

Therapeutic effects of transarterial infusion of lipiodol and ethanol in various ratios in a rabbit VX2 tumor model

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PURPOSE

We aimed to evaluate the therapeutic effect and safety of transcatheter arterial embolization with various volume ratios of lipiodol and ethanol in a rabbit VX2 tumor model to identify the optimal volume ratio.

METHODS

Eighteen adult male New Zealand white rabbits implanted with VX2 tumors in their left liver lobes were randomly divided into six groups based on volume ratios of lipiodol to ethanol: group A, 3:1; group B, 2:1; group C, 1:1; group D, 1:2; group E, 1:3; and group F, 1:4. Pre- and post-treatment unenhanced magnetic resonance imaging was used to detect tumor formation and evaluate tumor growth rates. Liver samples were harvested one week after the procedure, and apoptosis index of tumor tissues was evaluated by pathologic examination and TUNEL assay.

RESULTS

Tumor size decreased in groups B, C, and D, but increased in groups A, E, and F. Tumor growth rates in groups A–F were 0.40 ± 0.03 , -0.11 ± 0.21 , -0.08 ± 0.09 , -0.12 ± 0.07 , 0.06 ± 0.12 , and 0.05 ± 0.09 , respectively. The change in tumor size was significantly different in group A compared with the rest of the groups, but no significant difference was observed among groups B–F. Apoptosis indexes of the six groups were $4.7\pm 2.1\%$, $6.7\pm 2.1\%$, $11.7\pm 3.1\%$, $11.0\pm 2.0\%$, $10.7\pm 3.2\%$, and $12\pm 3\%$, respectively. Apoptosis index was significantly lower in group A compared with groups C–F ($P < 0.05$). Apoptosis index of group B was significantly lower than groups C and F. There was no significant difference among the other groups.

CONCLUSION

The volume ratios of lipiodol to ethanol ranging from 2:1 to 1:4 were equally effective, the ratios 2:1 and 1:3 had equal safety, and the ratios 1:1 and 1:2 indicated better long-term therapeutic effect. Increasing ethanol in the mixture caused more severe liver injury. Optimal efficacy and safety was achieved with a lipiodol to ethanol volume ratio of 1:1.

Hepatocellular carcinoma (HCC) is one of the most common solid malignancies in the world (1). Transarterial chemoembolization (TACE) is widely used to treat HCC patients who are not suitable candidates for curative treatments (2–5). The most common embolic agent used in TACE is lipiodol, which can be mixed with surgical glues (cyanoacrylates) or with ethanol for interventional procedures. Ethanol was confirmed to be effective in occluding the hepatic arterial system, but it can cause perisinusoidal fibrosis (6). Transarterial ethanol ablation (TEA) with a mixture of lipiodol-ethanol has been shown to be an effective treatment for HCC (7). Yu et al. (8) reported that the embolization efficacy and treatment effectiveness of TEA were probably superior to those of TACE for HCC, and a decreased proportion of ethanol (33% by volume) in the mixture was suggested. Lipiodol-ethanol mixtures with reduced ethanol proportions have been shown to be associated with decreased endothelial damage while maintaining effective delivery of the mixtures to tumor vasculature (9). However, the optimal ratio between lipiodol and ethanol that should be used for TEA remains controversial.

In the present study, we aimed to determine the efficacy and optimum volume ratio of lipiodol-ethanol mixture in a rabbit VX2 hepatoma model.

