Low-dose steroid pretreatment ameliorates the transient impairment of liver regeneration

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INTRODUCTION

Liver resection is still one of the best curative therapies for primary or secondary liver tumors in most cases with...
Steroid pretreatment ameliorates LR

no extrahepatic metastasis\(^2\). Various techniques and devices for liver resection have been employed to improve the perioperative outcome\(^3\), although the clamp-crush technique used by a skilled surgeon still has the most favorable outcome according to recent systematic reviews\(^9,8\). Liver surgeons focus on reducing bleeding during liver resection, which leads to shorter operative time. Heat-assist devices such as the harmonic scalpel\(^2\), Ligasure\(^2\), saline-linked monopolar cautery\(^5\), microwave coagulator\(^2\), and radiofrequency (RF) devices\(^6\), can seal vessels and bile ducts to avoid postoperative bleeding and bile leakage. Because these heat-assist devices can achieve firm sealing of vessels and bile ducts, unnecessary ties and clips can be avoided without any adverse events\(^6\). Some randomized trials have indicated the merits and demerits of using heat-assist devices\(^6\).

The greatest merit is the sealing effect to reduce perioperative morbidity\(^6,24\). A second merit is enhancement of the surgical margins of tumors located near the cutting surface of the liver\(^6\). On the other hand, a necrotic zone remains in the cutting surface of the residual liver\(^6\). Although cryoablation causes lethal systemic responses with high levels of cytokines\(^6,21\), RF ablation may be safe and result in only minimal release of soluble factors causing systemic responses. However, it is not known whether RF manipulation, and the residual necrotic tissue during liver resection, is beneficial or harmful to liver regeneration (LR). In addition, the molecular events of LR after the use of heating devices for liver resection are also unknown.

LR is regulated by sequential molecular events in which various humoral factors such as tumor necrosis factor (TNF-\(\alpha\)) and IL-6 prime and facilitate hepatocyte replication\(^20-22\). Although the humoral factors increase and influence each step of LR in a very short time\(^21\), each cytokine activates subsequent molecular signals to complete LR\(^22\). RF ablation, in particular, excessively increases plasma cytokines such as TNF-\(\alpha\) and IL-6 compared to simple liver resection\(^23,24\). Due to superphysiological stimulation of these cytokines, the heat effect of RF ablation may impair liver regeneration\(^23\). On the other hand, fast recovery of liver function in LR after RF ablation has been reported in major clinical hepatectomy\(^1\). Therefore, the exact effect of RF ablation on LR remains unclear.

Steroid administration has been proved to attenuate surgical stress following liver resection\(^26,27\). In addition, steroid pretreatment has been proved to decrease plasma cytokine levels and the therapeutic dose of the steroid does not inhibit hepatocyte proliferation\(^28\). Although previous investigations showed that steroid treatment could ameliorate excessive surgical stress of extended hepatectomy\(^29,30\), the exact benefits of steroid administration in clinical LR are largely unknown. The main aim of this study was to determine whether liver regeneration could be disturbed following RF ablation. The second was to determine the effect of steroid pretreatment on LR after RF ablation.

**MATERIALS AND METHODS**

Animal studies were performed in compliance with institutional and National Research Council guidelines for humane care of laboratory animals.

**Animals**

Adult female Fisher 344 rats (250-350 g) were obtained from Charles River Japan (Kanagawa, Japan). They were housed in a climate-controlled (21 °C) room under a 12 h light-dark cycle and were given tap water and standard laboratory chow. All operations were performed between 9:00 a.m. and noon under general (ether) anesthesia using a sterile surgical technique.

**Surgical animal models**

**Sham hepatectomy:** The sham hepatectomy consisted of laparotomy and mobilization of the liver.

**Partial hepatectomy:** The two anterior liver lobes were removed as previously described\(^29,30\). In this model, removal of the two anterior lobes (68% of the liver) is known to induce the optimal proliferative response in the remnant liver mass.

**Partial hepatectomy with radiofrequency ablation:** Preceding partial hepatectomy (PH), the two anterior liver lobes were ablated with saline-linked electric bipolar forceps (ERBE Elektromedizin GmbH, Tübingen, Germany). After complete ablation of the two anterior lobes, they were removed the same as PH operation.

