals were treated according to National Institutes of Health guide for the care and use of Laboratory animals and all efforts were made to minimize the number of rabbits used and their suffering.

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

Competing interests: There are no potential conflicts of interest to disclose.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81401494) and the Henan Medical Science and Technology (No. 201403059).

Authors' contributions

guarantor of integrity of the entire study: XHD, XWH, JZR; study concepts: XHD, XWH; study design: XWH, JZR; definition of intellectual content: XHD, XWH, JZR;

literature research: XWH, JZR, PFC; clinical studies: JZR, PFC, SJG; experimental studies: XHD, HL, XWH; data acquisition & analysis: SJG, DLK; statistical analysis: JZR, PFC; manuscript preparation: XHD, HL, XWH; manuscript editing: HL, XWH, JZR; manuscript review: XHD, HL, XWH, JZR. All authors read and approved the final manuscript.

Acknowledgements: Not applicable.

Tables

Table 1. Suzuki score of hepatic necrosis, tumor growth and necrotic rate on day 7 in different groups.

Groups (n)	Suzuki-score hepatic necrosis		
		Tumor growth rate (%)	Tumor necrotic rate (%)
TACE+RFA (5)	4.26±2.29[a]	116.5±36.4[b]	91.5±14.3[b]
TAE+RFA (5)	1.89±1.24[a]	122.9±52.3[b]	87.5±20.8[b]
TACE (5)	2.16±1.31[a]	334.4±53.1[b]	58.5±13.7[b]
RFA (5)	1.13±1.01[a]	242.1±48.3[b]	66.5±14.9[b]

a: Suzuki score of hepatic necrosis in TACE+RFA group was significantly different from the others groups ($P < 0.05$).

b: The tumor growth rate and the tumor necrotic rate in TACE+RFA and TAE+RFA group was significantly different from TACE and RFA group ($P < 0.05$).

There were no significant differences in tumor growth rate and necrotic rate between the TACE+RFA and TAE+RFA groups.

Table 2. Changes in serum ALT and AST levels before and after operation.

Group(n)	1 day preoperative		3 days postoperative		7 days postoperative	
	ALT	AST	ALT	AST	ALT	AST
TACE+RFA(5)	35.3±8.6	48.1±12.2	178.7±31.4	263.8±14.4	123.1±10.63	160.3±15.2
TACE(5)	33.1±9.4	46.8±13.9	136.1±25.4[a]	159.3±32.1[a]	86.7±23.1[a]	116.2±19.3[a]
TAE+RFA(5)	33.2±9.4	46.8±13.9	84.2±28.1[a/b]	125.1±27.5[a/b]	57.3±22.2[a/b]	81.2±25.5[a]
RFA(5)	36.2±10.6	49.3±10.5	49.3±12.5[a/b/c]	60.2±18.8[a/b/c]	56.7±18.8[a/b/d]	66.4±22.2[a/b/d]

ALT and AST levels in the 4 groups were not significantly different 1 day before the operation ($P > 0.05$). The TACE+RFA group showed the highest ALT and AST levels on days 3 and 7 ($^aP < 0.05$). TACE group had statistically significant compared with TAE+RFA and RFA groups on days 3 and 7 ($^bP < 0.05$) in ALT and AST levels. On day 3, the TAE+RFA group showed a marked increase in ALT and AST levels RFA group ($^cP < 0.05$), but no significantly different on day 7 ($^dP > 0.05$).

Table 3. Serum TNF-α level on days 1, 3 and 7 in different groups.

Group(n)	Day 1 postoperative	Day 3 postoperative	Day 7 postoperative
TACE+RFA(5)	1239.3±238.3	829.3±176.3[a]	539.3±133.2[b]
TACE(5)	867.8±151.2[c]	567.8±111.8[a/c]	367.8±132.4[b/c]
TAE+RFA(5)	755.3±257.9[c]	555.3±97.9[c]	355.3±257.9[c]
RFA(5)	689.5±176.2[c]	439.5±92.3[c]	323.5±57.3[c]

The peaked TNF-α level on day 1 were gradually dropped on days 3 and 7 in the four groups. In the TACE+RFA,TACE groups, the levels of TNF-α levels on day 1 was compared with days 3 ($^aP < 0.05$) and 7 ($^bP < 0.01$). TACE+RFA group had statistically significant compared with others groups ($^cP < 0.05$) on day 1, 3 and 7, respectively.