Methods

Animal models

Animal studies were performed according to the guidelines for the use of laboratory animals of the Ministry of Public Health of our country, and were approved by the Laboratory Animal Research Center. Eighteen adult male New Zealand white rabbits weighing 2.0–3.0 kg were used in this study. Rabbits were housed in separate cages, and the room temperature was maintained at 20°–23°C with 12 h light-dark cycles. Rabbits bearing VX2 tumor (derived from rabbit papilloma virus-induced squamous cell carcinoma) were provided by Tenth People's Hospital. Tumor implantation was performed as described in Lee et al. (10). In brief, rabbits were anesthetized by intramuscular administration of a mixture of ketamine hydrochloride 0.1–0.2 mL/kg, 2 mL (0.1 g) (Gutian Pharmaceutical) and Lumianning 0.1–0.2 mL/kg, 1.5 mL (dimethylamine thiazole and dihydroetorphine hydrochloride; Animal Health Products); and the skin was shaved and sterilized. The rabbits were fixed on a computed tomography (CT) examination bed in the supine position. The position of the left lobe of the liver was determined by scanning, and a puncture point was marked. Needle depth was measured by CT. VX2 tumor tissue was cut into fragments of 1–2 mm³. Suspension of tumor fragments in a total volume of 0.2 mL was injected by an 18G

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needle inserted into the left lobe of the liver at the marker point. Contrast-enhanced CT was performed two weeks after tumor implantation and before the TEA procedure to calculate the area of necrosis. Therapy was started two weeks after implantation, when the tumors reached 2–3 cm in diameter.

Animal grouping

Eighteen rabbits implanted with VX2 tumors were randomly divided into six groups having three rabbits per group. Volume ratio of lipiodol to ethanol was 3:1 in group A, 2:1 in group B, 1:1 in group C, 1:2 in group D, 1:3 in group E, and 1:4 in group F. The total volume was 1 mL in each group.

Imaging protocols

Two weeks after tumor implantation and before the procedure, magnetic resonance imaging (MRI) was performed using a 1.5 Tesla unit (Signa MR 1.5 T Echospeed Plus, GE Healthcare) to detect the diameter of the tumors. Conventional T1-weighted fast spoiled gradient recalled and T2-weighted fast recovery fast spin echo (TE 56 ms, TR 5000 ms, plies 20, slice thickness 3.0 mm, gap 0.5 mm, field of view 20 cm, matrix size 128×128, NEX 8.00) sequences were used. Diffusion weighted imaging (DWI) was performed using a non-breath-hold single-shot spin-echo echo-planar imaging sequence. The scanning parameters included b values of 100, 600, and 1000 s/mm². ADC maps were reconstructed and ADC values were determined using Function 2 software on a GE workstation. The ADC values were obtained from three slices containing the largest portion of the tumor. A circular region of interest (ROI) with an area of 15 mm² was drawn in the tumor periphery. Contrast-enhanced CT (16 Light Speed, GE Healthcare) was performed two weeks after tumor implantation and before the procedure to evaluate the tumor blood supply. Contrast medium (iodine alcohol, 2 mL/kg) was administered via ear vein at a rate of 1 mL/s. MRI was repeated one week after the TEA procedure to evaluate the therapeutic effects (11).

Transarterial ethanol ablation

TEA was performed using a Siemens C-arm unit (AXIOM ARTIS, Siemens). General anesthesia was induced with the mixture of ketamine hydrochloride and Lumianning administered intramuscularly. The right femoral artery was exposed. A 20G puncture needle was then inserted into the artery followed by a 3 F vascular sheath (Terumo Corporation) (12). A microcatheter (Echelon TM-14 microcatheter, Ev3 Inc.) was inserted into the tumor feeding artery under fluoroscopic guidance. A contrast medium (Iopamiron 300) was used to confirm the location and morphology of the VX2 liver cancer. Approximately 1 mL of the lipiodol-ethanol mixture was infused into the tumor feeding artery with an endpoint of complete antegrade stasis of blood flow. The microcatheter was then removed, and the femoral artery was ligated. Unenhanced CT scans were obtained to evaluate the distribution of the mixture (13).

Assessment of therapeutic effects and histopathology

MRI was repeated one week after treatment. Pre- and post-treatment tumor volumes ($ab^2/2$) were compared to evaluate the effectiveness of treatment. Tumor growth rate was calculated as $(V_{\text{post-TEA}} - V_{\text{pre-TEA}} / V_{\text{pre-TEA}}) \times 100\%$. Rabbits were euthanized; and liver tumors samples were obtained, fixed in 10% buffered formaldehyde, and paraffin-embedded. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was used to measure hepatocyte apoptosis in liver tissues (13). Five areas, where apoptosis was highest, were selected under light microscopy (40×), and apoptotic cells in these areas were counted at high magnification (400×). The apoptosis index was calculated for each specimen.

