Assessment of Effect of Intraperitoneal Tacrolimus on Liver Regeneration in Major (70%) Hepatectomy Model After Experimental Pringle Maneuver in Rats

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ABSTRACT

Aim. Small-for-size grafts have become more important, especially in living donor liver transplants. The Pringle maneuver, used to reduce blood loss, and the immunosuppressive medications used to prevent graft rejection in liver transplants have different side effects on liver regeneration. We researched the effect of situations where tacrolimus and the Pringle maneuver were applied or not on liver regeneration in rats with partial hepatectomy.

Material and Methods. This study was completed with 35 Wistar Albino rats. The subjects were randomly divided into 5 groups: Group 1 had the abdomen opened and no other procedure was performed; Group 2 underwent a 70% hepatectomy; Group 3 underwent a 15-minute Pringle maneuver + 70% hepatectomy; Group 4 underwent a 70% hepatectomy + 5 days of 1 mg/kg/day intraperitoneal tacrolimus; and Group 5 underwent a 150 minute Pringle maneuver + 0% hepatectomy + 5 days of 1 mg/kg/day intraperitoneal tacrolimus. All rats were sacrificed on the seventh postoperative day, remaining liver tissue was weighed, and weight indices created. The remaining liver tissue was stained with phosphohistone H3 and the mitotic index calculated.

Results. The groups that underwent the Pringle maneuver, 70% hepatectomy, and tacrolimus administration were compared with the control group in terms of mitotic index and weight index, but no statistically significant differences were identified.

Conclusion. Suppression of regeneration forms a risk after liver transplantation with small-volume grafts. As a result, research on the effect of tacrolimus combined with the Pringle maneuver is important, especially for transplantations using segmented liver grafts. In our study, we showed that the use of tacrolimus had no negative effect on liver regeneration.

THE LIVER is an organ with the capacity to regenerate, though the cells are renewed slowly. Though the liver is the organ with highest cell proliferation capacity, only 0.0012%–0.01% of hepatocytes experience mitosis in any period. This low cell cycle rate in healthy livers is higher than the 3% seen in situations with toxic liver damage or surgical resection. Surgical removal of part of the liver or loss due to the effect of toxic materials begins regeneration. Cell proliferation begins in the periportal region and reshapes cell series towards the center of the lobules. The regeneration response is typically linked to the proliferation of the acinar structure in the remaining liver tissue. In resection cases, this result is linked to hypertrophy of the

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remaining liver tissue rather than restoration of the resected lobe [1–4]. Though the hepatic segments remaining after partial hepatectomy are affected by increased portal perfusion and pressure, the best approach to ensuring liver regeneration not accompanied by cellular injury is shown by in-vivo regenerative response studies. Active cell replication begins within the first 24 hours after partial hepatectomy and continues until the organ reaches its initial weight. Significant degrees of regeneration form in the first 10 days and the process is completed within 4–5 weeks. The generally accepted view of hepatic regeneration is that it is a chain of perfectly organized events that continues until the liver reaches optimum size with the regulatory effect of signals from the organism.

The 2 cell groups responsible for growth in liver regeneration after resection are hepatocytes and progenitor cells known as oval cells. Oval cells (precursor cells) act as reserve compartments, while hepatocytes are the first differentiated cells in situations where the liver is injured or surgically resected. In repairing hepatic tissue after partial hepatectomy, hepatocytes are followed by ductus epithelial cells, Kupffer cells, stellate cells, and sinusoidal endothelial cells. Under normal conditions, dormant hepatocytes have high proliferative capacity in situations with regenerative stimulus. In situations where hepatocyte proliferation is inhibited, the oval cells in the reserve compartment are activated. Series biopsies after acute liver failure show that these progenitor cells are largely responsible for hepatocyte regeneration. The question of whether bone-marrow-sourced cells are involved in hepatocyte production after liver transplantation is controversial because these hepatocytes rarely exceed 1% of the total hepatocyte population in the livers of transplanted people. Furthermore, after partial hepatectomy in rodents, there is no evidence of the presence of bone-marrow-sourced hepatocytes in liver regeneration. Apart from hepatocytes and stem cells, other non-parenchyma cells play an important role in liver regeneration. The replication time of non-parenchyma cells is relatively well-tuned, like that of hepatocytes. Functionally, Kupffer cells, endothelial cells, and stellate cells (connective tissue cells) are important for normal hepatocyte proliferation because they are the sources of nearly all cytokines and growth factors required for hepatocyte replication [5–7].

