Original Paper

Impact of GLP-1 Analogue on Oxidative Damage and Hepatic Regeneration in Experimental 70% Hepatectomy Model

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ABSTRACT

Background/Aims: The purpose of our study is researching into impact of glucagon like peptide 1 (GLP 1) analogue on liver regeneration after major hepatectomy. **Methodology**: 24 wistar albino rats were consecutively divided into 3 groups. Group 1**:** Control (sham) group day 14 (n=8), Group 2: Liver resection group day 14 (n=8); 70% Liver resection was performed**,** Group 3: Study group day 14 (n=8); Subsequent to performing 70% liver resection, GLP-1 analogue was administered 2 times a day. (10 µgr/70 kg x 2 times). **After 14 day, rats were sacrificed. Oxi**dative stress and antioxidant enzymes and mitochondrial permeability transition, cytochrome-c, Bax, Bcl-2, caspase-3, caspase-8 and caspase-3 activity were examined. **Results: 70% Liver resection induced oxi**dative stress of liver tissue was ameliorated by GLP 1 induction. Administration of GLP increased Bcl-2 expression. Decreased expression of cytochrome-c was accompanied by a decrease caspase-3, caspase-8, and Bax expression and caspase-3 activity. **Conclusions:** Glp 1 induction plays a regenerative role in the major hepatectomy. This effect is dependent on modulation of the antiapoptotic and antioxidative pathways by GLP 1 expression.

Key Words: GLP-1, Liver resection, Oxidative damage, Regeneration.

INTRODUCTION

Major hepatectomy and advanced liver resections may be performed at many centers today. One of most important problems among surgical problems after liver surgery is state of residual liver volume. It is known that if a patient undergoes chemotherapy or existence of chronic liver disease is known, volume up to 40% is sufficient, for those with regular liver tissue up to 25% is sufficient (1). Despite all advanced diagnosis methods, during and after surgery, unexpected liver deficiency may emerge. Many patients may compensate this with early stage liver regeneration. Literature contains a lot of studies with the purpose of increasing liver regeneration. However, at the moment there is no effective agent used in our clinical practice.

GLP-1 analogues were discovered in 1923 at pancreas tissue. Afterwards, it was found that this analogue also exists at gastrointestinal system mucosa. Receptors are particularly located at pancreas islet cells, brain, kidney, heart and intestine mucosa. It has various effects and these effects are known in literature as "incritin effect" (2).

The most important effect is increasing concentration of peripheric insulin concentration. This effect is especially made by reducing insulin uptake of liver after oral glucose intake and increasing insulin quantity in circulation. At the same time, pancreas B cell functions increase. Other than this, incritins inhibit gastrointestinal system, pancreas secretion. They reduce intestine and stomach motility. Pancreatitis is reported as the most serious adverse effect. Today, GLP-1 analogues are used for the purpose of increasing insulin concentration for Type-II diabetes treatment (3-5).

Studies conducted allow us to know that insulin has significant hepatotropic effect on early stage liver regeneration (6). Our purpose in this study is to research impact of incritins, which are so effective at gastrointestinal and pancreas tissue, on liver generation and oxidative damage.

METHODOLOGY

The study was conducted from December 2012 to March 2013 at Istanbul University, Istanbul School of Medicine Experimental Medicine Research Center Laboratory with ethical review board approval dated 01.11.2012 and no. 2012/156.

Twenty-four male Wistar-albino rats weighing 250-300g were used in the study. All animals were housed in wiremesh bottomed cages in 12 hours light/12 hours dark cycle at a constant room temperature of 22 ± 2 ºC. They were fed a Standard chow diet and water. The study was approved by the local ethics review board.

Three equally numbered groups were created as follows:

Group 1: Control (sham) group day 14 (n=8).

Group 2: Liver resection group day 14 (n=8). 70% Liver resection was performed.

Group 3: Study group day 14 (n=8). Subsequent to performing 70% liver resection, GLP-1 analogue was administered 2 times a day. (10 µgr/70 kg x 2 times)

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TABLE 1. The results of Western Blot analysis.