**Experimental design**

Groups of sham hepatectomy (SH), PH, and partial hepatectomy with RF ablation (PHA) rats were euthanized in batches of six at 1, 3, 5 and 7 d after surgery. A separate experiment was designed to determine the effect of steroid administration. All animals were pretreated with dexamethasone at 30 min prior to the operation. Groups of steroid pretreated PH rats (S-PH) and PHA rats (S-PHA) were euthanized in batches of six at 1 d after surgery. One hour before euthanasia, 5-bromo-2-deoxyuridine (BrdU) was injected intraperitoneally (50 \(\mu g/kg\) body weight\(^31\)). When animals were killed, part of the liver tissue was immediately frozen in liquid nitrogen for molecular analysis and part of it was dipped into cold ethanol for immunohistochemical study.

**Blood chemistry and white blood cell counts**

Blood samples were analyzed for activity of alanine transaminase (ALT), aspartate transaminase (AST), total protein levels, and albumin (ALB) in a clinical laboratory. White blood cells were counted with an autocalculator in the laboratory.

**Multiple cytokine detection**

Serum obtained after euthanasia was kept at -80 °C until submission to a company (Upstate United States Inc., Charlottessville, VA, United States) for analysis. Briefly, multianalyte profiling was performed on a Luminex 100 system and the XY Platform (Luminex Corporation, Austin, TX, United States). Calibration microspheres for
classification and reporter readings as well as sheath fluid were obtained from Luminex Corporation. Acquired fluorescence data were analyzed using MASTERPLEX™ QT (Ver. 1.2, MiraBio Inc., South San Francisco, CA, United States). Serum concentrations of TNF-α, IL-6, IL-10, and monocyte chemoattractant protein-1 (MCP-1) were measured with an Upstate Beadlyte Mouse Multicytokine Bead master kit (Upstate United States, Inc.)[31]. All analyses were performed according to the manufacturers’ protocols.

**Immunohistochemistry for BrdU and BrdU labeling index**

The proliferative activity in the liver after heptectomy was determined by measuring incorporation of BrdU as previously described[28]. Briefly, a mouse anti-BrdU antibody (X 100 dilution: DAKO A/S, Copenhagen, Denmark) was used as the primary antibody, followed by the ABC method (DAKO Co., Carpinteria, CA). Both labeled and unlabeled hepatocytes were counted in 20 fields in three different sections per time point from five different animals. Data are presented as means ± SD from three independent experiments.

**Restitution of liver mass**

Growth of the residual liver lobes (right and omental lobes) was calculated as the ratio of residual liver weight/body weight (RLW/BW).

**Western blotting analysis**

Western blotting analysis was performed using the Invitrogen NuPAGE® electrophoresis system (Invitrogen, Carlsbad, CA, United States). The samples were homogenized in phosphate buffered saline and kept at -80°C until use. Briefly, nuclear proteins were extracted using the NE-PER® nuclear and cytoplasmic extraction protocol (Pierce Chemicals, Rockford, IL, United States). A BCA protein assay kit® (Pierce Chemicals) was used to measure the protein concentrations. Proteins (5 μg/lane) were separated on NuPAGE 4%-12% Bis-Tris gradient gels (Invitrogen). The gels were transferred to nitrocellulose membranes (Amersham Co., Buckinghamshire, United Kingdom) using an iBlot™ Gel Transfer Device (Invitrogen). Immunodetection of proteins was performed using a Western-Breeze® Chromogenic Immunodetection Kit (Invitrogen). Mouse monoclonal anti-proliferation cell nuclear antigen (PCNA) (Dako Co., Carpinteria, CA, United States) and rabbit polyclonal anti-ALB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as the primary antibodies (1:250). The ECL western blotting analysis system (Amersham Co.) was used to detect signals.

**Densitometric analysis**

Scanning densitometry was performed using a Macintosh G4 computer (Apple Computer, Cupertino, CA) and an EPSON GT-9600 scanner (Seiko Epson, Suwa, Japan). The signals were quantified using the NIH Image 1.55 Densitometric Analysis Program[32].