Five fields of tissue from each sample were observed to evaluate the severity of liver injury adjacent to the tumor at a magnification of 100×, according to the modified Suzuki criteria (14). Sinusoidal congestion, hepatocyte necrosis, and balloon degeneration were graded on a scale of 0–4, where the absence of necrosis, congestion, or ballooning

is scored as 0 (no liver injury), and the presence of severe congestion/ballooning and lobular necrosis of 60% or greater is scored as 4 (severe injury).

Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc.). All data were reported as mean±standard deviation. The tumor response in each group was compared by one-way analysis of variance (ANOVA) and least significant difference (LSD). Comparison of apoptosis index and liver injury among the six groups was performed using one-way ANOVA and LSD. Spearman correlation analysis was performed to evaluate the correlation between the volume ratio of lipiodol to ethanol and median Suzuki scores. A $P < 0.05$ was considered to be statistically significant.

Results

All rabbits had VX2 tumors in the left liver lobe as detected by MRI and contrast-enhanced CT (Fig. 1). No cholestasis was found. Two rabbits with subcutaneous growth tumors and one rabbit with multiple lesions were excluded. There was no significant difference in mean pretreatment tumor volumes among the six groups ($F=0.72$, $P=0.62$, Table 1). TEA was performed successfully in all animals, and no animals died during the experimental period.

Pretreatment contrast-enhanced CT showed that 14 tumors were supplied by the left hepatic artery, three by the left gastric artery, and one by the right hepatic artery; all tumors displayed rim-enhancement (Fig. 1). DWI showed tumor-restricted diffusion (high signal), while post-treatment T2-weighted MRI showed slightly higher signal of liver tissue infarction adjacent to the tumor (Fig. 1). The pretreatment mean ADC value showed no significant difference among the six groups, while the post-treatment mean ADC value showed significant differences between group A and the other five groups (Table 2); no significant differences were observed among groups B–F ($P > 0.05$).

Tumor growth rate was determined seven days after TEA in each group (Table 1). Tumor volume decreased in groups B, C, and D, but increased in