One of the most important factors affecting perioperative results during liver resection is loss of blood and associated blood transfusions [8–10]. Pringle, the first person to perform a liver resection described a technique in 1908 [11] that prevented perfusion from the hepatic artery and portal vein to the liver: he closed the vascular pedicle with an atraumatic clamp to minimize blood loss during liver operations (the Pringle maneuver). This technique is currently used for both traumatic injury and elective liver resections once or at intervals for longer periods [12]. The result of the Pringle maneuver is that ischemic injury and associated reperfusion injury occur at increasing rates with the duration of application [13].

Ischemia reperfusion injury is characterized by the onset of a severe inflammatory response to the temporary absence of perfusion to a tissue and then return of blood flow. Ischemia occurs due to arterial and/or venous blockage. Stasis then develops in the affected bed. Ischemia may be seen after shock, transplantation, and myocardial infarction. Apart from these, the main events causing ischemia in the liver are liver resection, hemorrhagic shock, and trauma [14–17]. The reason for organ dysfunction occurring after cessation of perfusion is the increased cell injury occurring after reperfusion and organ sensitivity. Characteristically, ischemia-reperfusion injury causes polymorphonuclear leukocyte activation [18], occurrence of free oxygen radicals [19], increased release of cytokines [20], increased production of eicosanoids [21], and activation of the complement system [22].

Tacrolimus (FK-506) is an immunosuppressive agent in the macrolide lactone group. It was first produced from a fungus called *Streptomyces tsukubaensis* in 1984. It is used to prevent rejection of tissue in those with organ transplants (eg, kidney, liver, heart, or pancreas) [23]. It inhibits calcineurin, activates specific T cells, and inhibits the release of inflammatory cytokines such as interleukin 4, interleukin 5, and mainly interleukin 2. Calcineurin is an enzyme that separates target proteins from phosphate groups and causes calcium activation. It plays a basic role in the transcriptional activation of cytokines and is a good target for immunosuppression. Tacrolimus passes the cell membrane and binds to the binding protein (tacrolimus binding protein). Tacrolimus binding protein is a cytosolic protein found commonly in T lymphocytes. The complex formed by tacrolimus and the binding protein inhibits calcineurin distant from phosphate active regions, physically interacts with the calcineurin-transcription factor, and prevents dephosphorylation. Finally, the synthesis and release of interleukin 2 and other cytokines is inhibited [24]. Experiments with in-vitro hepatocyte cultures have not identified any increase in mitosis in hepatocytes after the administration of tacrolimus to the culture. However, when tacrolimus is administered with rat serum, only those given rat serum have demonstrated significant rates of mitosis increase [25]. After partial hepatectomy, the effects of tacrolimus on liver regeneration are considered to occur by inhibiting the activation of T-lymphocytes and thus removing the suppressive effects of the immune system on hepatocyte mitosis or by the non-immune route of interacting with secondary messenger and signal transmission mechanisms of the growth control system [26].

In this study we aimed to research the effects of situations where tacrolimus and the Pringle maneuver are used or not used on liver regeneration in rats with partial hepatectomy.

MATERIAL AND METHOD

Our study used 35 8-week-old, 200–300 g male Wistar Albino rats obtained from Dokuz Eylül Health Science Institute Experimental Animals Laboratory as experimental animals. The rats used in these experiments were housed in cages, in standard laboratory conditions in accordance with the ethical rules of the Izmir International

Biotype and Genome Institute Experimental Animals Local Ethics Committee (DEU, IBG-HADYEK) directives. Subjects were housed in equivalent environmental conditions in the Experimental Surgery and Research Department Laboratory, with free access to YEMTA animal feed and water in a natural day-night (light-dark) environment. Before beginning the experiments, permission was granted by Dokuz Eylul University Experimental Animals Local Ethics Committee with protocol number 17/2017.