Table 4. The frequency of apoptotic hepatocytes surrounding zone of necrosis or coagulation was measured in 4 groups on different times.

Group(n=5)	Day 1 postoperative	Day 3 postoperative	Day 7 postoperative
TACE+RFA(5)	25.7±2.1	16.5±3.4	11.3±3.9
TACE(5)	17.2±1.3[a]	12.1 ±1.6[a]	5.8±2.2[a]
TAE+RFA(5)	10.3±3.9[a/b]	9.8± 2.4[a/b]	3.3±1.9[a]
RFA(5)	7.5±3.2[a/b]	6.9± 2.5[a/b]	1.5±0.6[a]

The percentage of hepatocyte apoptosis in the TACE+RFA group were the highest compared with the other groups on days 1, 3 and 7, respectively ($^aP < 0.05$). The percentage of apoptotic cells in TACE group was more than

TAE+RFA on day 1 and RFA group on day 1 and 3, respectively ($^{b}P < 0.05$).

Table 5. The percentage of Ki-67-positive hepatocytes surrounding the zone of necrosis or coagulation on days 1, 3 and 7 in different groups.

Group (n)	Day 1 postoperative	Day 3 postoperative	Day 7 postoperative
TAE+RFA (5)	15.3±3.9	24.8± 2.4	12.3±1.9
RFA (5)	9.5±3.2[a]	14.9± 2.5[a]	8.5±2.6[a]
TACE+RFA (5)	6.7±2.1[a/b]	11.5±3.4[a/b]	8.3±3.5[a]
TACE (5)	5.2±1.3[a/b]	8.1 ±1.6[a/b]	6.8±2.2[a]

The percentage of Ki-67-positive hepatocytes in liver tissue were increased on day 1, peaked on day 3 and dropped on day 7 in the four groups.

The frequency of proliferation cells in TAE+RFA group was significantly different from others on days 1, 3 and 7($^{a}P < 0.05$).

The frequency of proliferation cells in RFA group was significantly different from TACE+RFA and TACE groups on days 1 and 3 ($^{b}P < 0.05$).

