Minimally Invasive Extended Hepatectomy in Miniature Pigs and the Subsequent Hepatic Regenerative Response

Hua Zhang[1]

Jin jin Tong[1]

Zhao nan Zhang[1]

Yong hong Zhang[1]

Hong bin Wang[2]*

Lin shu Jiang[1]*

[1]Beijing Key Laboratory of Dairy Cow Nutrition,
Animal Science and Technology College, Beijing University of Agriculture,
Beijing 102206, China

[2]Department of Veterinary Surgery, College of Veterinary Medicine,
Northeast Agricultural University, Harbin150030, China

* Corresponding author: Hong-bin Wang, E-mail: hbwang1940@126.com;
+86 18810589234

Co-corresponding author: Lin-shu Jiang, E-mail: linshujiangBUA@163.com;
+86 13613613901

KEY WORDS: Laparoscopy; Extensive hepatectomy; Hepatic regenerative; Pigs

ABSTRACT

This study reported a surgical method of laparoscopic extended hepatectomy and changes in the regenerative response. A purely laparoscopic four-port approach was used to perform left hemihepatectomy combined with right lateral lobectomy in miniature pigs. The total incision length was 5.1 cm, the average operative time was 139 min (range: 128-152 min), and the estimated blood loss was 136 ml. The serum total proteinlevel decreased 1 day after surgery, reached its lowest level 3 days and gradually increased from 7 to 14 days after surgery. Aspartate aminotransferase, total bilirubin and γ-glutamyl transpeptidase levels peaked 1 day after surgery and then gradually decreased from 3 to 14 days after surgery. Histopathological examination revealed hepatocyte proliferation and increased hepatic sinus space in local hepatic lobules 1 day after surgery. Disordered proliferating hepatocytes were identified in the hepatic lobules 3 days after surgery. Seven days after surgery, interstitial fibrous tissue hyperplasia and inflammatory cell infiltration occurred in the liver tissue near the resection. The expression of PCNA in hepatocytes peaked 3 days after surgery and then decreased over time. The expression of the cyclin D1 gene in the liver tissue was significantly increased during the early stage after hepatectomy, with levels significantly higher 1 day after surgery than before surgery ($P<0.01$); this difference relative to preoperative levels persisted until 3 days after surgery ($0.01<P<0.05$). This minimally invasive extended

hepatectomy in swine could serve as a useful model for investigating liver diseases and regeneration, and offer preclinical information to improve hepatobiliary surgical procedures.

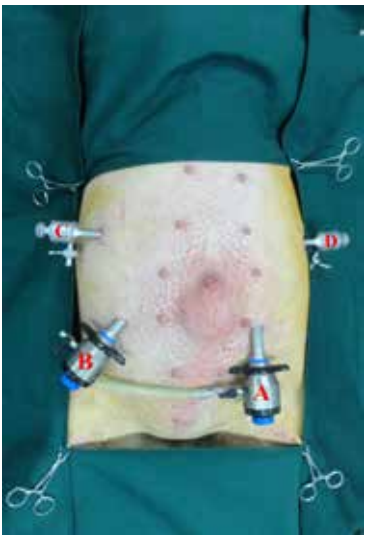
INTRODUCTION

Laparoscopic hepatectomy is a surgical procedure that is commonly performed in the management of liver cancer (Chen et al.,2018; Kanazawa et al.,2015) and during donor hepatectomy (Au et al.,2018;Chen et al.,2018). Liver regeneration after partial hepatectomy is a time-dependent process, and the failure of this complex process leads to posthepatectomy liver failure, which is associated with a high rate of mortality (Iguchi et al.,2017). Thus, in experimental studies, a porcine model of hepatectomy is an extremely important research tool for the evaluation of liver regeneration after various treatments. The successful establishment of hepatectomy models could promote technological advances and the development of new methods for the treatment of hepatic diseases.