The rats were administered ketamine (Ketalar, 10 mL, 50 mg/mL, Pfizer, New York City, NY, United States) (45 mg/kg, intraperitoneal) and xylazine (Alfazyne 50 mL, 20 mg/mL, Alfasan, Woerden, the Netherlands) (5 mg/kg, intraperitoneal) administered for general anesthesia. When necessary, 20 mg/kg ketamine was repeatedly administered intraperitoneally to maintain anesthesia depth based on reflex responses (painful stimulation to the foot with forceps-pedal reflex).

The experimental 70% hepatectomy model cited in the literature was first created by American scientists Higgins and Anderson [27] in the 1900s: they removed the left lateral and median lobes of the liver in a 70% hepatectomy in rats under ether anesthesia [28]. In studies, they observed that 75% of the liver weight loss was resolved within the first month.

In the experimental animal resection model, the regeneration response is maximized when 70% of the liver is resected. When smaller amounts of parenchyma are removed, the restoration proceeds more slowly, while resections above 70% disrupt DNA synthesis and mitotic activity. After a standard partial hepatectomy removing nearly 70% of the liver in rats, nearly 95% of hepatocytes divide during regeneration in young animals. In older animals, the liver repair is clearly slower and this rate falls to 70%. Many studies have shown that replication after partial hepatectomy begins after nearly 12 hours in rats [29,30].

In our study the hepatic resection method was performed at Dokuz Eylül University Health Sciences Institute Experimental Animals Laboratory with all surgical interventions to rats completed under aseptic conditions. All subjects were fasted from 24:00 before the operation with only water allowed. Rats were operated on in supine position under sterile conditions with a 3 cm midline abdominal incision. As described by Madrahimov [31], the left lateral (33.37 ± 1.90%) and median lobe (38.17 ± 1.45%) pedicles were tied with 4/0 polyglactin 910 sutures (Vicryl, Ethicon, Somerville, NJ, United States) and 70% hepatectomy was completed as described by Higgins and Anderson [27]. After the surgical procedure, fascia and skin were closed with 4/0 continuous silk sutures and cleaned with povidone iodine.

From the sixth postoperative hour, oral water and diet intake was allowed and the rats were housed in the laboratory with 12-hour light/dark cycle, noise insulation, and fixed temperature and humidity, with free access to food and water. In our study, the rats were divided into 5 groups according to the experimental protocol:

Group 1 (sham group) (n = 7): rats had the abdomen opened, with liver and hepatic pedicles revealed and no other procedure performed. This group was sacrificed after 7 days and livers were taken as pathology samples.
Group 2 (n = 7): Rats had 70% hepatectomy (left lateral lobe [LLL] + median lobe [ML]) performed. This group was sacrificed after 7 days and residual livers were taken as pathology samples.
Group 3 (n = 7): Rats had 70% hepatectomy (LLL + ML) performed with a 15-minute Pringle maneuver to the hepatic pedicles. This group was sacrificed after 7 days and residual livers were taken as pathology samples.
Group 4 (n = 7): Rats had 70% hepatectomy (LLL + ML) performed and 12 hours after hepatectomy, intraperitoneal tacrolimus (1 mg/kg/day) was administered for 5 days. This group was sacrificed after 7 days and residual livers were taken as pathology samples.
Group 5 (n = 7): Rats had 70% hepatectomy (LLL + ML) performed with a 15-minute Pringle maneuver to the hepatic pedicles (Fig 1). Twelve hours after hepatectomy intraperitoneal tacrolimus (1 mg/kg/day) was administered for 5 days. This group was sacrificed after 7 days and residual livers were taken as pathology samples.