Figures

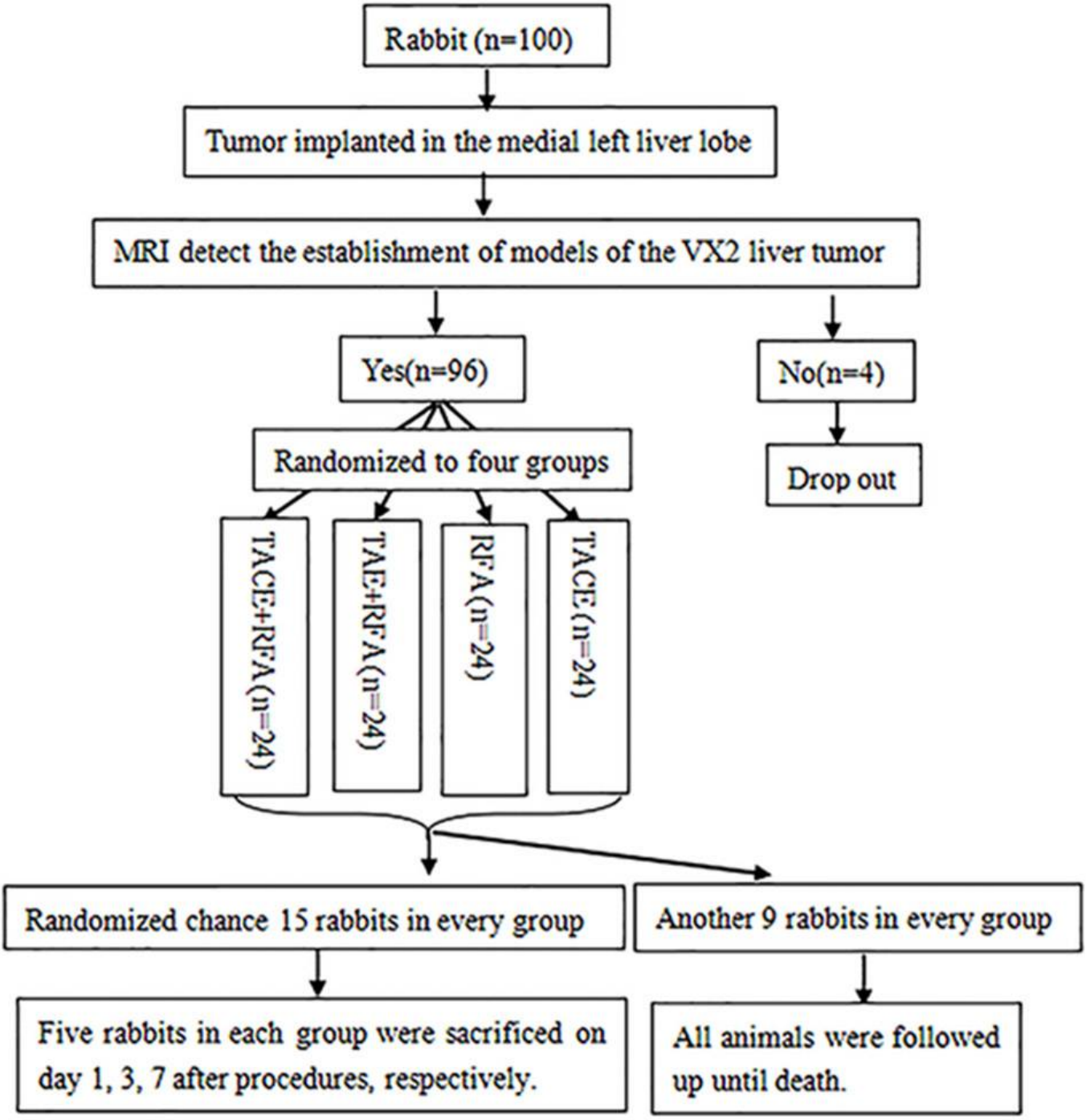

Figure 1

Flowcharts of treatment course and follow-up in the 4 groups.