Minimally invasive laparoscopic surgery results in minimal trauma to the abdominal wall, thus providing reduced pain and wound complications, faster recovery times, and a more rapid return to activity compared to those of patients who undergo conventional surgery (Gouda et al.,2015). However, there have been few reports of laparoscopic hepatectomy in veterinary medicine or of the establishment of an experimental model, and most of these reports required expensive instruments and had varying levels of procedural complexity (Gouda et al.,2011).

The expression of the cyclin D1 gene often acts as a marker of hepatocyte proliferation, and proliferating cell nuclear antigen (PCNA) has been used to quantify protein expression (Enkhbold et al.,2015; Lai et al.,2016). However, although studies have reported many different methods and devices effect on liver regeneration after hepatectomy in pigs, including vascular rings (Bucur et al.,2018), monoclonal antibodies (Bruha et al.,2015), and partial portal vein arterialization (Chen et al.,2012), these approaches have not been used to investigate the proliferative response in a model of laparoscopic extended hepatectomy.

Figure 1. *Locations of portals A, B, C, and D for laparoscopic liver resection.*



The purpose of this study was to establish a simple and reproducible technique of extensive hepatectomy by a purely laparoscopic technique in miniature pigs and to report the subsequent regenerative response.

MATERIALS AND METHODS

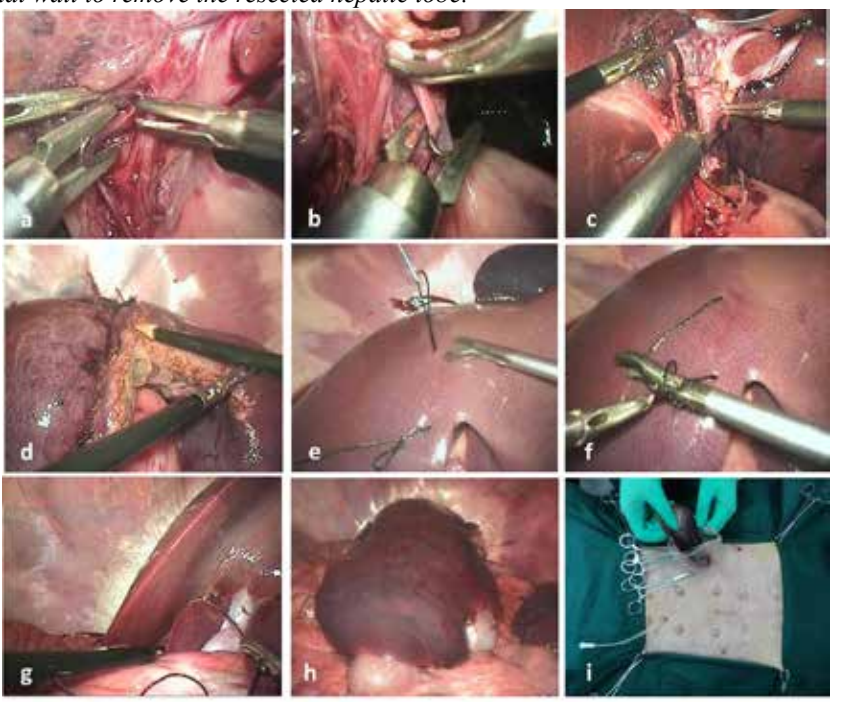
Animals

In this study, 14 Bama miniature pigs with an average age of 6 months (range: 4-8 months) and an average body weight of 18.5 kg (range: 15.8-23.5 kg) were used. The miniature pigs were randomly divided into the following two groups: the laparoscopic hepatectomy group (LH group, n = 7) and the sham (control) group (sham group; n = 7). The experimental protocol was approved by the Animal Ethics Committee of Northeast Agricultural University.