With the aim of assessing liver tissue regeneration using pathologic investigation and mitotic index identification, rats had phosphohiston H3 (PHH3) immunohistochemical staining applied to determine mitosis. For this, liver tissues were fixed in formalin and submerged in paraffin, and sections of 4 μm thickness were then prepared. Slices were left at 56°C for 60 minutes. A Benchmark ULTRA automatic immunohistochemical staining device (Ventana Medical Systems, Tucson, Ariz, United States) was used for immunohistochemical staining. Sections were deparaffinized with EZ Prep solution. To reveal the antigens, CC1 standard (Tris, Borate, EDTA, ph 8.4) was used at 95°C for 76 minutes. An ultraviolet inhibitor (3% H_2O_2) was then applied for 4 minutes at 37°C. Sections were incubated at 37°C for 60 minutes in primary antibody (PHH3 polyclonal, 1:500, Cell Marque, Rocklin, Calif, United States). Sections were then incubated for 37°C for 8 minutes with HRP UNIV MULT (peroxidase-labeled streptavidin, streptavidin biotin kit) and later had hydrogen peroxide (H_2O_2) combined with 3,3′-diaminobenzidine chromogen applied for 8 minutes. Then inverse staining was applied with Harris hematoxylin at 37°C for 4 minutes.

Sections with immunohistochemical staining were assessed by an expert pathologist for mitosis numbers. PHH3-stained sections for each subject were assessed in 10 different magnification areas at 40x magnification with a light microscope. Mitosis numbers were counted and the mitotic index was determined (Fig 2).

The weight index identification was calculated by using the ratio of rat liver to total rate body weight in Group 1 (sham group). All liver weights were found to be 5.67% of rat weight. The ratio of liver weight of sacrificed rats to the remaining liver weight after 70% hepatectomy was calculated with the value multiplied by 100 to find the weight index. Results are given as %.

The formula is as follows: weight index = [sacrificed liver weight/(total liver weight − resected liver weight)] × 100.

Statistical Analysis

All values were analyzed using SPSS 22.0 for Windows (IBM, Armonk, NY, United States). Groups 1–5 were compared using the Kruskal-Wallis test. Two-way comparisons used the Mann-Whitney U test. $P < .05$ was accepted as statistically significant.

RESULTS

After hepatectomy, there were no rats that died in Group 1 and Group 2. In Group 3, 1 rat died on the first postoperative day, in Group 4, 1 rat died on the operating day, and in Group 5, 1 rat died on the first postoperative day and 1 on the second postoperative day. All of the dead rats had an autopsy performed, none of which revealed any significant pathology. The measured and calculated parameters related to all groups are presented in Table 1.