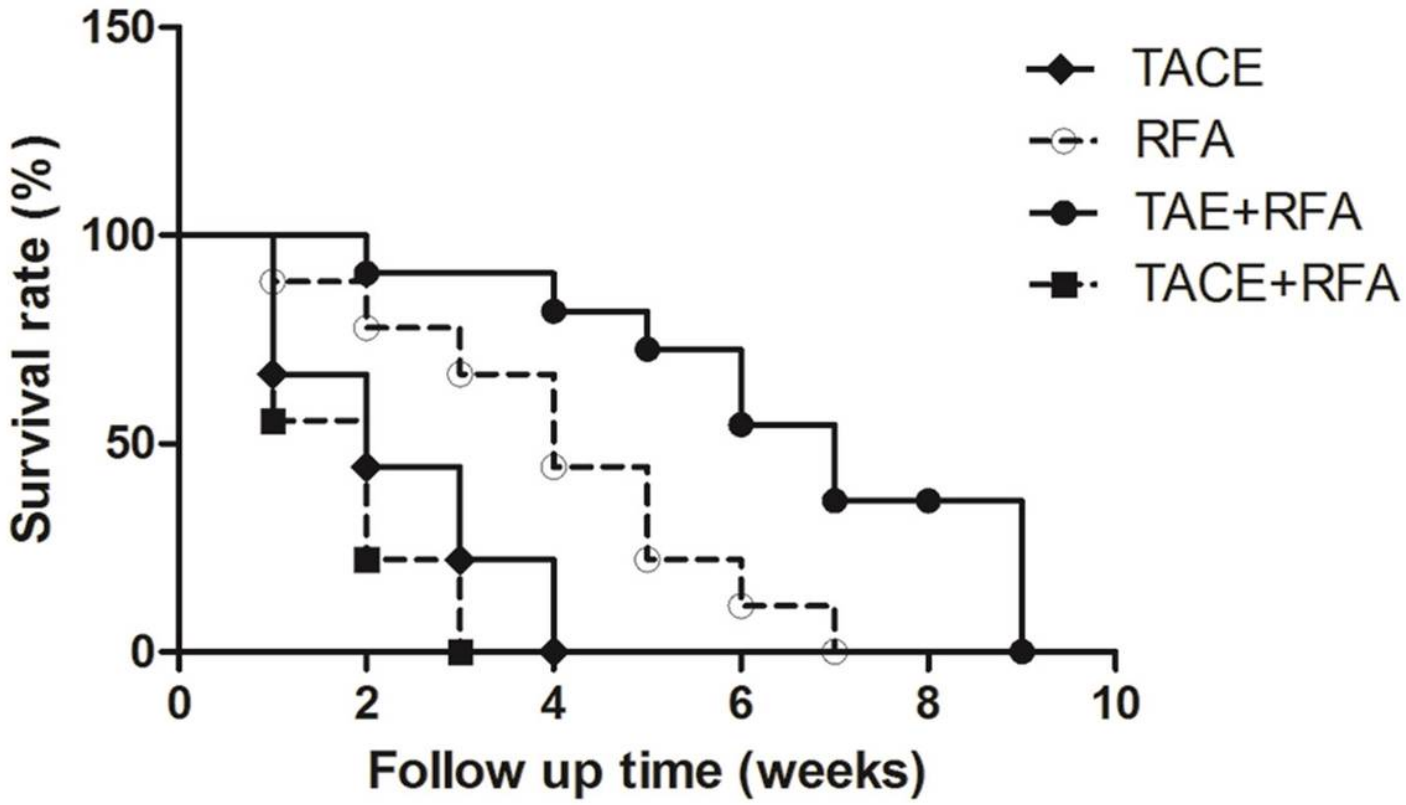

Figure 2

Kaplan-Meier analysis of animal survival in subgroup B following the treatment with TACE, RFA, TACE+RFA and TAE+RFA. The mean survival in RFA group (35.9 days ± 11.2) was higher than that in TACE group (26.5 days ± 7.9; $P < 0.05$) and TACE+RFA group (16.9 days ± 4.8; $P < 0.05$). The highest mean survival was observed in TAE+RFA group (50.2 days ± 16.5; $P < 0.05$).