Anesthetic protocol

The miniature pigs were fasted for 12 hours before the experiment, and water was withheld for 6 hours before surgery. Before surgery, atropine sulfate (0.05 mg/kg body weight, Xiantu; Harbin Pharmaceutical Group, China) was injected subcutane-

Figure 2. *Images during the laparoscopic hepatectomy*
a: Separation and clipping of the left hepatic duct. b: Separation and clipping of the left branch of the hepatic artery. c: Separation and clipping of the left branches of the portal vein. d: Two needles threaded with one silksuture were used to penetrate the left hepatic lobe. e: The suture is knotted using both ends. f: Transfixing ligation of the base segment of the right lateral hepatic lobe. g: A high-frequency electric scalpel is used to divide the liver parenchyma. h: Intra-abdominal image immediately after surgery. i: Small incision in the right abdominal wall to remove the resected hepatic lobe.



ously, and then ketamine hydrochloride was administered intramuscularly (i.m.) (10 mg/kg body weight, Hansen Pharma Co., Ltd., Changsha, China) 15 minutes later. After the experimental pig was sedated, propofol (5 mg/kg body weight) was slowly injected through the ear margin vein to induce anesthesia. Then, endotracheal intubation was performed with a 6.0-7.5-mm endotracheal tube under laryngoscope guidance, and anesthesia was maintained via inhalation of 1.5-3% isoflurane (Lishi; Jiupai Pharmaceutical, China) using an anesthesia machine. A heated air pad was used to maintain the body temperature of the miniature pig at 36-38°C. During surgery, normal saline (3 ml·kg-1·h-1) was infused through the ear margin vein, and various physiological parameters were continuously monitored.

Surgical technique

The miniature pig was placed in the supine position and secured on the operating table, which was tilted at 10-30° with the head higher than the tail. The anterior abdomen was shaved, disinfected, and draped for laparoscopic surgery. A Veress needle was used to create an artificial pneumoperitoneum by filling the abdominal cavity with CO2 (to 12 mm Hg). Four trocars were used for this procedure. One trocar (Portal A) was used for placement of the laparoscope, and the other three trocars (Portals B, C, and D) were used for surgical instrument access (Figure 1).

Portal A (laparoscope) was located in the left abdomen below the third nipple from the tail side and 3-5 cm from the midline. A 10-mm trocar was placed and a

30° laparoscope was inserted. Portal B was located approximately 5 cm lateral to the third nipple from the tail side in the right abdomen, and a 10-mm trocar was placed for laparoscopic surgical instrument access. Portal C was located 3-5 cm below the right costal margin, and a 5-mm trocar was placed for laparoscopic surgical instrument access. Portal D was located on the left side of the abdominal midline opposite to portal C, and a 5-mm trocar was placed for laparoscopic surgical instrument access.

First, scissors were used to divide the round ligament, the sacral ligament, the left and right triangular ligaments, and the coronary ligament to fully mobilize the liver. Then, under laparoscopy, the left hepatic duct, the hepatic artery, and the branches of the portal vein were separated, clipped, and divided with titanium clips (Figures 2a to 2c).

A high-frequency electrosurgical scalpel was used to divide the liver parenchyma (Figures 2d) along the middle liver fissure (the boundary between the left and right medial segments) after double-row transfixing ligation of the left hepatic lobe (the left lateral and medial lobe) (Figure 2e and 2f) and the base segment of the right lateral hepatic lobe (Figure 2g).

The incised liver section was examined to confirm that no bleeding was present(Figure 2h). A specimen bag was placed into the abdominal cavity through a trocar incision. The resected liver was placed into the bag. The right abdominal trocar incision was extended to remove the resected liver (Figure 2i).

An abdominal drainage tube was placed through a trocar incision into the abdomen and secured. The abdominal wall was closed with conventional suture.

Changes in hepatic function

The levels of serum total protein (TP), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), and total bilirubin (T-Bil) were determined with a spectrophotometer (Mindray Biomedical Electronics Co., Ltd., Shenzhen, China) and a liver function test kit (Yapp Biological Technology Co., Ltd., Shanghai, China) before surgery and at 1 day, 3 days, 7 days, 14 days and 30 days after surgery.

Observation of hepatic histology

Liver tissues obtained 1 day, 3 days, and 7 days after surgery were fixed in a 10% formaldehyde solution, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The sections were then observed under a light microscope.