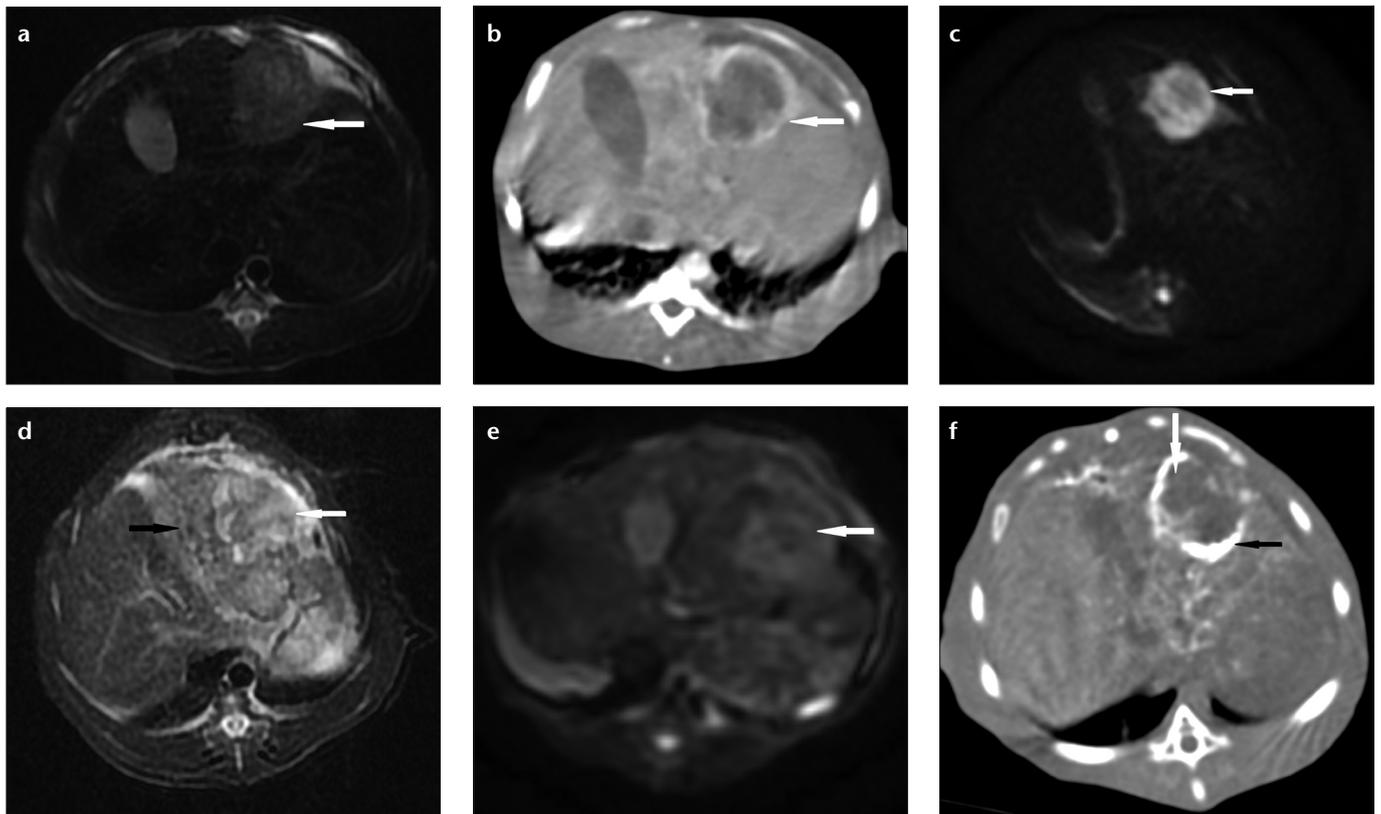


Figure 1. a–f. T2-weighted image before transarterial ethanol ablation (a) shows an oval shape of slightly higher signal intensity in the left lobe of the liver which represents the VX2 tumor (arrow). Contrast-enhanced CT scan (b) shows a tumor with rim-enhancement (arrow). The central portion of tumor is heterogeneously enhanced. Diffusion-weighted imaging (DWI) ($b=600$ s/mm²) scan (c) shows tumor-restricted diffusion (arrow). T2-weighted image one week after procedure (d) shows heterogeneously high signal intensity in the tumor (white arrow) and slightly high signal intensity in the normal liver tissue adjacent to the tumor (black arrow). DWI ($b=600$ s/mm²) scan one week after the procedure (e) shows restricted diffusion at tumor margins (arrow). Unenhanced CT one week after procedure (f) shows iodized oil accumulation in the tumor margin and scattered oil in the adjacent liver tissue (black arrow). The central portion of tumor has a low density without iodized oil accumulation (white arrow).

Table 1. Tumor volume and growth rates

Group	Lipiodol:Ethanol	V _{pre-TEA} (mm ³)	V _{post-TEA} (mm ³)	Growth rate
A	3:1	7679.6±1207.4	10714.7±1691.1	0.40±0.03
B	2:1	5673.5±1022.0	4950.2±924.5	-0.11±0.21
C	1:1	4617.7±2326.0	4290.5±2199.6	-0.08±0.09
D	1:2	5152.5±2961.3	4387.7±2233.3	-0.12±0.07
E	1:3	5718.0±3381.8	5813.8±2797.7	0.06±0.12
F	1:4	4527.0±2289.1	4648.3±1994.7	0.05±0.09
F value		0.72	4.32	8.61
P		0.62	0.018	0.001

Comparison of tumor growth rates by the least significant difference test: Groups A vs. B, A vs. C, A vs. D, $P < 0.001$; Groups A vs. E and A vs. F, $P = 0.004$. TEA, transarterial ethanol ablation.

groups A, E, and F. Growth rate was significantly different in group A compared with the other five groups (Table 1), but no significant differences were observed among groups B–F.

