Diltiazem Increases the Liver Regeneration in Rats by Inhibiting TGF-β1

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Abstract

Transforming growth factor beta-1 (TGF- β1) is the most important inhibitory cytokine during the hepatic regeneration process. Diltiazem is a L-type calcium channel blocker that has inhibitory effect on TGF- β1. The aim of the present study was to determine the effect of diltiazem on hepatic regeneration. Sixty female Wistar Albino rats were used. Three groups were created; the control, low dose diltiazem and high dose diltiazem groups, each consisting of 20 rats. After partial liver resection (70% hepatectomy), saline

was introduced to control group, 5 mg/kg diltiazem was introduced to low dose group and 15 mg/kg diltiazem to high dose group intraperitoneally. Ten rats in each group were sacrificed on the first postoperative day and the remaining rats on the fifth day. Liver weight, mitotic rate and the Ki-67 ratio were measured for determining hepatic regeneration. Liver regeneration rate on the fifth postoperative day was significantly higher both in the low dose and high dose diltiazem groups than the control group (Low diltiazem *vs* control: $P<0.001$; High diltiazem *vs* control: $P<0.001$). No significant difference was found between the groups regarding the number of mitoses on the first and fifth days following partial hepatectomy ($P>0.05$). The Ki-67 ratio on the first postoperative day was significantly higher both in the low dose and high dose diltiazem groups than the control group (Low diltiazem *vs* control: $P<0.001$; High diltiazem *vs* control: $P<0.001$). Diltiazem increases liver regeneration by inhibiting TGF-β1.

Keywords: Partial hepatectomy, liver regeneration, diltiazem

Introduction

One of the most important limitations in hepatic surgery is the functional capacity of the remaining liver tissue following major hepatic resection. Many studies have been conducted on the complex regeneration process of the liver, which is affected by various different cytokines and hormones (Diehl & Rai,1996, Bucher,1991, Noguchi,1991, LaBrecque,1994). During the regeneration process, some cytokines act to increase the regeneration, whereas the other some have inhibitory effects. A number of model have been proposed for the study of liver regeneration. The most common studied model is partial hepatectomy (Mao SA,2014).

It has been previously demonstrated that TGF- β1, which source has been proven to be the spleen, is the most important inhibitory cytokine during the hepatic regeneration process (Akahoshi, Hashizume, Tanoue, et al.2002, Tomikawa, Hashizume, Highashi et al.1996, Zhong, Tsukada, Rehman, et al.2010, Houck & Michalopoulos 1989, Ueda, Yamanoi, Hishikawa, et al.2003, Friedman, Flier, Underhill 1993). It is also known that splenectomies increase hepatic regeneration (Perez,1983, . Perez & Romero,1958).

Diltiazem is a L-type calcium channel blocker, which is used in the treatment of hypertension and arrhythmia (Kayaalp,1990). Several studies have shown that diltiazem prevents renal interstitial fibrosis and peritoneal fibrosis by inhibition of TGF-β1(Kesgin, Oktay, Yavascaoglu, Simsek et al, 2002). It has been also demonstrated that diltiazem causes TGF-β1 inhibition in liver cell cultures (Crenesse, Tornieri, Laurens, et al,2002).

Although diltiazem causes TGF-β1 inhibition in various tissues, its effect on hepatic regeneration is still unknown. The aim of the present study was to determine the effect of diltiazem on hepatic regeneration.

Material and Methods

After approval of the ethical committee of ****** University, the study was conducted in November 2012 in the Experimental Animal Research Laboratory at ***** University's Medical Faculty. Sixty 8-10-week-old Wistar Albino type female rats, weighting 200-250 were used for the study.

The rats were kept at 23 °C, with 50% humidity, and a 12 h light and dark cycle for 2 weeks, with free access to water and food. Regular replacement of cage and clean bedding before the experiments were provided. Three groups were formed, including 20 rats in each group:

Control group (Saline (0.09% NaCl) introduced by intraperitoneal way after 70% partial hepatectomy)

Low dose diltiazem group (5 mg/kg introduced by intraperitoneal way after 70% partial hepatectomy)

High dose diltiazem group (15 mg/kg introduced by intraperitoneal way after 70% partial hepatectomy)