Immunohistochemical staining for PCNA

The protein expression of PCNA was measured by immunohistochemical staining, and the cell proliferation index was calculated for paraffin sections of liver tissues that were obtained on the day before surgery and at 1 day, 3 days, and 7 days after surgery. The presence of dark brown nuclei indicated PCNA-positive staining. Five microscopic fields were selected for each sample under a high-power field (400×). The average number of cells with positive staining per 100 cells in the 5 fields was recorded as the number of PCNA-positive cells.

Real-time PCR for cyclin D1 gene expression

From the liver tissues harvested at each time point (before surgery and 1 day, 3 days, and 7 days after surgery), 50 mg of liver sample that had been frozen in liquid nitrogen was obtained, and total RNA was extracted from the sample for cDNA synthesis. The primers were designed using Primer Premier 5.0 and Oligo 6 software, and the sequences of the designed primers are shown in Table 1.

Fluorescence-based quantitative PCR was performed using the Real-Time PCR System (Applied Biosystems, USA) and the Premix Ex Taq Kit (Takara Biomedical Technology, Beijing, China). The reaction volume was 20 µl. The reaction buffer was prepared on ice. The composition of the reaction buffer is shown in Table 2.

The real-time PCR program comprised the following three stages: Stage 1: predenaturation at 95°C for 30 s; Stage 2: PCR at 95°C for 5 s, 60°C for 20 s, and 72°C for 20

Table 1. *Genes and primers for fluorescence-based quantitative real-time PCR*

Gene	Sequence (5' -3')	Product Size (bp)
β-actin	Sense: CTCCSTCSTGSSGTGCGSCGT	114
	Antisense: GTGATCTCCTCCTGCATCCTGTC	
Cyclin D1	Sense: AGGAACAGAAGTGCGAGGAG	192
	Antisense: GGATGGAGTTGTCGGTGTAGA	

s, repeated for 35 cycles; and Stage 3: dissolution curve at 95°C for 0 s, 60°C for 15 s, and 95°C for 0 s.

The Ct values for each gene from quantitative real-time PCR were used to calculate the expression of the cyclin D1 gene in the liver tissue in the LH group relative to that in the control group according to the 2-ΔΔCt method.

Statistical analysis

The operative time, estimated blood loss, length of the incision, weight of the resected hepatic lobe, and intraoperative and postoperative complications were documented. The operative time was the time from the beginning of the skin incision to the closure of the trocar incision. The amount of blood loss was estimated by the total amount of liquid in the suction container minus the amount of washing liquid. SPSS statistical software 17.0 (version, 17.0; SPSS Institute, Cary, NC, USA) was used for statistical analysis. One-way analysis of variance was used to compare the differences between and within groups at different time points. Data are expressed as the mean ± SD. $P < 0.05$ was considered to indicate significant differences.

Table 2. *Reagent composition for fluorescence-based quantitative real-time PCR*

Reagent	Volume
Forward primer	0.8 μl
Reverse primer	0.8 μl
cDNA template	2 μl
RNase-free dH_2O	6.4 μl
Dye	10 μl

RESULTS

Surgical results and clinical outcomes

In this study, the four-trocar method (two 5-mm trocars and two 10-mm trocars) was used to successfully establish a miniature pig model of laparoscopic extensive hepatectomy in 7 pigs. No major intraoperative or postoperative complications occurred. The total length of the incision was 5.1±0.1 cm, the average operative time was 139 min (range: 128-152 min), and the estimated blood loss was 136±63.2 ml. Resected liver lobe volumes were determined by the measuring cup immersion method and averaged 157 ± 16 cm3. The average resected liver was 343.9±69.1g .

All experimental pigs showed signs of mild discomfort on the day of surgery and were able to drink a small amount of water; no symptoms of pain, such as bowing or trembling, were observed. On day 1 after surgery, the mental state and appetite of the pigs returned almost to normal levels, and a small amount of fluid feeding began. After surgery, the drainage fluid gradually became clearer, and the abdominal drainage tube could be removed after 3-5 days. Sutures were removed 7-9 days after surgery, and the trocar incisions were essentially healed. Laparoscopic exploration was performed one month after surgery and revealed adhesions between the liver tissue near the resection and the omentum or the stomach wall. Otherwise, no abnormalities were found in the abdominal cavity (Fig. 3).