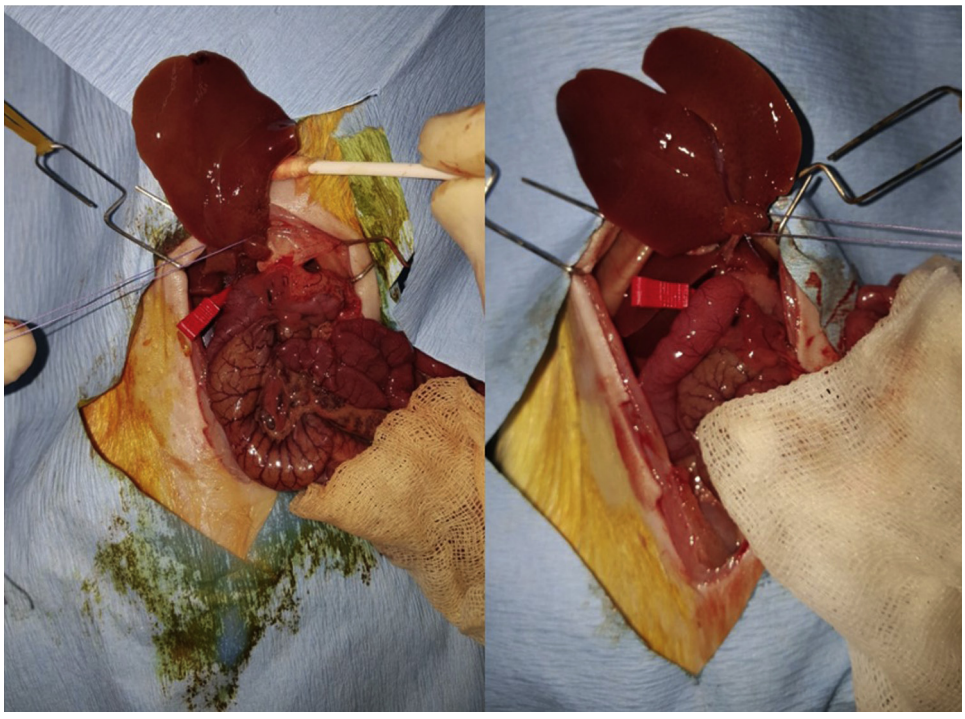

Fig 1. Pringle maneuver to the hepatic pedicles and suspension of portal pedicles before resection of left lateral lobe (on left) and median lobes (on right).

Assessment of Mitotic Index Results

When the Kruskal-Wallis test was applied to Groups 1–5, there was no statistically significant difference observed in terms of mitotic index ($P = .575$). The mitotic indices in the groups are presented in Figure 3.

When Group 2 and Group 1 (sham group) were compared in terms of mitotic index with the Mann-Whitney U test, there was no statistically significant difference identified ($P = 1.000$).

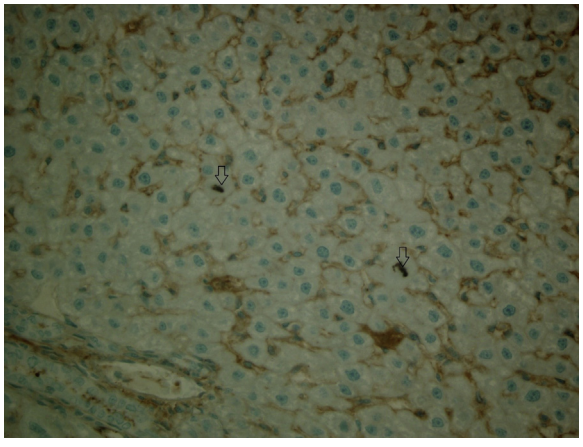

Fig 2. Mitotic figures with positive staining for PHH3 immunohistochemistry (white arrows) (×40).

When Group 3 and Group 1 were compared in terms of mitotic index with the Mann-Whitney U test, there was no statistically significant difference identified ($P = .179$).

When Group 4 and Group 1 were compared in terms of mitotic index with the Mann-Whitney U test, there was no statistically significant difference identified ($P = .581$).

When Group 5 and Group 1 were compared in terms of mitotic index with the Mann-Whitney U test, there was no statistically significant difference identified ($P = .861$).

Investigation of Weight Index Results

When the Kruskal-Wallis test was applied to Groups 2–5, there was no statistically significant difference between the groups in terms of weight index ($P = .431$). The weight indices identified for the groups are presented in Figure 4.

When Group 3 and Group 2 (only 70% hx) were compared using the Mann-Whitney U test in terms of weight index, there was no statistically significant difference identified ($P = .668$).

When Group 4 and Group 2 were compared using the Mann-Whitney U test in terms of weight index, there was no statistically significant difference identified ($P = .775$).

When Groups 5 and 2 were compared using the Mann-Whitney U test in terms of weight index, there was no statistically significant difference identified ($P = .570$).