**STAT3 DNA-binding activation assay**

STAT3 activation was quantified using a TransAM™ STAT3 Kit (Active Motif, Funakoshi Co., Tokyo, Japan)[32]. Briefly, 10 g/well of the nuclear cell extract from whole liver tissue (containing an activated transcription factor) was incubated in a 96-well plate on which double-stranded oligonucleotides containing the consensus sequence for the STAT3 DNA-binding site (5’-TTCCCGGAA-3’) were immobilized. The primary antibody used to detect STAT3 recognized epitopes on both the alpha and beta forms of STAT3, which are accessible only when STAT3 is activated and bound to its target DNA. After incubation with horseradish peroxidase, absorbance was recorded at 450 nm using a reference wavelength of 655 nm.

**Statistical analysis**

The unpaired Student’s t-test, Welch’s t-test or one-way analysis of variance (ANOVA) was used as appropriate. Data are given as mean ± SD. Statistical analysis was performed using the StatView 5.0 program (SAS Institute, Cary, NC, United States) and the difference between the means was considered significant when P < 0.05.

**RESULTS**

All rats tolerated the operative procedures well and recovered uneventfully from anesthesia. Samples were collected immediately after each animal was euthanized.

**Liver regeneration after ablation**

Although the liver was ablated within a short time and most necrotic tissue was removed in the PHA group, postoperative serum AST and ALT levels in this group at one day after operation were significantly higher than in the PH group (Table 1). On the other hand, the white blood cell counts were not significantly different among the groups (Table 1). Total protein and albumin levels at two, three, and five days after operation in the PHA group were significantly lower than in the PH group.

Serum cytokine and chemokine levels are shown in Figure 1. TNF-α levels in the PHA group at one and two days after operation were significantly higher than in the PH group (Figure 1A). IL-6 (Figure 1B) and MCP-1 (Figure 1D) levels in the PHA group at two, three, and five days after operation were significantly higher than in the PH group. IL-10 levels in the PHA group at two and three days after operation were significantly higher than in the PH group (Figure 1C).

DNA synthetic activity was determined by immunohistochemistry for BrdU (Figure 2A-J) and labeling indices (LIs) (Figure 2K). The LI at one day in the PHA group was significantly lower than in the PH group (12.17 ± 3.43 vs 29.02 ± 8.47, P = 0.001). On the other hand, the LIs at two days and three days after operation in the PHA group were significantly higher than in the PH group (15.85 ± 4.18 vs 7.05 ± 1.54, P < 0.001, and 12.55 ± 3.14 vs 6.03 ± 2.11, P = 0.002, respectively). Although DNA synthetic activities between the groups are significantly different, RLW/BW ratio was not greatly significantly different among the groups (Figure 2L).

Protein expression of nuclear PCNA and cytosolic
Table 1  Alterations of blood cell counts and laboratory tests in partial hepatectomy and partial hepatectomy with radiofrequency ablation models

| WBC (× 10^3 μL) | Day 0       | Day 1       | Day 2       | Day 3       | Day 5       | Day 7       |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|
| PH              | 4.95 ± 0.74 | 4.70 ± 0.81 | 6.55 ± 1.26 | 6.15 ± 0.81 | 7.43 ± 0.99 | 5.48 ± 1.14 |
| PHA             | 5.80 ± 1.09 | 5.70 ± 0.84 | 7.68 ± 1.32 | 6.60 ± 1.49 | 7.65 ± 1.41 | 6.73 ± 0.81 |
| P values        | NS          | NS          | NS          | NS          | NS          | NS          |
| TP (g/dL)       |             |             |             |             |             |             |
| PH              | 5.55 ± 0.24 | 4.71 ± 0.29 | 4.87 ± 0.29 | 5.18 ± 0.21 | 5.67 ± 0.81 | 6.78 ± 0.34 |
| PHA             | 5.62 ± 0.17 | 4.43 ± 0.25 | 4.95 ± 0.21 | 4.68 ± 0.24 | 5.31 ± 0.36 | 4.80 ± 0.15 |
| P values        | NS          | NS          | NS          | NS          | NS          | NS          |
| ALB (g/dL)      |             |             |             |             |             |             |
| PH              | 4.10 ± 0.14 | 3.73 ± 0.21 | 3.58 ± 0.21 | 3.80 ± 0.22 | 4.08 ± 0.15 | 4.08 ± 0.15 |
| PHA             | 4.20 ± 0.18 | 3.55 ± 0.21 | 3.18 ± 0.17 | 3.10 ± 0.22 | 3.78 ± 0.28 | 3.78 ± 0.28 |
| P values        | NS          | NS          | 0.022       | 0.002       | 0.019       | NS          |
| TP (U/L)        |             |             |             |             |             |             |
| PH              | 77.3 ± 3.3  | 591.8 ± 111.8 |
| PHA             | 79.3 ± 4.6  | 2441.8 ± 501.8 |
| P values        | NS          | NS          | 0.001       | 0.001       | 0.001       | 0.001       |
| ALT (U/L)       |             |             |             |             |             |             |
| PH              | 48.5 ± 13.4 | 734.3 ± 187.4 |
| PHA             | 49.3 ± 9.6  | 1603.3 ± 313.7 |
| P values        | NS          | NS          | 0.003       | 0.025       | NS          | NS          |

