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UNSPECIFIED

ORIGINAL ARTICLE

Ultrasound-guided percutaneous implantation of rabbit VX2 carcinoma, using a coaxial technique and gelfoam pellet injection combination to establish a rabbit liver tumor model

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PURPOSE
We aimed to investigate the safety and tumor seeding rate of a coaxial implantation technique combined with injection of a gelfoam pellet in establishing a VX2 liver tumor model in rabbits.

METHODS
A VX2 liver tumor model was established in 60 male New Zealand white rabbits, which were randomly divided into 3 groups (20 in each group) based on implantation technique (all performed under ultrasound guidance): group A, single needle only; group B, single needle with injection of a gelfoam pellet; or group C, coaxial technique with injection of a gelfoam pellet. The rates of liver tumor formation and tumor seeding to extrahepatic tissues were compared 2 weeks after implantation. Data were also collected regarding procedure time, number of punctures, occurrence of complications, and mortality rate.

RESULTS
A VX2 liver tumor model was established in all 60 rabbits (100%, 60/60). Ectopic implantation rate was 70% (14/20) in group A, 35% (7/20) in group B, and 5% (1/20) in group C, with significant difference among the groups ($P < .001$). Post hoc analysis showed significant difference between group A and group C ($P < .001$). However, there were no significant differences between group B and group A or group C ($P = .027$, $P = .048$, respectively). There were no significant differences among the groups in terms of procedure time ($P = .405$) or number of punctures ($P = .612$). No complications or deaths occurred.

CONCLUSION
A coaxial technique with injection of a gelfoam pellet is an effective and safe method for VX2 liver tumor implantation in rabbits, and this technique can reduce the risk of tumor seeding to the abdominal wall and omentum.

VX2 carcinoma is an anaplastic squamous cell carcinoma derived from a virus-induced papilloma in rabbits, which was first identified by Shope and Hurst in 1933.[1] A rabbit VX2 liver tumor model is frequently used in research regarding human hepatocellular carcinoma (HCC), especially in studies of diagnostic imaging[2–4] and minimally invasive treatments.[5–7] This VX2 liver tumor model is a useful animal model of HCC in part because of its easy growth on implantation, its hypervascular nature, and the presence of a main blood supply from the hepatic artery.[8] In addition, rabbits are large enough to undergo catheterization, whereas rats and mice are not.[8, 9]

Research has shown that implantation of VX2 liver tumor fragments is more reliable than injection of a cell suspension in terms of establishment of a tumor model, with success rates of 84% to 95% with implantation versus 35% to 47% with injection.[10, 11] An implanted rabbit VX2 liver tumor model can be established through either open surgery or an image-guided minimally invasive approach.[12–14] Open surgery is a reliable technique for establishing the tumor model, with a success rate ranging from 95.5% to 100%,[10, 11, 15, 16] but this method is traumatic, requires prolonged procedure times, and carries the risk of tumor seeding, infection, and bleeding.[13] Image-guided percutaneous implantation has largely replaced open surgery because of its minimally invasive nature and its lower complication rate coupled with its high rate of technical success (92%–100%).[13, 16] However, the risk of extrahepatic tumor seeding with image-guided percutaneous implantation

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remains a problem, with a reported tumor seeding rate as high as 38% .[13, 16] Extrahepatic tumor seeding negatively affects researchers' ability to assess the efficacy of various treatments in VX2 tumor models;[17] therefore, an implantation technique that decreases the risk of extrahepatic tumor seeding is needed.

In this study, we sought to investigate the safety and tumor seeding rate of a coaxial implantation technique combined with the injection of a gelfoam pellet in the establishment of a VX2 tumor model in rabbits, comparing this technique with two other implantation procedures.

Methods

Experimental animals

This study was performed in accordance with protocols approved by the Animal Ethics Committee and in compliance with institutional guidelines (Animal Experiment Ethical Inspection Form of Guizhou Medical University, No. 1900932).