Changes in liver function

Serum TP levels decreased in the early days after surgery and then increased. No obvious change was noted in the control group. In

Figure 3. *Abdominal exploration image 30 days after the laparoscopic surgery*

the LH group, the TP level began to decrease 1 day after surgery and reached its lowest level at 3 days after surgery. Then, the TP level gradually returned to normal from 7 to 14 days after surgery. Compared to the TP level before surgery, an extremely significant difference was observed 3 days after surgery ($P<0.01$), and a significant difference was observed between the TP levels at 1 day and 7 days after surgery ($0.01<P<0.05$). The difference was not significant at other time points ($P>0.05$).

AST levels first increased and then decreased, and there was a large range in the values measured. The AST levels peaked 1 day after surgery and then gradually decreased 3 days, 7 days and 14 days after surgery. Compared to the AST level before surgery, extremely significant differences were observed 1 day and 7 days after surgery ($P<0.01$). A significant difference relative to presurgery levels was also observed 7 days after surgery ($0.01<P<0.05$). This difference was not significant at other time points ($P>0.05$). The difference in AST levels between the two groups was extremely significant ($P<0.01$) at 1 day and 3 days after surgery; this difference was also significant 7 days after surgery ($0.01<P<0.05$) but not at the other time points ($P>0.05$).

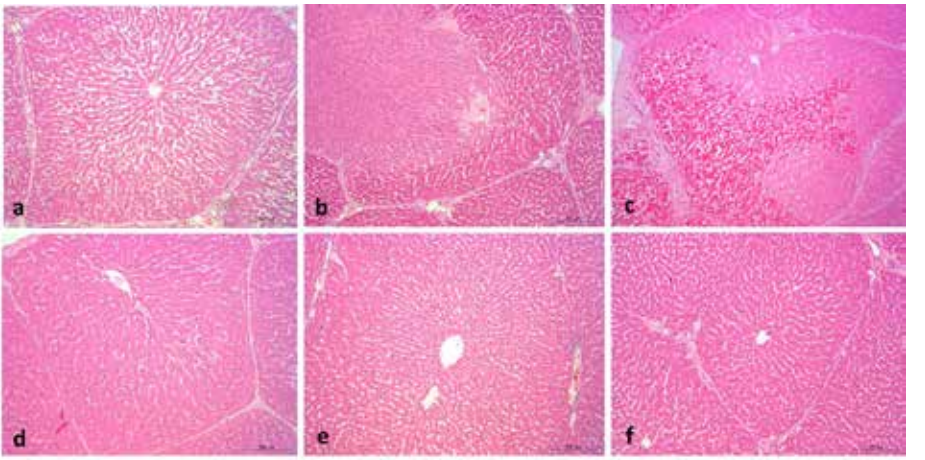
GGT levels were slightly elevated after surgery. In the LH group, the GGT level was significantly different from the level before surgery at 1 day and 7 days after surgery ($0.01<P<0.05$). This difference was not significant at other time points ($P>0.05$). The

Table 3. *The change in liver function in the LH model (X(—)±SD, n=7)*

Time point	Group	Monitoring parameters			
		TP (g/L)	AST (U/L)	GGT (U/L)	T-Bil (μmol/L)
Pre-op	LH	77.5±3.11	76.25±15.97	42.75±2.22	2.48±0.39
	Con	74.0±3.16	58.25±14.38	32.5±5.92	2.66±0.50
POD1	LH	64.75±4.57*▲▲	998.75±24.01**▲▲	64.5±9.80*	4.64±0.42*▲
	Con	73.75±0.96	199.75±18.92*	37.25±7.23	2.91±0.38
POD3	LH	59.0±7.52**▲	601.25±21.3**▲▲	53.5±4.10*▲	3.15±1.07
	Con	72.5±3.32	102.25±23.86*	31.0±3.37	2.47±0.85
POD7	LH	63.25±8.05*▲	234.5±12.14*▲	46.25±10.7	2.06±0.80
	Con	77.5±4.35	65.50±14.92	34.75±6.89	2.23±0.42
POD14	LH	69.5±8.73	71.75±10.24	39.0±8.40	2.11±0.37
	Con	76.5±4.79	62.75±11.11	37.25±6.40	2.45±0.48
POD30	LH	74.25±4.78	68.5±11.82	39.25±2.21	2.00±0.12
	Con	75.0±3.74	56.75±12.65	33.5±6.65	2.42±0.50