One-way analysis of variance was used for statistical analysis and P < 0.05 is considered to be significant. WBC: White blood cell; PH: Partial hepatectomy; PHA: Partial hepatectomy with radiofrequency ablation; TP: Total protein levels; ALB: Albumin; AST: Aspartate transaminase; ALT: Alanine transaminase; NS: Not Significant.

Figures:
- Figure 1  Changes in serum tumor necrosis factor-α (A), IL-6 (B), IL-10 (C), and monocyte chemoattractant protein-1 (D) levels after the operations. *P < 0.05 between groups. TNF-α: Tumor necrosis factor-α; MCP-1: Monocyte chemoattractant protein-1.

albumin is shown in Figure 3A. Densitometric analysis of the expression of each protein is shown in Figure 3B. The pattern of PCNA expression was similar to the immunohistochemistry for BrdU and LIs. The peak of BrdU expression in the PH group was seen at one day after operation and in the PHA group at two and three days after operation. ALB expression in the PH group dropped at one and two days after operation and recovered thereafter. In contrast, ALB expression in the PHA group dropped at three and five days after operation. STAT3 DNA-binding activity was also consistent with the results of BrdU, LIs, and PCNA expression (Figure 3C).
The peak of STAT3 DNA-binding activity in the PH group was seen at one day after operation and in the PHA group at two and three days after operation.

Response of liver regeneration after ablation with steroid administration

We found that LR was disturbed after RF ablation in hepatectomy, with high cytokine/chemokine induction. Because steroid treatment could block cytokine elevation after hepatectomy, we tested the effects of S-PH and S-PHA groups.

Immunohistochemistry values for BrdU (Figure 4A-P) and LIs (Figure 4Q) at 1 d after steroid administration at different concentrations are shown in Figure 4. BrdU uptake in the S-PH group (Figure 4A-H) gradually decreased when the steroid dose was increased. In contrast, that in the S-PHA group (Figure 4I-P) gradually increased when the steroid dose was increased by 0.04 mg/kg and then it decreased with further dosage. STAT 3 DNA-binding activities (Figure 5B) were also consistent with the BrdU staining, LIs and PCNA expression.

DISCUSSION

We investigated the influence of RF ablation during hepatectomy on LR. We found that LR after RF ablation was disturbed, with high serum cytokine/chemokine induction. Low-dose steroid administration nearly restored LR after RF ablation during hepatectomy, and STAT3 DNA-binding activity supported this finding.

Surgical model to investigate influence of radiofrequency ablation on liver regeneration

The method of liver resection should take into account...
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**A**

| MW (kDa) | SH | PH (d) | PHA (d) |
|----------|----|--------|--------|
| 30 kDa   | a  | a      | a      |
| 65 kDa   | a  | a      | a      |

**B**

| % PCNA (%) | Days after surgery |
|------------|--------------------|
| 0          | 1 2 3 5 7          |

**C**

| % ALB (%) | Days after surgery |
|-----------|--------------------|
| 0         | 1 2 3 5 7          |

**Figure 3** Proliferation cell nuclear antigen expression in the nuclear protein and albumin expression in the cytosol after the operations. A: Western blotting for proliferation cell nuclear antigen expression (PCNA); B: Densitometric analysis of each protein signal; C: STAT3 DNA-binding activity in the nuclear protein after the operation. *P < 0.05 between groups. SH: Sham hepatectomy; PH: Partial hepatectomy; PHA: Partial hepatectomy with radiofrequency ablation; MW: Molecular weight; C.B.S.: Coomassie blue staining; ALB: Albumin.