Western blot expressions	Group 1 (control)	Group 2 (%70 hepatectomy)	Group 3 (glp 1 treatment)
cytochrome-c in cytosol	11.8±2	147.17±16	95.4±11
Bax	101.14±23	157.56±36	56.5±14
Bcl-2	93.5±13	38.7±7	167.56±31
Bax/Bcl-2	1.8±0.3	3.4±1.2	0.4±0.1
Caspase-3	98.4±15	172.44±38	101.34±14
Caspase- 8	87.38±22	156.65±42	98.47±21

Values with different letters have significance according to ANOVA test. BAx/ccl, The significance between control and group is p<0.01; group 2 and 3.

Surgical procedure

The surgical procedure was carried out under sterile conditions on the 2nd and 14th day of the therapy received by the experiment group. All rats were anesthetized by intramuscular administration of 80 mg/kg of ketamine hydrochloride (Ketalar, Eczacıbası) and 8 mg/kg of xylazin (Rompun, Bayer). Thus a general anesthesia and spontaneous respiration were maintained. The abdominal region was cleaned with povidone iodine.

Liver resection technique

After shaving abdomens, laparotomy was performed with midline incision, approximately 3 cm in length. After freeing pedicles of liver left lateral and median lobs, coronary, left lateral and gastrohepatic ligaments, they were tied with 3/0 silk and 70% hepatectomy was performed as described by Higgins and Anderson (7).

For the purpose of daily liquid supplement to all resected animals, subcutaneous SF 5 cc was performed. After surgery, all animals were sacrificed at day 2 and 14. Liver tissue samples were collected for bio-chemical and histological examination. 5cc of blood was drawn into an appropriate sample tube suitable for the investigation of biochemical parameters. Tissue samples were obtained for biochemical and histopathological investigations and transferred into 0.9% NaCl solution or into 10% formaldehyde solution. Blood samples were centrifugated and preserved at −80 º C for biochemical investigations. Residual liver tissue after surgery was weighed with precision balance. All results were statistically compared.

Preparation of postmitochondrial and cytoplasmic extracts

Preparation of postmitochondrial and cytosolic fractions was performed as previously described (8). Homogenates were centrifuged at 600 X *g* for 15 min at 4°C, and the supernatants were then centrifuged at 14 000 X *g* for 15 minute (min) at 4°C. Supernatants were used for HO-1, Bcl-2, Bax, caspase 3, and caspase 8 western blot analysis and oxidative parameters. For cytochrome-c analysis, the remaining supernatants were centrifuged at 100 000 X *g* for 1 hour at 4°C.

Western blot analysis

The mitochondrial and cytosolic fractions were lysed in modified RIPA buffer (50 mM Tris HCl, pH 7.4, 0.25% Na-deoxycholate, 10 % SDS, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 1% Glycerol, 1 µg/ml of aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml soybean trypsin inhibitor, 0.5 mM dithiothreitol, and 1 mM NaF). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed using the Bio-Rad Mini Protean III gel system according to Laemmli's method (9). Equal amounts of protein from each sample (50 mg/well) were loaded onto a 10% SDS–PAGE and proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. Following incubation, membranes were washed with PBS containing 0.01% Tween 20 (PBS-T) and then exposed to secondary antibodies. After washing, blots were visualized using the enhanced chemiluminescence (ECL) kit from Amersham (*Pharmacia Biotech., NJ, USA*) according to the manufacturer's protocol. The relative densities of the bands were quantified using the Vilber Lourmat-Bio-Profil imaging system. (*Vilber Lourmat Biotechnology, Marne-la-Vallée, France*). The following primary antibodies were used: mouse monoclonal anti-Bcl-2 (*SC-7382, Santa Cruz Biotechnology, CA*)**,** mouse monoclonal anti-Bax (*SC-7480, Santa Cruz Biotechnology, CA*)**,** Mouse monoclonal anti-cytochrome-c (*GTX13575, GeneTex, Inc., SA*), rabbit polyclonal anti-caspase 8 (*FLICE, Ab-4 Lab Vision Corporation, UK*), mouse monoclonal anti- α actin (*SC-32251, Santa Cruz Biotechnology, CA*), rabbit polyclonal anti-caspase 3 (*CPP32, Ab-4, Lab Vision Corporation, UK*). Secondary antibodies were used goat anti-mouse IgG-HRP **(***SC-2005, Santa Cruz Biotechnology, CA*)**,** goat anti-rabbit IgG-HRP (*SC-2030, Santa Cruz, Biotechnology, CA*), goat anti-rabbit IgG Peroxidase Conjugated, H+L (*AP124P,Chemicon, CA*).