*Note: Pre-op: preoperation; POD: postoperative day; WBC: white blood cell; CRP: C-reactive protein; IL-6: interleukin-6; COR: cortisol. Compared to preoperative values, * $0.01 < P < 0.05$, ** $P < 0.01$; Compared to the OH group, ▲$0.01 < P < 0.05$, ▲▲$P < 0.01$; one-way ANOVA.*

Figure 4. *Histopathological changes after laparoscopic hepatectomy.*



GGT levels of the two groups were significantly different 3 days after surgery ($0.01<P<0.05$), but this difference was not significant at other time points ($P>0.05$).

Serum T-Bil levels first increased and then decreased. In the LH group, the T-Bil level at 1 day after surgery was significantly different from that before surgery ($0.01 <P <0.05$), but there were no significant differences at any other time points ($P> 0.05$). The difference in T-Bil levels between the two groups was significant 1 day after surgery ($0.01<P<0.05$) but was not significant at any other time points ($P>0.05$) (Table 3).

Liver histology changes

On day 1 after surgery, the light microscopic pathology evaluation showed that the LH group had increased hepatic sinus space with mild atrophy of liver cells (Fig. 4a). In the LH group, disordered proliferating hepatocytes were observed in the hepatic lobules on day 3 after surgery (Fig. 4b). Interstitial fibrous tissue hyperplasia occurred in the liver tissue near the resection, and inflammatory cell infiltration occurred on day 7 after surgery (Fig. 4c). There were no significant changes in liver structure or hepatocytes at 1, 3 and 7 days after surgery in the control group (Fig.4d, 4e, and 4f).

Changes in PCNA protein expression in liver tissues

The PCNA expression level of the hepatocytes in the LH group peaked at 3 days after surgery and then decreased over time (Figs. 5a, 5b, 5c, and 5d). In the control group, PCNA staining was weakly positive, and a few PCNA-positive hepatocytes were observed (Figs. 5e, 5f, 5g, and 5h). The PCNA index at 1 day and 3 days after surgery in the LH group was significantly different ($P<0.01$) than that before surgery, and there were no significant differences at the other time points ($P>0.05$). There were no significant differences in the PCNA index between any time points after surgery and the presurgery level in the control group ($P>0.05$). The PCNA index was significantly higher in the LH group (strong positive expression) than in the control group (weak positive expression) 1 day after surgery ($P<0.01$). The PCNA index was significantly higher in the LH group (positive expression) than in the control group (weak positive expression) 3 days after surgery. The PCNA index was not significantly different between the control group and the LH group (weak positive expression in both groups) 7 days after surgery ($P>0.05$) (Fig. 6).

Changes in cyclin D1 gene expression in the liver tissue

The expression of the cyclin D1 gene in liver tissues was significantly increased during the early stage after hepatectomy, and it was significantly higher at 1 day after surgery than before surgery ($P<0.01$).

Figure 5. *Changes in PCNA protein expression in liver tissues.*
a: A few PCNA-positive hepatocytes were observed (400×) in LH group (before surgery). b: A large number of proliferating PCNA-positive hepatocytes and biliary epithelial cells were observed (400×) in LH group (1 day after surgery). c: PCNA staining was strongly positive in liver parenchymal cells (400×) in LH group (3 days after surgery). d: a small number of PCNA-positive liver parenchymal cells were observed (400×) in LH group (7 days after surgery) e-f: A small number of hepatocytes with weakly positive PCNA staining were observed (400×) before surgery, 1 day,3 days and 7days after surgery.