Both perioperative safety and oncological curability. RF ablation is one of the less invasive strategies for small liver tumors and can be used for hemostasis during liver resection. The one concern is that some necrotic tissue will remain in the residual liver after RF ablation and may affect LR. In addition, thermal energy during the operation also may affect it. Most investigations have used large animal models to study the effects of RF ablation after hepatectomy on humoral and oncological activities. The use of murine models to investigate RF ablation has been limited. Large animal models can be ideal to simulate the human response; however, they are time consuming and it is difficult to examine the molecular details compared to murine models. Although our model did not totally reproduce the human clinical situation, the postoperative course was very similar, as serum transaminases were strongly elevated at one day after operation. Furthermore, high serum cytokine levels after ablation have been reported in human studies. These clinical postoperative alterations of laboratory tests supported the idea that our model could represent the clinical phenomena of hepatectomy using RF ablation. Therefore, our model was suitable to examine the thermal effect of RF ablation after hepatectomy.

**Effect of radiofrequency ablation on the liver**

Effects of RF ablation on cancer cells in the liver could modulate the systemic immune responses, including cytokine/chemokine production and the proliferative activity of the cancer cells. Necrotic tissue after RF ablation could also modulate the systemic immune response to specific cancer cells. On the other hand, Meredith et al. reported that RF ablation itself did not accelerate tumor growth. In our study, RF ablation delayed the liver regenerative response, which was consistent with a previous study. These differently reported proliferative responses may be due to the differences between cancer cells and normal cells. Other reasons could be differences in the amount of necrosis and the duration of the ablation. We could not distinguish between the exact effects of necrosis and ablation, but our results indicated that the heat effect of RF ablation, not necrosis, could delay LR, because most ablated tissues were removed in both groups, and the amounts of necrosis in the PH and the PHA groups were comparable in our model. Serum cytokines such as IL-6, IL-8, and IL-10 are elevated after RF ablation, which is also consistent with our results. Therefore, regardless of how much necrotic tissue was removed after RF ablation, the heat effect during the ablation itself could activate cytokine/chemokine responses.

**Cytokine/chemokine signals in liver regeneration**

LR after RF ablation was delayed without any difference in the R/LW/BW ratio. This indicated that volume recovery after PH did not represent parenchymal cell proliferation itself, which was consistent with a previous report. Delayed LR after RF ablation affected the serum albumin level. Albumin production was suppressed when hepatocytes began to proliferate. The time lag between DNA synthesis and the serum albumin level could be due to the long half-life of serum albumin. Even though there was no critical event in our model, we need to pay attention to the albumin level, which could decrease in LR after RF ablation and be associated with delayed LR in the clinical setting.

A systemic cytokine/chemokine response was activated by RF ablation even within the short time during operation. We could not determine the specific cytokine/chemokine that disturbed hepatocyte proliferation. Excessively high levels of cytokines such as TNF-α and...
Figure 4  Immunohistochemistry for 5-bromo-2-deoxyuridine staining in the partial hepatectomy with dexamethasone pretreatment group (A-H) and partial hepatectomy after radiofrequency ablation with dexamethasone pretreatment group (I-P) at 1 d after the operations. Animals were pretreated with 0 mg/kg (A and I), 0.002 mg/kg (B and J), 0.02 mg/kg (C and K), 0.04 mg/kg (D and L), 0.2 mg/kg (E and M), 0.4 mg/kg (F and N), 1 mg/kg (G and O), or 2 mg/kg (H and P) dexamethasone. Q: The brown nuclei are positive for 5-bromo-2-deoxyuridine. The labeling indices were calculated from 20 fields in three different sections per treatment for five different animals; R: Serum tumor necrosis factor-α levels at 1 d after the operations. The horizontal axis presents each concentration of dexamethasone pretreatment. *P < 0.05 between groups. S-PH: Steroid pretreatment in the partial hepatectomy group; S-PHA: Steroid pretreatment in the partial hepatectomy after radiofrequency ablation group. LIs: Labeling indices.
IL-6 are desensitized from growth stimuli\textsuperscript{[43]}, although knockout murine models targeting TNF-\(\alpha\) receptor and IL-6 genes have demonstrated that these cytokine signals are necessary to accomplish LR\textsuperscript{[44,45]}. The lack of DNA-binding activity of STAT3 in our results supported the finding of growth suppression in the PHA group. Even though the peaks of most cytokine/chemokine levels in the PHA model were between two days and five days after operation, DNA synthesis in PHA continued in these periods. The only distinctive alteration seen was in the TNF-\(\alpha\) level, which gradually decreased. In addition, steroid pretreatment in the PHA group showed that only the TNF-\(\alpha\) level was different between the PH group and PHA group at one day after operation. Therefore, DNA synthesis after RF ablation could be more affected by TNF-\(\alpha\) than by other cytokines/chemokines. Thus, TNF-\(\alpha\) activation should be observed within a short time after simple hepatectomy\textsuperscript{[21]}. However, it could be prolonged in LR after RF ablation, as shown in our results. Though we could not determine the mechanism of the TNF-\(\alpha\) activation after RF ablation, our results strongly suggested that the TNF-\(\alpha\) could play a major role in LR after RF ablation. Further study is needed to determine whether TNF-\(\alpha\) could be a molecular target to control LR in the clinical setting.