Caspase-3 activity assay

Caspase-3 activity was measured with a colorimetric caspase-3 assay kit (Sigma-Aldrich, St. Louis, MO). The activity of the enzyme was expressed as the amount of p-nitroaniline liberated per mg protein per minute.(28)

Oxidant and antioxidant status

The levels of malondialdehyde (MDA) and glutathione (GSH) and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione transferase (GST) were determined in the post-mitochondrial fraction. The levels of MDA were measured with a thiobarbituric acid test (9). GSH levels were measured with 5,5-dithiobis-(2-nitrobenzoate) at 412nm.[21] SOD activities were assayed by its ability to augment the effect of riboflavin-sensitized photooxidation of orthodianisidine. GSH-Px and GST activities were measured using cumene hydroperoxides and 1-chloro-2,4-dinitrobenzene as substrates, respectively. The carbonyl content, used to determine the extent of oxidative damage to proteins, was measured according to the method by Reznick and Packer based on the spectrophotometric

detection of the reaction between 2,4-dinitrophenylhydrazine and protein carbonyls to form protein hydrazone (10).

Histopathological Methods

Mitotic index, inflammation and fibrosis level were investigated at liver tissue. During the study, number of mitosis was sought at 10 large growth sites. Ishak scoring was used for inflammation and fibrosis at liver (11).

Statistical analysis

Data were analyzed using SPSS 16.0 for Windows. Results with normal distribution were expressed as mean ± SD. Comparisons of normal distribution data were done by one-way ANOVA test. Results were considered statistically significant when the two tailed p value was less than 0.05.

RESULTS

During first 24 hours before sacrifice, we lost 2 rats from group 2. Early autopsy was performed on these rats. Intense acid was found inside abdomen. These rats were excluded from study and replaced with new animals. No mortality was determined at groups administered with GLP 1 analogue.

During evaluation of liver tissues which regenerate histopathologically, no significant difference in terms of fibrosis was found.

Upon comparing control group and experiment group with respect to mitosis index, significant difference was determined at mitosis index at group where GLP 1 analogue was used (group 3) in comparison with group where GLP 1 was not used on group 2. (**Figure 1**)

There was no significant difference in histopathological comparison of inflammation level among groups. Bio-chemical examination results of groups are presented in **Table 1**.

Oxidant and antioxidant status

The levels of MDA and protein carbonyl were found to be significantly higher in group 2 compared with group 1 (3.8±0.5 nmol/mg protein and 4.7±0.7 nmol/mg protein vs. 2.5±0.1 nmol/mg protein and 2.05±0.4 nmol/mg protein, p<0.01). The activities of GSH, GST, GSH-Px, and SOD were found to be significantly lower in group 2 compared with group 1 (16.1±2.6 nmol/mg protein, 135.2±20 nmol/min/mg protein, , p<0.01). GLP 1 administration significantly increased antioxidant enzymes and decreased oxidative damage compared with group 2 (**Figure 1**).