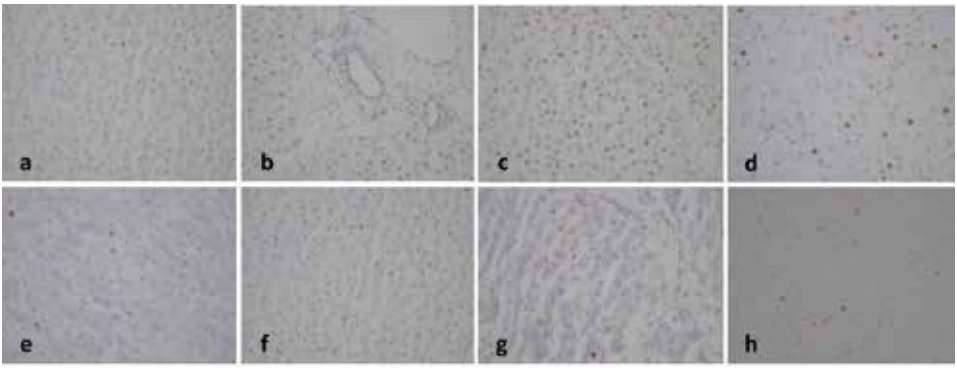


Figure 6. *Changes in PCNA expression in liver tissue.*

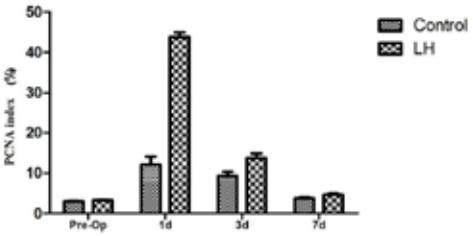
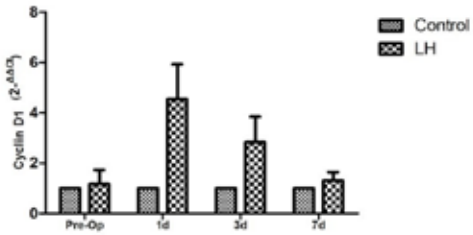


Figure 7. *Changes in cyclin D1 mRNA expression in liver tissue.*



This significant difference (0.01<P <0.05) persisted for 3 days after surgery. Cyclin D1 gene expression was not significantly different at other time points (P>0.05). There was no significant difference between the cyclin D1 expression before and after surgery in the control group (P>0.05). The difference in cyclin D1 gene expression between the two groups was extremely significant (P<0.01) at 1 day after surgery, and this difference remained significant at 3 days after surgery (0.01<P<0.05). This difference was not significant at other time points (P>0.05) (Fig. 7).

DISCUSSION

To date, multiple procedures for successful partial hepatectomy have been reported in porcine models. However, most of these procedures require expensive equipment, such as Ligasure, an ultrasonic scalpel and an Endo-GIA Stapler (Jersenius et al., 2007; Gehrig et al., 2013; Katagiri et al., 2017). In this study, the liver parenchyma was laparoscopically ligated with double-row transfixing sutures, and a combined left hemihepatectomy and right lateral hepatic lobectomy was successfully performed using a titanium clip and a high-frequency electrosurgical scalpel. This simplified method for creating an animal model of partial hepatectomy does not require other special instruments or equipment, and it can reduce the difficulty of establishing the model and facilitate its replication and application.

During the operation, the ligaments around the liver should be divided first. Care should be taken when dividing the coronary ligament, which should not be completely divided to avoid injury to the posterior vena cava. Unlike the human hepatic anatomy, in pigs, the hepatic vein near the diaphragm is buried in the liver parenchyma. Dissection of the hepatic vein at this location can cause massive bleeding, air embolism, and even death (Court et al., 2003). Therefore, the hepatic vein was not dissected in this study; instead, the liver parenchyma was ligated at the base segment of the liver using a transfixing ligation along the fissure between the liver lobes, and the liver parenchyma was then directly divided with a unipolar electrocoagulation hook. According to our experience, once the liver lobes have been fully mobilized, the resection can be performed in the following order: the left hepatic lobe is ligated first but not removed, the right lateral lobe is then ligated, and the right lateral hepatic lobe and the left hepatic lobe can then be removed together. This approach can improve the efficiency of surgery and facilitate the operation. During the division of the liver parenchyma, the liver lobe should be retracted as far as possible toward the posterior abdominal cavity to prevent the contraction and burning of the diaphragm muscles by the high-frequency electrosurgical current. Such burning of the diaphragm muscles may cause pneumothorax. When using a high-frequency electric scalpel to divide the liver parenchyma, the resection line should be 1-2 cm away from the ligation line to avoid damaging the sutures and causing bleeding in the area of resection. In the case of bleeding, electrocoagulation, large titanium clips and the transfixing ligation technique can be used for hemostasis, depending on the conditions.