Sixty-one male New Zealand white rabbits were purchased from the animal center of Guizhou Medical University (License: syxk [XXX] 2018-0001); the rabbits were 10 weeks old, with a mean weight of 2±0.5 kg. They were raised in the Animal Laboratory Center of Guizhou Medical University by trained personnel. The rabbits were allowed to eat and drink freely.

Preparation of VX2 tumor implantation tissue

The VX2 tumor cell line (Ginnio Biotech Company) was inoculated into both hind legs of a donor New Zealand rabbit.[14] Two weeks after implantation of the cell line, an ultrasound examination was performed to assess the status of tumor growth in the legs;[18] the tumors were ready to be harvested when they reached approximately 2 cm in diameter. After the tumors were explanted from both hind limbs, the rabbit was euthanized with carbon dioxide.

The tumor specimens were placed into a sterile dish. The necrotic tissues, fascia, and connective tissues were removed from the specimens and the VX2 tumors were cut into small pieces (0.5–1 mm^3) (Figure 1a). The VX2 tumor tissue was then loaded into an 18 G angiocatheter needle sheath (Wenzhou Huali Medical Equipment Co., Ltd.) for tumor inoculation (Figure 1b).

Establishment of rabbit VX2 liver tumor model

Before the inoculation procedure was performed, the rabbits were fasted for 8 hours (with the exception of water). After general anesthesia was administered, the rabbits were placed in the supine position with their limbs fixed to the corners of the operating table. The subxiphoid area was shaved and the overlying skin was prepared and draped using sterile technique. All procedures were performed percutaneously under ultrasound guidance (IU-22, Philips Medical Systems), and tumor tissues were all implanted into the left lateral lobe of the liver.[19]

The 60 rabbits were randomly divided into 3 groups, with 20 rabbits in each group. In group A, an 18 G angiocatheter needle (Wenzhou Huali Medical Equipment Co., Ltd) was used for direct puncture; the introducer needle (stylus) of the angiocatheter was removed once it was confirmed that the correct target had been reached. Next, the tip of the 18 G angiocatheter sheath (prefilled with tumor cells) was inserted into the hub of the puncturing needle sheath (18 G) (Figure 2a), and the stylus of the 18 G angiocatheter was inserted into the 18 G angiocatheter sheath to push the tumor tissue into the puncture needle sheath. The angiocatheter sheath was then removed, and the stylus was inserted into the puncture needle sheath to deliver the tumor cells into the liver parenchyma.[13] Finally, the puncture needle sheath was removed, and the overlying tissue was compressed manually for 3 minutes. An ultrasound examination was then performed immediately to assess for bleeding.

For group B, all of the procedural steps were identical to those used for group A, with one addition: a gelfoam pellet (Zhong Qiang Strength Co., Ltd.)[3] was inserted during removal of the puncture needle sheath.

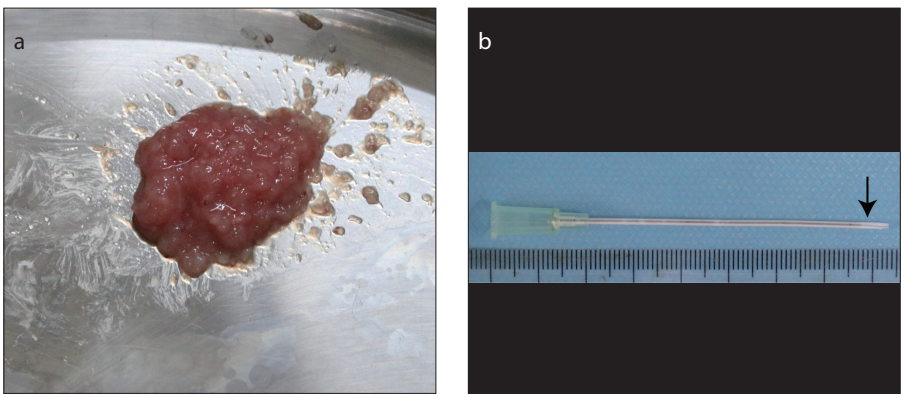

Figure 1. a, b. Panel **(a)** shows the tumor cut into small pieces (0.5–1 mm^3). The tumor tissue was then loaded into an 18 G angiocatheter sheath **(b)**, leaving approximately 5 mm of unfilled space at the tip of the needle sheath to avoid contamination of the needle tip *(black arrow)*.