**Steroid administration and liver regeneration**

Steroids have been demonstrated to inhibit LR by inhibiting excessive TNF-\(\alpha\) and IL-6 production\textsuperscript{[46-48]}, although moderate stimulation by TNF-\(\alpha\) and IL-6 is necessary to complete LR\textsuperscript{[21]}. Our results also showed that cytokine/chemokine levels decreased gradually depending on steroid administration in the S-PHA group. On the other hand, steroids can inhibit the DNA synthesis of hepatocytes directly\textsuperscript{[42]}. The reason why DNA synthesis recovered after low-dose steroid administration in the S-PHA group could be related to the balance between the suppression of excessive cytokine production and the direct inhibition of DNA synthesis. In other words, LR after low-dose steroid administration could recover, escaping from the excessive cytokine production, and be nearly free from the direct inhibition by the steroid. Our results indicated the presence of an optimal threshold of the steroid concentration that facilitated LR when cytokines/chemokines were excessively activated. Therefore, our results strongly suggest that we need to pay careful attention to the clinical steroid concentration because the effects of steroid administration could be altered depending on the clinical condition.

In conclusion, LR was disturbed after RF ablation, with high serum cytokine/chemokine induction. Low-dose steroid administration could improve LR after RF ablation with TNF-\(\alpha\) suppression. Further clinical study is needed to confirm that low-dose steroid administration has a clinical benefit for LR after RF ablation.

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COMMENTS

Background
Liver resection is still one of the best curative therapies for primary or secondary liver tumors. Various techniques and devices for liver resection have been employed to improve the perioperative outcome. Radiofrequency (RF) devices can seal vessels and bile ducts to avoid postoperative bleeding and bile leakage. Although some of its merits have been reported, its demerits are largely unknown.

Research frontiers
The greatest merit of an RF device is the sealing effect to reduce perioperative morbidity. A second merit is enhancement of the surgical margins of tumors located near the cutting surface of the liver. On the other hand, a necrotic zone remains in the cutting surface of the residual liver. The research hotspot is whether RF manipulation, and the residual necrotic tissue during liver resection, is beneficial or harmful to liver regeneration (LR).

Innovations and breakthroughs
The present study showed LR after RF ablation delayed the regenerative response with high serum cytokine/chemokine induction, and low-dose steroid administration could improve LR after RF ablation with TNF-α suppression. The results indicated that the heat effect of RF ablation, not necrosis, could delay LR, because necrosis was removed. Serum cytokines such as IL-6, IL-8, and IL-10 were elevated after RF ablation, which is consistent with previous studies. Therefore, regardless of how much necrotic tissue was removed after RF ablation, the heat effect during the ablation itself could activate cytokine/chemokine responses.

Applications
This study provides insights into the mechanism by which RF ablation could activate cytokines/chemokines in LR and found that steroids can be used for controlling LR. The results strongly suggest that they need to pay careful attention to the clinical steroid concentration because the effects of steroid administration could be altered depending on the clinical condition.

Peer review
The aim of this study focused on liver regeneration after RF ablation is clear and interesting, and these data may provide a basis for RF ablation studies in the future. The impact of this study in this field is moderate.

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