As the regenerative responses change daily, the operations were conducted in the first half of the day. The rats to be operated were on fasting one night prior. For anesthetic purposes, 80/12 mg/kg ketamine HCl (Ketalar, Eczacıbaşı) / xylene HCL (Rompun, Bayer) were intramuscularly administered. After controlling for sufficient anesthesia depth by determining the loss of cornea and gag reflexes, the rats' abdomens were shaved and they were attached to the operation board by their four extremities. The abdomen was cleaned with 10% povidone iodine and an abdominal laparotomy was performed with a midline incision of 2.5 cm. The pedicles of the left lateral and median lobes were ligated with 4/0 silk. A approximately 70% partial hepatectomy was performed by resection of median and left lobes of the liver, as defined by Higgins and Anderson (Higgins & Anderson.,1931). The removed liver tissue was weighed and recorded for each animal. After this stage, before closure of the abdomen, Saline solution (0.09% NaCl) was given to the control group, 5 mg/kg diltiazem (Diltizem–L® enjektabl 25 mg, Mustafa Nevzat, Istanbul, Turkey) was administered intraperitoneally in the low dose diltiazem group, and 15 mg/kg diltiazem in the high dose diltiazem group. Then the laparotomies were closed with a single layer of continuous sutures.

The weight of regenerated liver

The wet weight of the removed left and median liver lobes were determined after partial hepatectomy (RA) by assuming that 70% of whole weight of the liver was resected, and total weight of liver before resection was calculated (TA=RA/0.7). Total weight of liver on the day of sacrificing (SA) was divided by the calculated assumed weight before resection (TA) and the amount of resection was calculated with the Kwon formula (SA/TA x 100)(Kwon, Uetsuji, Yamamura et al, 1990). From another perspective, this formula provides information about the weight proportion reached on the day of sacrifice compared to the weight before resection.

Histological and immunological assessment

Histological and immunological assessments were performed in the Pathology Laboratories of ******** Teaching and Research Hospital. The obtained tissue samples were fixed in buffer formalin (10%). Five µm sections were taken from the prepared paraffin blocks. The mitoses were counted under 10x large magnification field stained with hematoxyline & eosin (Olympus, C x 31).The proportions were given as percentage(Selzner & Clavien, 2000).

To determine Ki-67 expression, 5µm thick sections were taken to lame with 'Poly- L-Lysine'. Ki-67 (SP6) (Neomarkers, USA) was administered using the immunohistochemical staining procedure with ready-for-use rabbit monoclonal antibody. The sections that were left at 37ºC for one night were held at 60 ºC for 60 minutes on the next day. For deparafinization, they were held in xylazine for 5 minutes, repeating three times, and in alcohol for 5 minutes, repeating three times, as well (first absolute, then 96%). To rehydrate, they were held in distilled water for 5 minutes, repeating three times. For antigen retrieval, pressured vapor was administered for 5 minutes to thelame, which were placed into EDTA buffer (pH:8) and diluted at a ratio of 1/10 with distilled water. The sections, which were cooled at room temperature, were washed with distilled water. To inhibit endogenous peroxidase activity, they were incubated in 3% hydrogen peroxide for 20 minutes. Then, the sections were washed with distilled water for 5 minutes, repeating three times. They were washed with PBS (phosphate buffer saline) for 5 minutes, repeating three times, as well. Furthermore, they were washed in blocking solution for 5 minutes. They were incubated with Ki-67 monoclonal antibody at room temperature for 60 minutes. After washing with PBS solution for 5 minutes, repeating three times, the routine staining procedure with HRP AEC (Horseradish peroxidase - 3-amino-9-ethyl-carbazole) method was completed (Wintzer, Zipfel, Mönting, et al. 1991). While evaluating the Ki-67 staining pattern, the nuclear staining in 1000 cells were counted. The proportion of the positively stained nucleuses was given as percentage.

Statistical Analysis

The data were analyzed using SPSS for Windows 11.5 package program. The distribution of continuous variables was determined to be normal or not with the Shapiro-Wilk test. The descriptive statistics were demonstrated as median (minimum-maximum). The significance of the difference between groups in terms of median values was evaluated with the Kruskal-Wallis test. When the results of the Kruskal-Wallis test statistics were significant and non-parametric, the multiple comparison test was used to determine the factors causing the difference. Values of $P<0.05$ were accepted as statistically significant. However, Bonferroni's correction was used to control for Type I errors in all possible multiple comparisons (Conover WJ, 1980).