Table 1. Statistical Data Related to Rat Groups

Rat Group	Initial Rat Weight (g)	Sacrificed Rat Weight (g)	Resected Liver Weight (g)	Sacrificed Liver Weight (g)	Weight Index (%)	Mitotic Index
1						
n	7	7		7		7
Mean ± std. deviation	272.85 ± 27.35	287.00 ± 28.29		16.42 ± 3.58		0.09 ± 0.11
Median (min-max)	274.00 (228.00–300.00)	286.00 (247.00–317.00)		18.20 (11.00–20.00)		0.10 (0.00–0.30)
2						
n	7	7	7	7	7	7
Mean ± std. deviation	274.29 ± 16.77	264.29 ± 23.7	6.46 ± 1.52	14.49 ± 1.74	162.44 ± 34.21	0.09 ± 0.11
Median (min-max)	275.00 (247.00–300.00)	258.00 (231.00–300.00)	6.20 (4.40–8.80)	13.80 (12.60–17.80)	149.91 (123.30–213.70)	0.10 (0.00–0.30)
3						
n	7	6	7	6	6	6
Mean ± std. deviation	267.14 ± 16.85	261.83 ± 19.78	6.09 ± 0.84	15.20 ± 0.96	173.34 ± 8.58	0.23 ± 0.25
Median (min-max)	270.00 (241.00–290.00)	262.00 (235.00–283.00)	6.20 (4.80–7.40)	15.40 (13.80–16.20)	173.30 (162.63–183.62)	0.15 (0.00–0.70)
4						
n	7	6	7	6	6	6
Mean ± std. deviation	291.43 ± 9.31	266.17 ± 34.03	6.86 ± 1.30	14.97 ± 2.05	154.16 ± 27.22	0.12 ± 0.24
Median (min-max)	293.00 (279.00–300.00)	269.00 (225.00–323.00)	7.20 (5.20–8.80)	14.20 (12.80–17.80)	148.27 (127.92–201.65)	0.00 (0.00–0.60)
5						
n	7	5	7	5	5	5
Mean ± std. deviation	270.29 ± 16.20	212.60 ± 8.08	6.97 ± 1.02	14.08 ± 1.48	165.53 ± 20.06	0.24 ± 0.36
Median (min-max)	268.00 (252.00–300.00)	213.00 (203.00–221.00)	7.20 (5.80–8.20)	13.40 (12.60–16.40)	159.67 (148.44–199.53)	0.00 (0.00-.80)
Total						
N	35	31	28	31	24	31
Mean ± std. deviation	275.20 ± 19.16	260.97 ± 33.10	6.59 ± 1.18	15.09 ± 2.24	163.74 ± 24.53	0.14 ± 0.22
Median (min-max)	275.00 (228.00–300.00)	262.00 (203.00–323.00)	6.30 (4.40–8.80)	14.60 (11.00–20.00)	164.14 (123.30–213.70)	0.10 (0.00–0.80)