Main points

- A VX2 liver tumor model can be safely and effectively established in rabbits through the use of ultrasound-guided percutaneous approaches.
- Implanting small pieces of VX2 liver tumor through a single needle is associated with a high rate of extrahepatic tumor seeding.
- Using a coaxial technique combined with injection of a gelfoam pellet for VX2 liver tumor implantation can reduce the risk of tumor seeding to both the abdominal wall and the omentum.

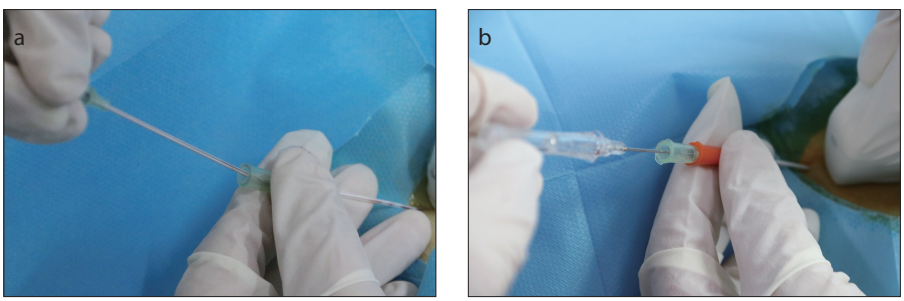

Figure 2. a, b. Panel **(a)** shows the tip of the sheath prefilled with tumor cells inserted into the hub of an 18 G puncturing sheath. Panel **(b)** shows an 18 G needle sheath prefilled with tumor cells inserted coaxially through the 14 G needle sheath.

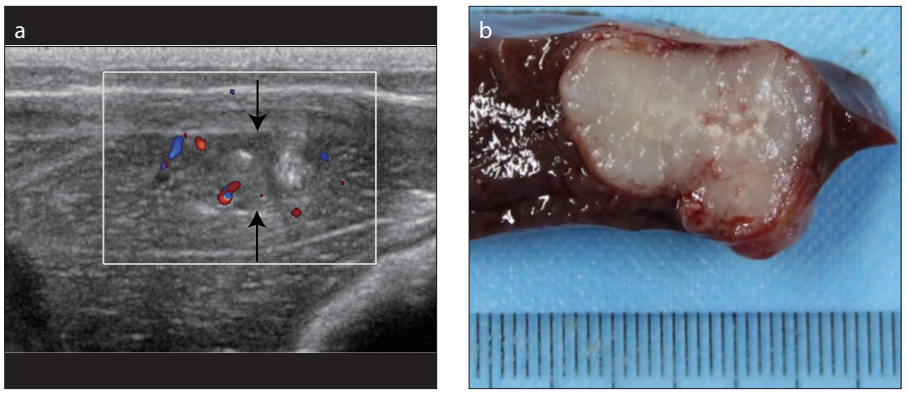

Figure 3. a, b. Ultrasound examination of the liver demonstrated short, rod-shaped blood flow signals surrounding the VX2 tumor *(black arrows)*. Gross specimens from abdominal wall **(a)** and omentum **(b)** demonstrate hard pale nodules *(black arrows)*.

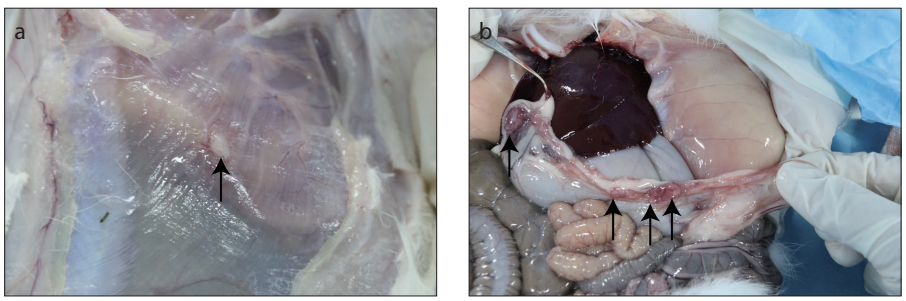

Figure 4. a, b. Gross specimens from abdominal wall **(a)**, liver and omentum **(b)** demonstrate hard pale nodules *(black arrows)*.