Compared to traditional open surgery, minimally invasive laparoscopic surgery has obvious advantages, such as minimal exposure of the abdominal cavity and operation under laparoscopic vision (Beard et al., 2017). However, the effect of extensive hepatectomy on liver regeneration has not been fully evaluated or reported. PCNA and cyclin D1 are considered to be useful for evaluating the extent of the regenerative response (Enkhbold et al., 2015). In this study, after 70% liver resection in miniature pigs, the patterns of the changes in PCNA expression (immunostaining) and cyclin D1 gene expression (PCR) were basically the same. During the early stage after surgery (1 day after surgery), liver regeneration had started, and the expression of proteins and genes related to cell proliferation was significantly elevated. Gaglio PJ et al.(Gaglio et al.,2000) performed 60% hepatic lobe resection with liver biopsies for Ki-67 staining at 1, 2, 7, 14 and 30 days after surgery. Their results showed that all pigs can tolerate extensive hepatectomy and revealed a significant level of proliferating liver cells 2 days after surgery. This finding suggests that a larger percentage of liver resection is associated with a more pronounced level of liver proliferation.

Another study reported that rats subjected to 70% liver resection could survive for a long period of time without postoperative death. The liver function of these rats was significantly impaired from 5 days to 7 days after the surgery. The AST, GGT and T-Bil levels of these rats were significantly higher than those in the control group at time points within 7 days after surgery. Moreover, the AST level peaked at 12 hours after surgery, decreased rapidly, and returned to its preoperative level on the 14th day after surgery (Polaneo et al.,2008). In the current study, the AST level, which is a liver function index, peaked at 1 day after surgery and then gradually decreased at 3 days, 7 days and 14 days after surgery. The AST level was significantly higher than that in the control group at 1 and 3 days after surgery, and it returned to normal 7 days after surgery. In the LH group, the level of serum T-Bil first increased and then decreased; this level was significantly higher 1 day after surgery ($0.01 < P < 0.05$) than before surgery. There were no significant differences at the other time points ($P > 0.05$). Based on the results described above, the changes in liver func-

tion in rats and miniature pigs after extensive liver resection are virtually the same. Therefore, this model can be applied to the following studies: the accelerated recovery of liver function after liver resection, the molecular mechanism of liver function compensation and the therapeutic study of stem cells in liver injury.

In summary, this study describes an animal model of combined laparoscopic left hemihepatectomy and right lateral hepatic lobectomy using the 4-trocar method in miniature pigs. The resulting postoperative outcomes convincingly demonstrate the promise of our operative procedure. Moreover, from the perspectives of histopathology and molecular biology, this study has revealed the pattern of early liver regeneration after extensive hepatectomy. This porcine model could serve as a useful model for investigating liver diseases and regeneration, and it could offer preclinical information for improving hepatobiliary surgical procedures.

Funding

This study funded by the National Natural Science Foundation of China (Grant no. 31702302, 31472245), and the Da Bei Nong Foundation for Young Teachers (15ZK006), Young Teacher Research Fund of BUA (SXQN2016105), Beijing Education Commission Science and Technology Project (SQKM201710020011) and Open Project of Beijing Key Laboratory of Dairy Cow Nutrition, Beijing University of Agriculture.

Conflict of interest

The authors have no conflict of interest to declare.

Ethical standards

The experiments protocol approved by the Animal Ethics Committee of Northeast Agricultural University. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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