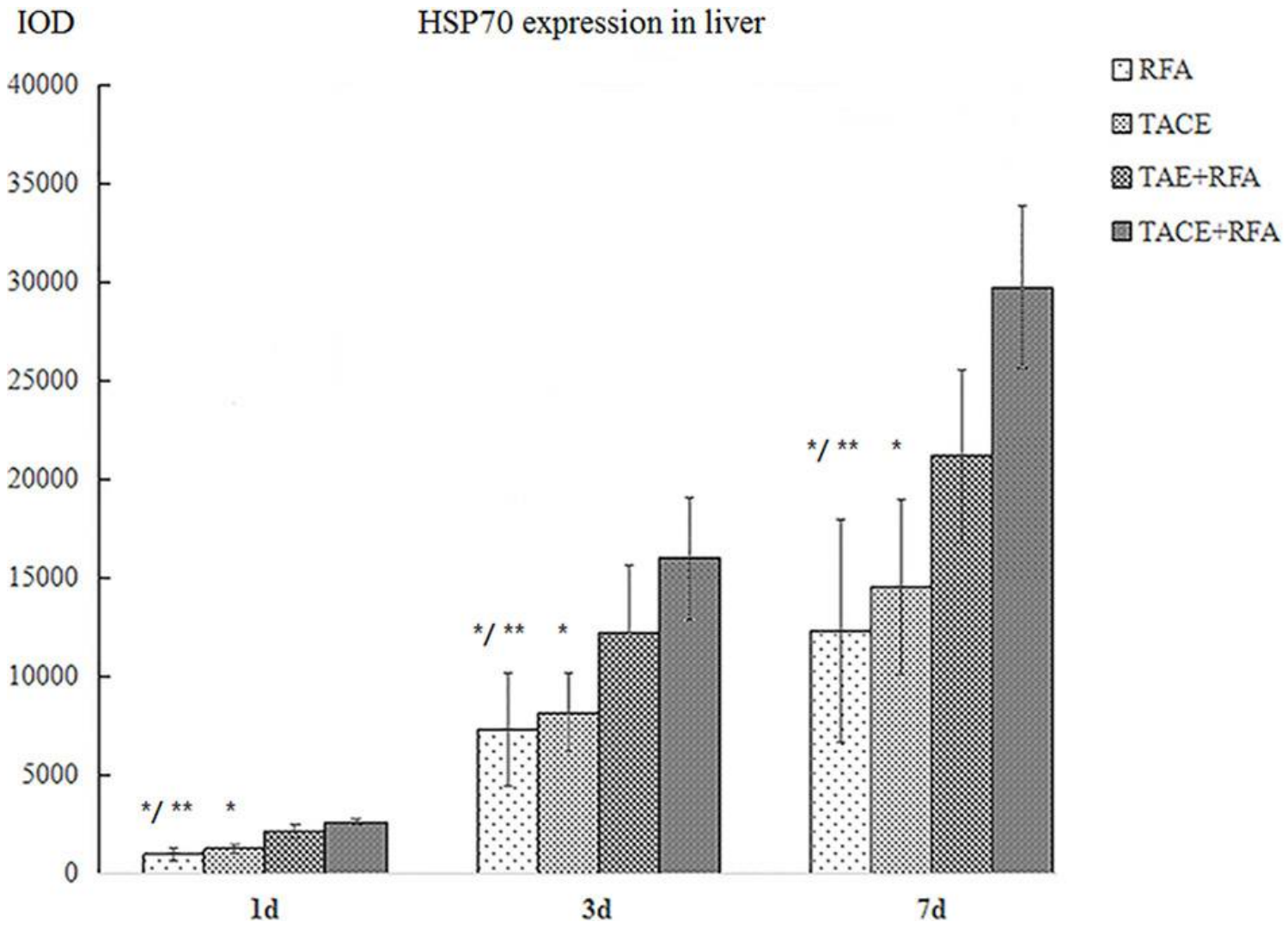

Figure 3

IOD of HSP70 expression in the transitional zone of ablation in liver on days 1, 3 and 7 postoperative. IOD of HSP70 expression in TACE+RFA group was significantly different from others on days 1, 3 and 7, respectively. Its day 7 expression was significantly different from its days 1 and 3 ($P < 0.05$). **: IOD of HSP70 expression in TACE group was significantly different from RFA group on days 1 and 3 ($P < 0.05$). *: IOD of HSP70 expression in TAE+RFA group was significantly different from TACE and RFA groups on days 1 and 3 ($P < 0.05$), respectively.