Hematoxylin-eosin (H-E) staining showed various degrees of hepatic

necrosis and sinusoidal congestion of liver tissues adjacent to the tumor in each group (Fig. 2). Median Suzuki scores were significantly lower in groups A and B compared with the other groups (Table 3). A significant difference was observed between group

A and the other groups, except group B ($P < 0.05$). Also, there were significant differences between groups B and E, and between groups B and F. There were no significant differences among the other groups. There was a positive correlation between groups and median Suzuki scores as shown in Fig. 3 ($R^2=0.954$, $F=82.376$, $P = 0.001$).

Apoptosis of tumor cells was evaluated by TUNEL staining (Fig. 2). The average apoptosis indexes are shown in Table 4. There were significant differences between group A and the other groups, but not between groups A and B ($P < 0.05$). Significant difference was also found between groups B and C, and between groups B and F. No significant difference was found among the other groups.

Discussion

This study evaluated the therapeutic effect and safety of transcatheter arterial embolization with various vol-

Table 2. Tumor ADC value

Group	Pre-TEA ADC	Post-TEA ADC
A	1.18±0.11	1.81±0.18
B	1.18±0.03	2.23±0.13
C	1.18±0.10	2.30±0.13
D	1.21±0.12	2.24±0.09
E	1.19±0.14	2.15±0.08
F	1.20±0.08	2.14±0.19
F value	0.04	4.78
P	1.00	0.01

Comparison of post-TEA tumor ADC values by the least significant difference test: Group A vs. B and A vs. D, $P = 0.003$; Group A vs. C, $P = 0.001$; Group A vs. E, $P = 0.011$; Group A vs. F, $P = 0.013$. TEA, transarterial ethanol ablation; ADC, apparent diffusion coefficient.

Table 3. Liver injury adjacent to the tumor

Group	Suzuki score
A	2.33±0.58
B	2.67±0.58
C	3.33±0.58
D	3.33±0.58
E	3.67±0.58
F	4.00±0.0
F value	4.16
P	0.02

Comparison of liver injury adjacent to the tumor by the least significant difference test: Group A vs. B, $P = 0.45$; Group A vs. C, $P = 0.04$; Group A vs. D, $P = 0.04$; Group A vs. E, $P = 0.01$; Group A vs. F, $P = 0.002$; Group B vs. C and B vs. D, $P = 0.15$; Group B vs. E, $P = 0.04$; Group B vs. F, $P = 0.01$.

Table 4. Apoptosis index

Group	Tumor tissue apoptosis index (%)
A	4.7±2.1
B	6.7±2.1
C	11.7±3.1
D	10.7±3.2
E	10.7±3.2
F	12 ±3
F value	4.00
P	0.02

Comparison of tumor tissue apoptosis by the least significant difference test: Group A vs. B, $P = 0.37$; Group A vs. C, $P = 0.007$; Group A vs. D, $P = 0.01$; Group A vs. E, $P = 0.02$; Group A vs. F, $P = 0.005$; Group B vs. C, $P = 0.04$; Group B vs. D, $P = 0.06$; Group B vs. E, $P = 0.09$; Group B vs. F, $P = 0.03$.