In group C, a 14 G intravenous catheter (Wenzhou Huali Medical Equipment Co., Ltd.) was used to puncture the targeted liver parenchyma (rather than the 18 G needle used in groups A and B). The 14 G stylus was then removed and an 18 G needle sheath (prefilled with tumor cells) was inserted coaxially through the 14 G needle sheath (rather than through the hub of the 18 G needle sheath). The stylus of the 18 G angiocatheter needle was then inserted to deliver the tumor tissue into the liver parenchyma (Figure 2b). Once the 18 G needle sheath had been removed, a gelfoam pellet was injected into the 14 G needle as the needle was slowly removed.

The procedure time, defined as the time from the initiation of general anesthesia to the time when anesthesia was discontinued, was recorded for each subject. The number of punctures for each subject was also recorded, and data were collected regarding the occurrence of any complications (such as bleeding or infection) or death after tumor implantation.

Ultrasound examination

Tumor growth was evaluated with ultrasound examinations at 1 week and 2 weeks after tumor implantation. The length, width, and depth at the greatest dimension of each tumor were measured in each group using conventional grayscale sonography. Sonography was also used to determine the number of tumors and to collect information regarding each tumor's location, echogenicity, boundaries, and blood flow. Two sonographers examined each rabbit independently, and a consensus was reached by a third sonographer in case of any disagreements.

Necropsy

After the ultrasound scan at 2 weeks, all rabbits were euthanized with carbon dioxide, and full abdominal necropsies were performed. The location and number of intrahepatic tumors and extrahepatic tumor implantations were recorded.

Histopathology

The tissues were fixed in 10% formalin solution and embedded in paraffin to create 4 μm transverse tissue sections. Hematoxylin and eosin (H&E) staining was performed, and the intrahepatic and extrahepatic specimens were examined using light microscopy.

Statistical analysis

SPSS software (IBM SPSS Statistics for Windows, Version 22.0) was used for the statistical analysis. The Shapiro-Wilk test was used to verify the normality of quantitative data and mean ± standard deviation was used to express data that obeyed a normal distribution. Nonparametric variables were given median (minimum-maximum) and were tested with the Kruskal-Wallis one-way ANOVA. For categorical data, frequencies with percentages were reported. The chi-square test was used for categorical data, with $P < .05$ considered to indicate statistically significant difference. In case of significant results, pairwise comparisons were performed with Bonferroni correction and the level of statistical significance was adjusted ($P < .008$).

Results

In this study, 60 rabbits, half male and half female, successfully tolerated VX2 tumor inoculation. The procedure times were 8.48±1.25 min in group A, 8.88±1.36 min in group B, and 8.53±1.24 min in group C. The operation time of the groups was not significantly different ($P = .405$, one-way ANOVA). The median number of punctures for each group was 1 (range, 1–3), with no significant difference among the groups ($P = .612$, Kruskal-Wallis one-way ANOVA). No cases of infection, bleeding, or death were observed immediately after inoculation or 2 weeks later.

One week after the procedure, ultrasound examination demonstrated intrahepatic tumors in the left liver that had heterogeneous echogenicity, with high echogenicity in the center and low echogenicity in the periphery. The nodules were regular in shape, with clear boundaries and short, rod-shaped blood flow signal at the edge of the tumors.

Two weeks after the procedure, ultrasound examination again demonstrated intrahepatic tumors in the left liver that had heterogeneous echogenicity, mainly hypoechoic, with a regular shape and clear boundaries and with short, rod-shaped blood flow signal. Some tumors demonstrated hyperechoic areas (Figure 3).