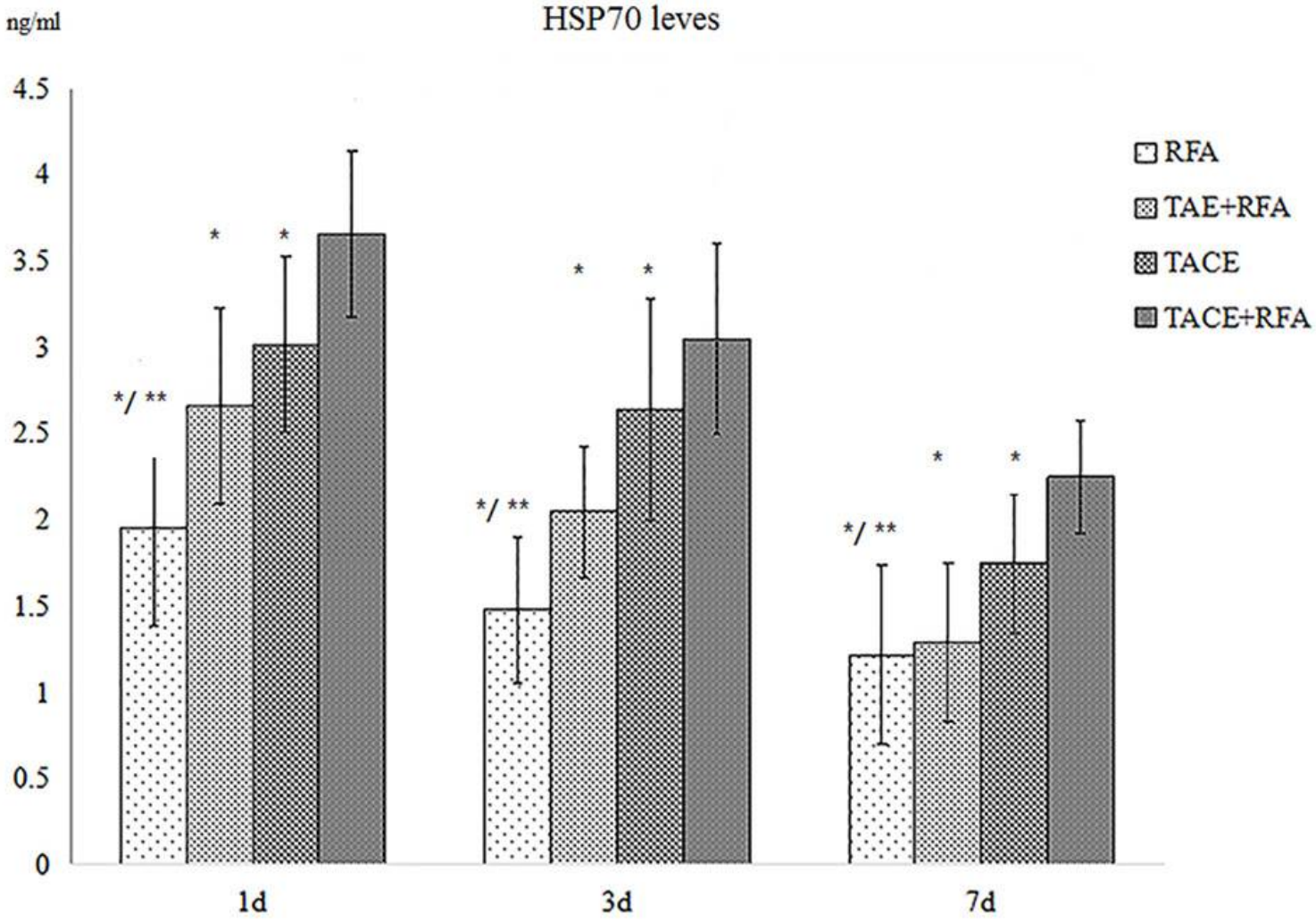

Figure 4

The levels of serum HSP70 (ng/ml) on day 1, 3 and 7 in different groups. The levels of serum HSP70 in the TACE, TACE+RFA and TAE+RFA groups on day 1 were significantly different from theirs day 3 and day 7 ($P < 0.05$). *: The levels of serum HSP70 in TACE+RFA group were significantly different from other groups ($P < 0.05$) on days 1, 3 and 7, respectively. **: The differences of serum HSP70 on days 1, 3 and 7 between the TACE and RFA were significant ($P < 0.05$).