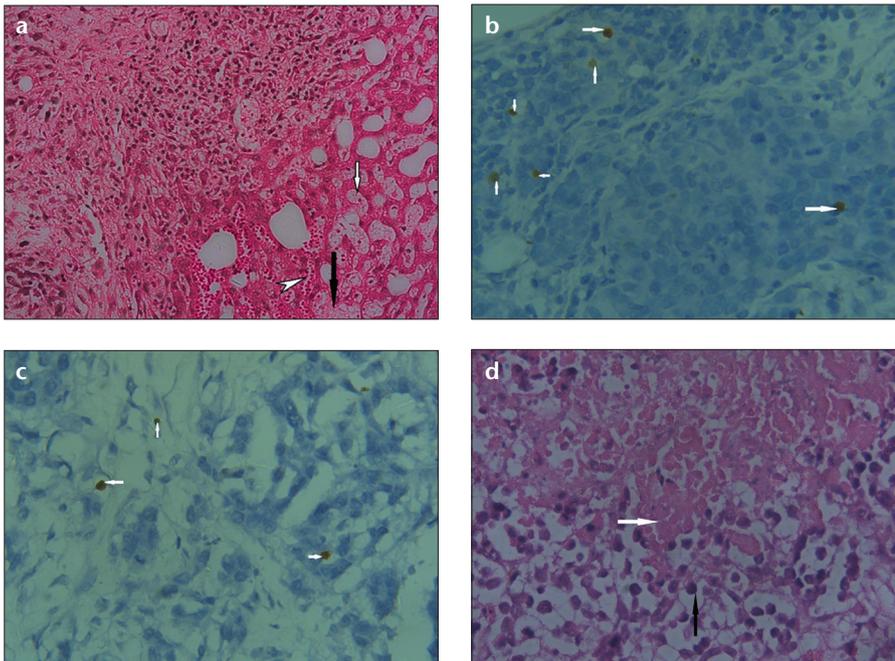


Figure 2. a–d. Hematoxylin and eosin (H-E) staining (a) shows hepatic necrosis (white arrow), ballooning degeneration (black arrow), and sinusoidal congestion (arrowhead) of liver tissues adjacent to the tumor (H-E; original magnification, 200×). TUNEL assay (b, c) shows apoptosis of tumor cells on day 7 (arrows) (original magnification, 400×). H-E staining (d) shows necrosis of tumor cells (white arrow) and residual tumor cells (black arrow) (H-E; original magnification, 400×).

ume ratios of lipiodol and ethanol in a rabbit VX2 tumor model. Our results demonstrated that the volume ratios of lipiodol to ethanol from 2:1 to 1:4 were equally effective, and that increasing ethanol in the mixture caused more severe liver injury.

In this study, we chose conventional MRI and DWI to evaluate the therapeutic effectiveness in the study, so

post-treatment CT was not performed. In contrast to CT, MRI has the advantage of excluding the impact of iodized oil. Conventional MRI can provide tumor morphological information, while DWI can clearly distinguish viable from necrotic tumors (15, 16). In the present study, post-treatment mean ADC value showed significant differences between group A and the other five groups, but

no significant differences were observed among groups B–F. Similarly, tumor growth rate was significantly different in group A compared with groups B–F, suggesting that groups B–F had equal therapeutic effect.

Kan et al. (9) performed a study on lipiodol-ethanol mixtures in ratios of 5:1, 4:1, 3:1, 1:1, and 1:0 injected into the rat hepatic artery to create dual, complete arterial, and portal venous embolization. Lobar ablation effects were achieved in the 5:1, 4:1, and 3:1 ratio groups. Cheung et al. (17) reported that TEA with a lipiodol-ethanol mixture ratio of 1:3 was an economical, safe, and feasible method for treating HCC. Gu et al. (18) suggested that TEA with a lipiodol-ethanol mixture in a volume ratio of 1:1 was an effective therapy for patients with HCC, and might be more effective than TACE for treating refractory disease. In the present study, the volume ratios of lipiodol-ethanol mixture ranging from 2:1 to 1:4 were found to be equally effective. Adding ethanol to lipiodol can enhance embolic effects by damaging the vascular endothelium, retaining embolic material in the liver, and preventing resumption of arterial flow. Pure iodized oil has little effect because iodized oil passes through the sinusoid (19). The decreased proportion of ethanol in group A (3:1) likely resulted in a reduction of this effect.