Results

Regeneration

No significant difference was found between the groups on the first postoperative day according to the weight measurements of the liver remnant tissues ($P>0.05$). However, the liver regeneration rate on the fifth postoperative day was significantly higher both in the low dose and high dose diltiazem groups than the control group (Low diltiazem *vs* control: $P<0.001$; High diltiazem *vs* control: $P<0.001$). The difference between high dose and low dose diltiazem groups was not significant ($P>0.05$).

Table 1. Regeneration Rates of Groups at the first and 5th days after partial hepatectomy

Groups	**First day (n=10) (%, min-max)**	**5th day (n=10) (%, min-max)**
Control	35.85 (24.1-54.7)	42.7 (38.9-57)[b,c]
Low Dose	45.35 (34.3-55.2)	66.45(41.4-97.6)[b]
High Dose	50.3 (43.2-60.2)	97.9 (62.1-125.4)[c]
*P*value[a]	>0.05	<0,001

a $P<0.025$ was considered statistically significant according to the Bonferroni Correction
b Control group *vs* Low dose Diltiazem group ($P<0.001$)
c Control group *vs* High dose Diltiazem group ($P<0.001$)

Mitosis

No significant difference was found between the groups regarding the number of mitoses on the first and fifth days following partial hepatectomy ($P>0.05$), (Table 2).

Table 2. Number of Mitotic Rates of Groups in the 1th and 5th days after partial hepatectomy

Groups		1th day (n=10)	5th day (n=10)
Control	**[(number, median (min-max)]**	1 (0-2)	0 (0-1)[b]
Low Dose	**[(number, median (min-max)]**	3 (0-12)	0.5(0-7)[c]
High Dose	**[(number, median (min-max)]**	1 (0-2)	0.5 (0-2)[b,c]
p-value[a]		>0.05	>0.05

a According to Bonferroni correction p<0,025 was accepted statistically significant.
b The difference between Control and low dose group was not statistically significant. (p>0.05)
c The difference between control and high dose group was not statistically significant (p>0,05)

Ki-67 ratio

The Ki-67 ratio on the first postoperative day was significantly higher both in the low dose and high dose diltiazem groups than the control group (Low diltiazem *vs* control: *P*<0.001; High diltiazem *vs* control: *P*<0.001). However, no significant difference was found between the groups on the fifth postoperative day (*P*>0.05), (Table 3). The Ki-67 did not significantly alter in the control group between the first and fifth postoperative days (*P*>0.05). On the other hand, the Ki-67 ratio significantly decreased on the fifth postoperative day both in the Low dose (*P*=0.008) and High dose (*P*=0.002) diltiazem groups when compared with the first postoperative day.

Table 3. Ki-67 ratio of Groups in the 1th and 5th days after partial hepatectomy

Groups		1th day (n=10)	5th day (n=10)
control	**[%, median (min-max)]**	0.5 (0-5)[b,c]	2 (1-8)
Low Dose	**[%, median (min-max)]**	27.5(14-43)[b]	2.5(0-26)
High Dose	**[%, median (min-max)]**	9 (6-28)[c]	0.5 (0-4)
p-value[a]		<0,001	>0.05

a According to Bonferroni correction p<0,025 was accepted statistically significant
b The difference between Control and low dose group was not statistically significant. (p>0.05)
c The difference between control and high dose group was not statistically significant (p>0,05)

Discussion

The liver is the only organ in humans and animals that has the capacity to grow and regenerate (Akcan, Kucuk, Ok et al.2006). Hepatic resections are performed due to many benign and malignant causes. Although individuals with healthy liver tissue have the ability to tolerate hepatectomy up to 80%, functional insufficiency may develop sometimes until the remaining liver tissue regenerates (Makuuchi, Takayasu, Takuma,1984).

Many cytokines play a role during the regeneration process that begins with lipopolysaccharides and cytokines from intestinal origins. Tumor necrosis factor- alpha (TNF-α) and interleukin-6 (IL-6) induce hepatocytes from G0 phase to G1 phase. On the other hand, hepatic growth factor (HGF)