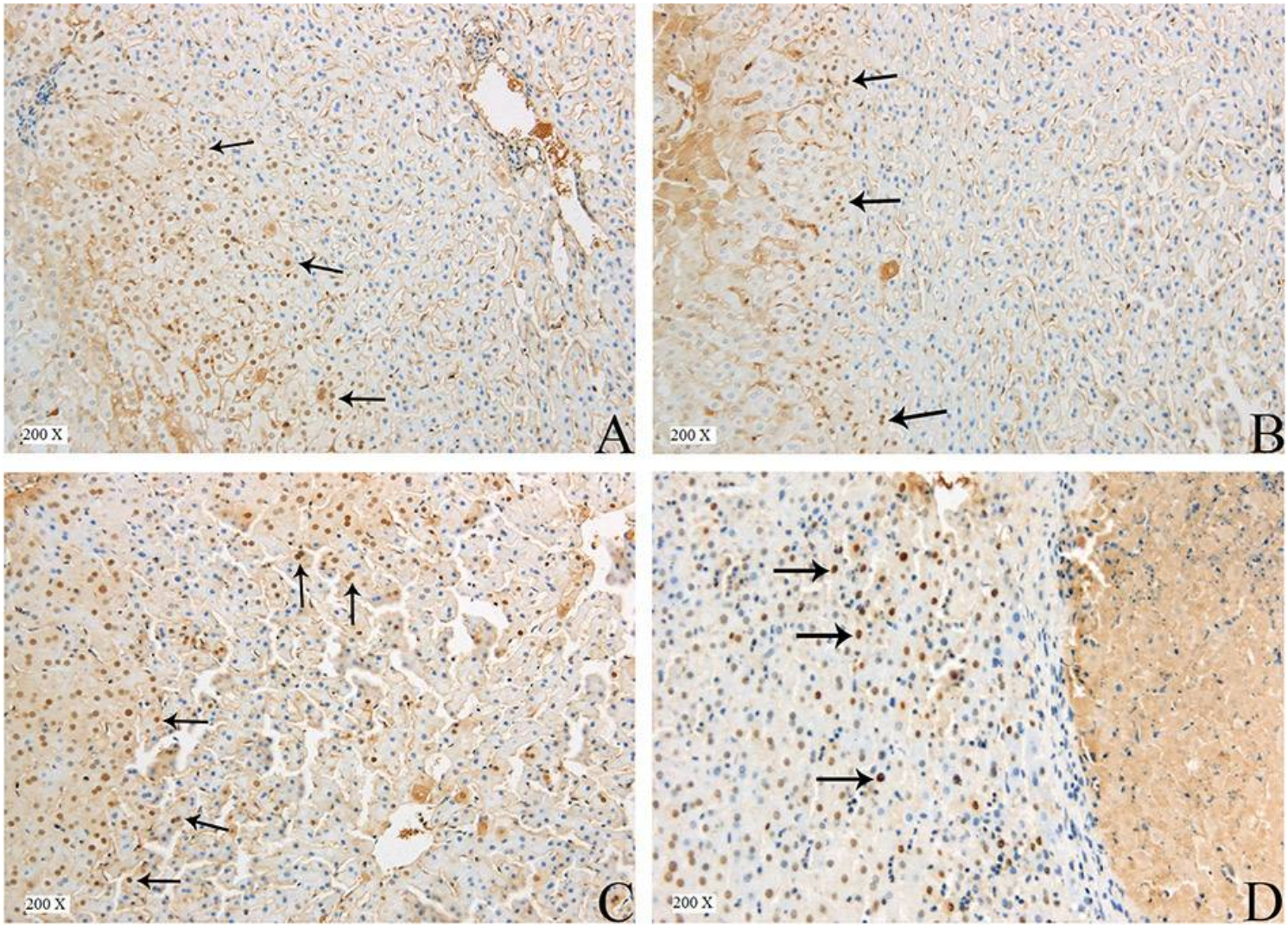

Figure 5

A representative TUNEL staining image (arrows, on day 1 post-procedure) of surrounding the zone of necrosis or coagulation in liver: TACE (A), RFA (B), TACE+RFA (C) and TAE+RFA (D) groups (200 × magnification).