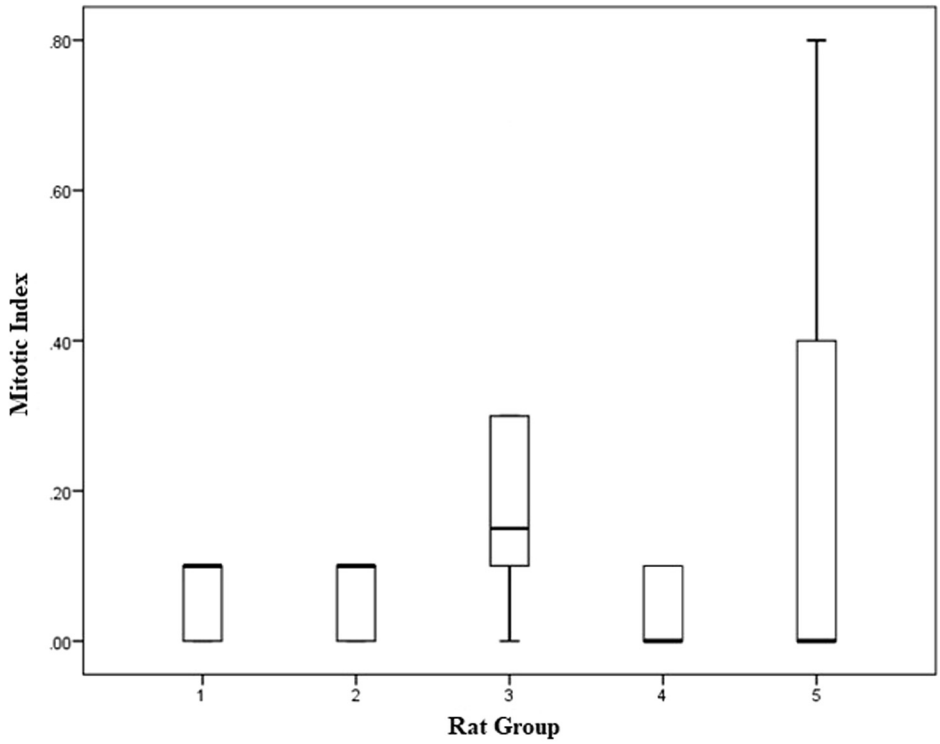

Fig 3. Mitotic indices in rat groups.

DISCUSSION

Clinically, the best examples of liver growth observed are the growth of donor and receiver livers in living donor transplantations, growth of the receiver liver after split liver transplantation, and liver growth after liver injury or partial hepatectomy. Currently, split liver grafts are not only used in children, but are increasingly used for adults too.

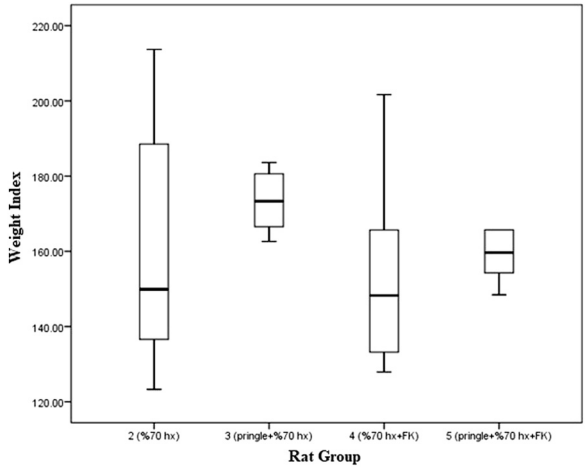

Fig 4. Weight indices in rat groups.

Immunosuppressive medications transformed treatments after solid organ transplants; however, some immunosuppressive medications used after liver transplantation suppress hepatic regeneration. The suppression of regeneration constitutes a significant risk after liver transplantation with small-volume grafts. Tacrolimus is an effective immunosuppressive medication commonly used after liver transplantation. There are a variety of publications related to the effect of tacrolimus on liver regeneration. A study by Chavez et al [32] administered 70% hepatectomy to rats and administered 1 mg/kg/day tacrolimus to rats from the third day before the operation to the seventh day after the operation. The results showed a 431.8% regeneration in the group with 70% hepatectomy and tacrolimus administration (n = 9) and 358% regeneration in the group with only 70% hepatectomy (n = 4); this difference was statistically significant ($P < .0001$).

The most comprehensive pioneering study related to the effect of tacrolimus on liver regeneration was by Francavilla et al in 1989 [33], who performed 40% (n = 8) and 70% (n = 15) hepatectomy in rats and identified that the group administered tacrolimus for 4 days before the operation had statistically significant regeneration compared to the control group (higher on the postoperative first day). This effect was not observed in rats that did not undergo hepatectomy. They considered that the suppressing effect of tacrolimus on T-cell functions removed the inhibiting effect of the immune system on regeneration. Francavilla et al published

another study in 1991 [26] where they performed 70% hepatectomy (n = 10) on T-cell deficit rats (investigating the direct effect of only tacrolimus by removing the effect of the cellular immune system). The rats were administered daily tacrolimus beginning on the third day before the operations and continuing afterwards. Rat liver tissue from rats sacrificed on the first postoperative day was identified to have statistically significant rates of regeneration increase compared to the control group. An in vitro study by Francavilla et al also found that tacrolimus administered directly to hepatocyte culture did not increase regeneration, but when administered to hepatocyte culture with rat serum regeneration increased. They showed that when tacrolimus is administered with rat serum in particular, regeneration increased further. After the study, they considered that tacrolimus increased regeneration with a non-immune mechanism [26].