Regarding tumor size as measured on ultrasound examinations, no significant differences were seen among the groups at 1 week or 2 weeks after the procedure (Table).

Two weeks after the procedure, ultrasound examination also demonstrated new-onset nodules on the abdominal wall

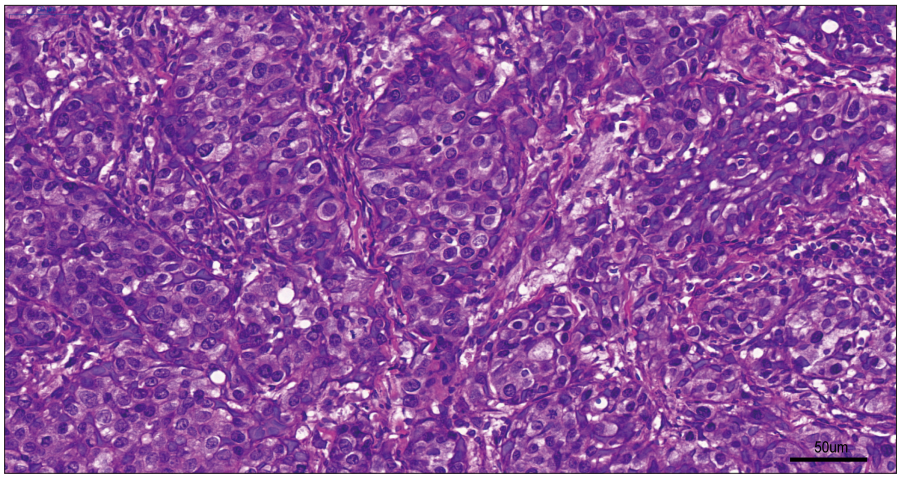

Figure 5. Light microscopy image shows VX2 tumor cells. Scale bar indicates 50 μm.

Table. Tumor size (cm) in each of the 3 groups

Time after procedure	Measurement	Group A	Group B	Group C	*P*
1 week	Length	0.68±0.14	0.62±0.16	0.59±0.11	.159
	Width	0.51±0.17	0.47±0.07	0.48±0.13	.597
	Depth	0.48±0.1	0.53±0.14	0.46±0.09	.140
2 weeks	Length	1.67±0.34	1.71±0.29	1.62±0.32	.660
	Width	1.11±0.3	1.14±0.19	1.18±0.2	.635
	Depth	1.03±0.15	1.11±0.17	1.12±0.12	.153

and omentum in some subjects. The abdominal wall nodules were hypoechoic, had uniform internal echogenicity, were regular in shape, were round or oval, and had clear boundaries; color Doppler ultrasound imaging demonstrated short strip-like blood flow signal within the nodules. The omental nodules manifested as round hypoechoic nodules with clear boundaries, with color Doppler imaging again demonstrating short strip-like blood flow signal within the nodules.

The success rate of intrahepatic tumor inoculation was 100% (60/60). Intrahepatic tumors appeared as gray fish-like nodules without a capsule in the left liver, with a small necrotic area in the center (Figure 4).

Abdominal wall and omental tumors, similar in appearance to the liver tumors, were observed in all 3 groups; however, there was no obvious necrosis in the center of these tumors.

Abdominal wall tumors occurred in 65% of animals (13/20) in group A, 35% (7/20) in group B, and 5% (1/20) in group C. The seeding rate of abdominal wall tumor was significantly different among the groups ($P < .001$, chi-square). As for the post hoc analysis, significant statistical difference existed between group A and group C ($P < .001$). However, no significant differences were observed between group B and groups A or C ($P = .058$, $P = .048$, respectively).

Omental tumors were present in 55% of animals (11/20) in group A, and 20% (4/20) in group B. No omental tumor was observed in group C. The seeding rate of omental tumors was significantly different among the groups ($P < .001$, Pearson chi-square). Post hoc analysis showed statistically significant difference between group A and group C ($P < .001$). However, there were no significant differences between group B and groups A or C ($P = .022$, $P = .114$, respectively).