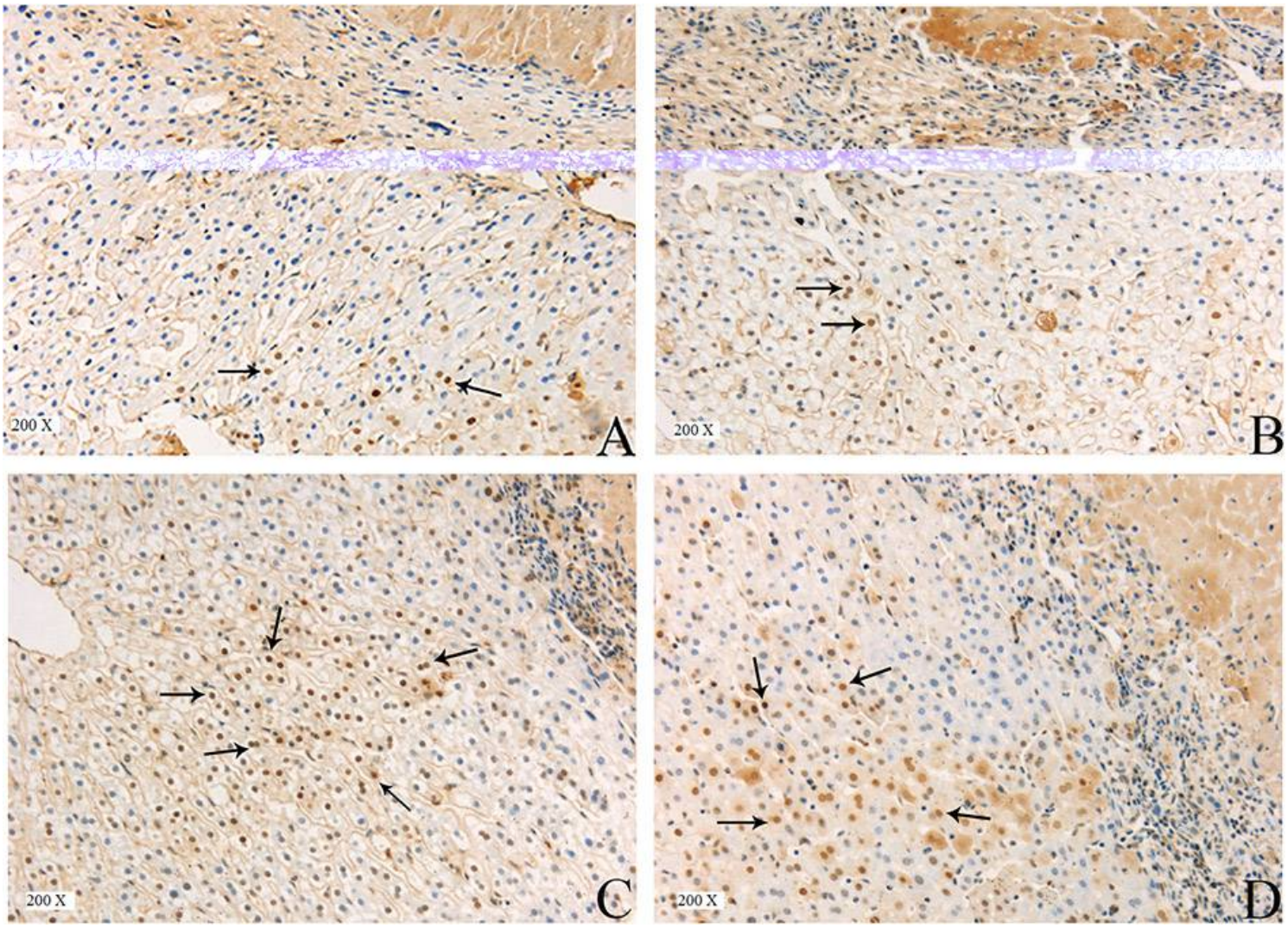

Figure 6

A representative anti-Ki-67 staining image (arrows, on day 3 post-procedure) of surrounding the zone of necrosis or coagulation in liver: TACE (A), RFA (B), TACE+RFA (C) and TAE+RFA (D) groups (200 × magnification).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVECHECKLIST.pdf