and epidermal growth factor (EGF) stimulate the progression of the cell cycle and regeneration(Roy-Chowdur,2006). The most important inhibitor cytokine for regeneration is TGF-β1(Zhong, Tsukada, Rehman et al, 2010, Tao,Wang,Chen et al, 2017 Houck&,Michalopoulos, 1989, Ueda, Yamanoi, Hishikawa et.al, 2003). Previous studies have shown that the spleen-derived TGF-β1 plays inhibitory role in hepatic regeneration and induces fibrosis (Akahoshi, Hashizume, Tanoue, et al.2002, Zhong, Tsukada, Rehman, et al. 2010). On the other hand, several studies have demonstrated that TGF-β1 monoclonal antibodies increase hepatic regeneration through TGF-β1 blockage (Akcan, Kucuk, Ok et al. 2006, Makuuchi, Takayasu, Takuma 1984). TGF-β1 shows this effect by direct inhibition of cell proliferation and induction of apoptosis (Zhong, Tsukada, Rehman, Parsons CJ, et al,2010, Ueda, Yamanoi, Hishikawa, et al.2003).

Nakamura et al. demonstrated in a cirrhotic rat model that TGF-β1 inhibition prevents liver fibrosis and increases the hepatocyte regeneration. Further, Akahoshi et al (Akahoshi, Hashizume, Tanoue, et al. 2002). showed the spleen as a source of TGF-β1. Ueda et al. proved the same effect of spleen-derived TGF-β1.

Diltiazem, which is a L-type calcium channel blocker, is a widely used in clinical practice due to its anti-hypertensive and anti-arrhythmic properties. It does not produce hypotension in either normotensive individuals or rats; furthermore, its inhibitory effect on TGF-β1 is higher than other calcium channel blockers (Kayaalp O. 1990, Wang, Tao, Yuan et al. 2010, Fang, Yen, Chen, et al. 2006, Cuschieri, Gourlay, Garcia, et al. 2002). It has been demonstrated that diltiazem has various effects in many tissues by inhibiting TGF-β1 at the level of receptor and intracellular conductive pathways (Kesgin, Oktay, Yavascaoglu, et al..2002, Wang, Tao, Yuan, et. Al. 2010) . Moreover, it prevents ischemic-reperfusion injury related apoptosis by inhibition of the TGF-β1-jnk pathway in hepatocyte cell cultures (Crenesse, Tornieri, Laurens, et.al,2002) .

It has been determined that best liver regeneration response in rats is following 70% partial hepatectomy (Higgins & Anderson, 1931). Active cell replication starts within 24 hours following partial hepatectomy and it continues until reaching the initial weight of the organ. A significant amount of regeneration occurs within the first ten days and this process is completed in 4-5 weeks(Michalopoulos& DeFrances,1997) . However, in humans, the liver reaches its previous size in 3-6 months. In the presence of cirrhosis, this period may increase up to 9-15 months (Nagasue, Yukaya, Ogawa, et.al. 1987).

Ki-67 is a nuclear protein that is used to measure the tissue proliferation index, as it is directly proportional to cell proliferation at a high rate. It is correlated with ribosomal RNA transcription. During the interphase,

it could be observed in the cell nucleus at a high rate. Although it is most commonly observed during the interphase, it can be seen in all active phases of the cell cycle. However, it is not present in cells during the resting phase (G0) (Gerdes, Lemke, Barsch, et. al, 1984) In the present study, the Ki-67 ratio was to be found highest in the low dose diltiazem group. Additionally, the Ki-67 ratio also was higher in the high dose diltiazem group than the control group.

It has been previously shown that most of the DNA synthesis was done by hepatocytes on the first day following partial hepatectomy. Similarly, when we compared with the control group, the Ki-67 ratios both in the low dose and high dose diltiazem groups were significantly higher on the first day than the fifth day. The DNA synthesis occurs in biliary ductal cells and Kupffer cells at the 36th hour (Michalopoulos & DeFrances, 1997, Perek & Kapan, 2000) . In the present study, DNA synthesis decreased in all three groups after the second day.

In the present study, when all three groups were evaluated in terms of the number of mitosis, no significant difference was found. However, - although statistically not significant- mitosis was higher observed in the low dose diltiazem group on the first day than the other groups. It is known that mitosis in rats is set to zero on the 10th day following partial hepatectomy (Michalopoulos & DeFrances, 1997). Again depending on the dose, most of the mitosis in the liver is completed before the first day following partial hepatectomy; thus, no difference was observed between three groups on the first and the 5th days, in terms of the number of mitoses.

In conclusion, diltiazem increases hepatic regeneration by inhibition of TGF-β1 in a partially hepatectomized rat model. However, more experimental and clinical studies are required for its routine use in clinical practice for humans.

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