Tamura et al [34] performed 70% hepatectomies on rats and administered 1 mg/kg tacrolimus per day for 3 days before and 2 days after the operation, sacrificing rat groups every 12 hours after the operation. They identified that DNA synthesis speed reached maximum values in the thirty-sixth hour. The DNA synthesis speeds in the 36th and 48th hours were statistically significant in favor of the tacrolimus group when compared to the control group (mean 2.2 times faster). Their investigation demonstrated that the effect of tacrolimus did not occur by changing hepatocyte growth factor and transforming growth factor β1 values but by reducing natural killer cell activity.

In the literature, a number of studies have investigated the effects of tacrolimus related to ischemia reperfusion injury. For example, Kawano et al [35] administered 1 mg/kg/day tacrolimus to rats for 4 days, administered 60 minutes normothermic ischemia to the liver, and allowed reperfusion. The 7-day survival rates were 80% in the group given tacrolimus and 50% among those not given tacrolimus ($P < .05$). The serum tumor necrosis factor and aspartate transaminase values in the tacrolimus group were found to be significantly lower.

In their study Wakabayashi et al [36] administered 1 mg/kg/day tacrolimus to rats for 4 days and then clamped the hepatoduodenal ligament for 30 minutes before allowing reperfusion. Investigations using 31P magnetic resonance spectroscopy showed the liver energy improvement status was better compared to the control group and histologic investigation showed that there was less ischemic injury in the group given tacrolimus. Clinically, ischemia reperfusion injury is observed in the liver after the Pringle maneuver is performed to control bleeding during elective liver tumor surgeries and liver transplantation operations. After liver transplantation operations completed with a graft at the margin with the Pringle maneuver applied, the regeneration capacity of the graft liver is vitally important. A variety of medications have been tested to increase this capacity; Tanemura et al [37] administered thrombomodulin to rats, Taki-Eldin et al [38] administered triiodothryonine, Yirmibesoglu et al [39] administered lisinopril, and Huranuma et al [40] administered edaravone. Then the researchers induced ischemia and reperfusion with the Pringle maneuver in partial hepatectomy models and showed that the negative effects of ischemia reperfusion were reduced.

In our study, we investigated the effect of the potent immunosuppressive medication commonly used after liver transplantation of tacrolimus on hepatic regeneration in an experimental 70% hepatectomy model. From the day after the operation, intraperitoneal 1 mg/kg dose of tacrolimus was administered for 5 days and the effects were examined at the end of the first postoperative week. Rats with and without Pringle maneuver applied were compared to control groups and we observed that tacrolimus did not cause a significant difference in the mitotic index and weight index. This result shows that despite the fact that tacrolimus has not been shown to have an effect on increasing regenerative capacity of the liver, it is important in terms of not reducing the regenerative capacity. Therefore, after partial graft liver transplantation, we conclude that tacrolimus, when administered to ensure effective immunosuppression in patients, can be used without considering the side effect of reducing regeneration.

As demonstrated by Tang et al [41], most studies investigating liver regeneration show mitosis peaks on the second day after hepatectomy and then gradually reduce. In our study, rats were sacrificed on the seventh postoperative day, which may have caused our mitotic index data to be lower than those of other studies.

Histologic assessment of liver regeneration in our study used phosphohistone H3 immunohistochemistry to obtain mitotic index results. There are many immunohistochemical methods used to measure antibody development against endogenous molecules such as proliferating cell nuclear antigen, Ki-67, anti-phosphoamino acid, and anti-ribonucleotide reductase for assessment of liver regeneration. As demonstrated by Furuta et al [42], proliferating cell nuclear antigen shows cell proliferation and is a stronger parameter for showing regeneration. Therefore the assessment of liver regeneration in our study may not have identified data as defined in other recent studies.

In conclusion, in light of the data we obtained, we can say that tacrolimus can be reliably used as an effective immunosuppressive treatment without reducing hepatic regeneration in partial liver graft receivers.

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