In the current study, apoptosis was evaluated one week after the TEA pro-

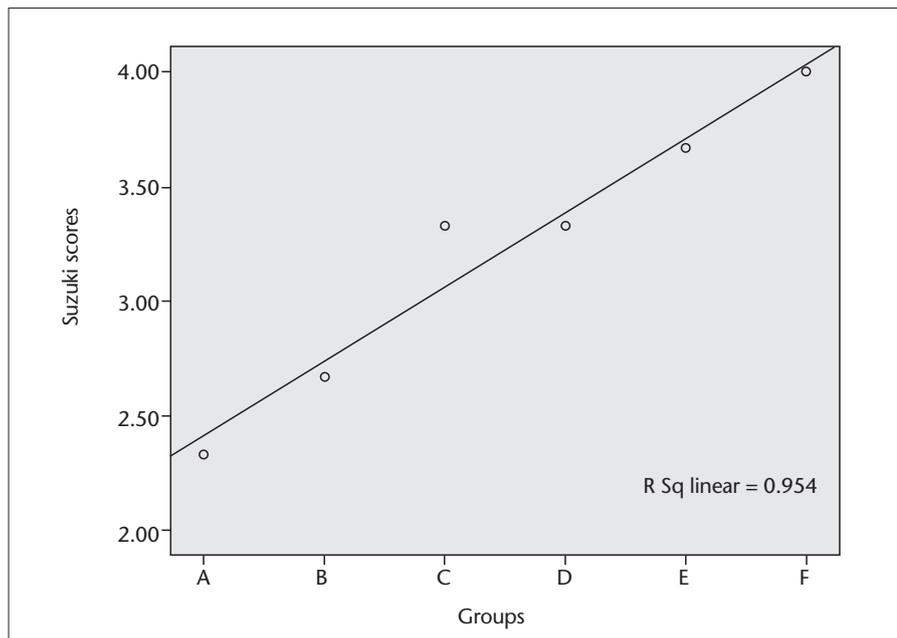


Figure 3. Comparison of the median Suzuki scores ($R^2=0.954$, $F=82.376$, $P=0.001$). Lipiodol-ethanol volume ratios were as follows: group A, 3:1; group B, 2:1; group C, 1:1; group D, 1:2; group E, 1:3; group F, 1:4.

cedure. Some studies have reported that embolization induces apoptosis (20, 21), which can be observed from 24 hours to 21 days after the procedure (22). Ethanol has been shown to cause liver tissue apoptosis (23) and liver fibrosis. In the current study, the apoptotic index of tumor tissue increased when the volume ratio of ethanol was increased. However, there was no significant difference in apoptosis index among the groups with lipiodol to ethanol ratios of less than 2:1.

In terms of liver injury adjacent to the tumor, we found that in group A (3:1) and group B (2:1), median Suzuki scores were lower than the other groups. The volume ratio of lipiodol to ethanol and median Suzuki scores were positively correlated. Increasing ethanol in the mixture caused more severe liver injury. Increasing ethanol in the mixture has been shown to cause vascular spasm, and to limit delivery of agents (9). Ethanol is a strong protein precipitant producing permanent embolic material. However, it can cause severe injury to normal liver tissue and vessels (24). In addition, increased ethanol volume ratios are difficult to monitor, and can result in reflux. Groups C, D, E, and F displayed higher apoptosis index in tumor tissues and increased thera-

peutic effects, suggesting better long-term outcomes. However, increased ethanol volume ratios also produced more severe liver injury, as observed in those four groups.

There are several limitations in this study. First, to ensure VX2 tumor diameters of 2–3 cm, animals with smaller tumors were excluded, resulting in only three cases for each group. Nevertheless, the current results did show significant differences between the groups. The lack of cirrhosis in this model is another limitation of this study. However, the VX2 tumors have a rich blood supply, and in this respect they were similar to human HCC. Finally, the rabbits were sacrificed to obtain the liver specimens one week after treatment. Therefore, survival data are not available, and these types of survival experiments are planned for the future.

In conclusion, the volume ratios of lipiodol to ethanol from 2:1 to 1:4 were found to be equally effective. A ratio of 3:1 demonstrated better safety results but poor therapeutic effect, and the ratios between 2:1 and 1:3 had equal safety and therapeutic effect, while the ratios 1:1 and 1:2 indicated better long-term therapeutic effect as shown by higher apoptosis indexes.

As increasing ethanol in the mixture caused more severe liver injury, optimal efficacy and safety was achieved with a lipiodol and ethanol volume ratio of 1:1.

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Conflict of interest disclosure

The authors declared no conflicts of interest.

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