Overall, the rate of ectopic implantation was 70% (14/20) in group A (10 rabbits had both abdominal wall and omental tumors, 3 rabbits had only abdominal wall tumor, and 1 rabbit had only omental tumor), 35% (7/20) in group B (4 rabbits had both abdominal wall and omental tumors, and 3 rabbits had only omental tumor), and 5% (1/20) in group C (abdominal wall tumor). Column proportions demonstrated again that the ectopic implantation rate was significantly different among the three groups ($P < .001$, Pearson chi-square). As for the post hoc analysis, significant statistical difference existed between group A and group C ($P < .001$). However, there were no significant differences between group B and groups A or C ($P = .027$, $P = .048$, respectively).

After H&E staining, a large number of abnormal cells were seen on the intrahepatic and extrahepatic specimens; these samples demonstrated disordered structure, dense arrangement, large cell volume, large nucleus, deep staining, and imbalance of nucleoplasm ratio (Figure 5).

Discussion

The VX2 tumor model is frequently used by interventional radiologists for studies of liver,[5–7] kidney,[20] uterine,[21] and lung[22] tumors. The rabbit VX2 liver tumor model in particular has been extensively used in research regarding various aspects of liver cancer and is commonly recognized as the liver tumor inoculation model for liver cancer. Although an implanted rabbit VX2 liver tumor model can be established through either open surgery or via a percutaneous approach, percutaneous implantation is generally favored over open surgery because it is minimally invasive with a lower complication rate and high rate of technical success. Even with this technique, however, extrahepatic tumor seeding can still occur.

In this study, 100% of cases demonstrated successful VX2 liver tumor implantation using percutaneous, ultrasound-guided approaches. However, the rate of extrahepatic tumor seeding was significantly lower in rabbits implanted with tumor using the coaxial technique with the insertion of a gelfoam pellet than in those implanted using other techniques. Previous studies have used coaxial technology to implant rabbit VX2 liver tumor, finding that the rate of extrahepatic tumor seeding was significantly lower with this technique.[13,16] However, no previous studies have assessed the use of this coaxial technique plus insertion of a gelfoam pellet in the creation of a rabbit VX2 liver tumor model, although this combination has previously been tried in clinical biopsies of the liver,[23] lung,[24] and spleen.[25] With this technique, the coaxial needle protects the puncture tract while gelfoam pellets prevent bleeding and leakage of tumor fragments.[26] In this study, only 1 rabbit (5%) implanted with VX2 liver tumor using this technique demonstrated the presence of an extrahepatic (abdominal wall) nodule.

In this study, we chose to implant minced tumor fragments rather than single 3-4 mm^3 tumor fragments.[13, 27] We chose this approach because minced tumor fragments were much easier to pass through the needle than single tumor fragments. However, the tract seeding we observed with this method was higher than that seen in previous research (70% in group A vs. 20.8%)[16] and was similar to that seen with implantation via tumor cell suspension.[11] This high seeding rate may have been caused by the smaller tumor pieces more easily leaking from the liver puncture site. However, the addition of the gelfoam pellet seemed to effectively contain the tumor fragments, leading to a much lower rate of tract seeding.

This study had several limitations. We used a small number of rabbits (n=20) in each group similar to the previous studies,[10, 28] and only male rabbits were included. No subjects were implanted via open surgery or via percutaneous coaxial approach without gelfoam; thus, comparisons could not be made regarding these other approaches. The short follow-up period (2 weeks) prevented us from assessing any long-term effects of intrahepatic or extrahepatic tumor implantation. No cases of lung metastasis were observed in this study, perhaps because of the short follow-up period.

In conclusion, a coaxial technique combined with injection of a gelfoam pellet was 100% technically successful in creating a VX2 liver tumor model in rabbits while also reducing the rate of extrahepatic tumor seeding to both the abdominal wall and the omentum. This technique was safe, with no complications or deaths seen among the study subjects.

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Conflict of interest disclosure

The authors declared no conflicts of interest.

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