The results of Western Blot analysis

The results of western blot analysis were summarized in **Table 1**. Cytochrome-c expression in the cytosol: Cytochrome-c expression was found to be significantly higher in group 4 compared with group 1, p<0.001. In group 3, cytochrome-c expression was found to be increased significantly compared with all other groups including group 3, p<0.001. Bax expression: The expression levels of the proapoptotic protein, Bax, in the liver tissue were found to be significantly higher in group 2 compared with group 1, p<0.001. In group 3, Bax expression was found to be increased significantly compared with all other groups including group 2, p<0.001. Bcl-2 expression: The levels of the antiapoptotic protein, Bcl-2 was found to be significantly lower in group 2 compared with group 1, p<0.001. GLP 1 administration significantly increased the Bcl-2 expression in the liver tissue compared with group 2, p<0.05. The ratio of Bax/Bcl-2: The ratio of Bax/Bcl-2 was found to be significantly higher in group 2 compared with group 1, p<0.01. GLP 1 treatment significantly decreased the Bcl-2 expression in the liver tissue compared with all other groups including group 2, p<0.001. Caspase-3 and caspase-8 expression: The expression of caspase-3 and caspase-8 in the liver tissue was found to be significantly higher in group 2 compared with group 1, p<0.001. In group 3, caspase-3 and -8 expressions was found to be increased significantly compared with all other groups including group 2, p<0.001.

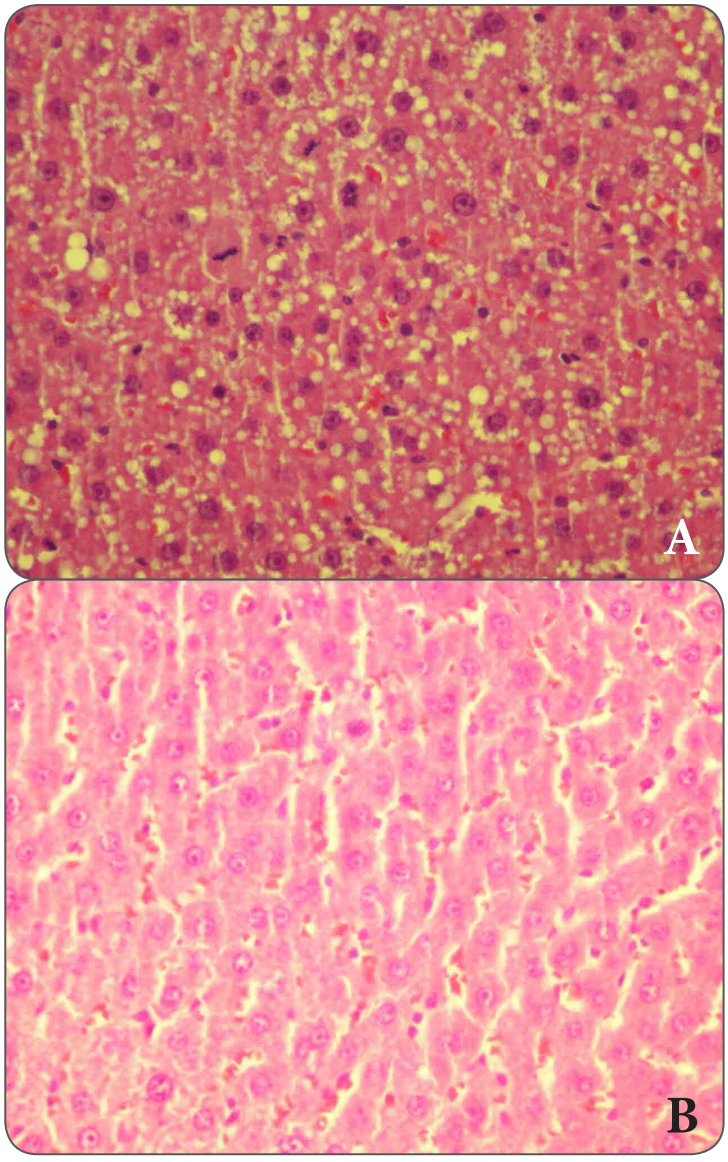

FIGURE 1. A) Macro and microvesicular fattening at liver, expansion at sinusoids and a great number of mitosis H/E X400 (GLP KC 1)**. B)** Expansion at sinusoids, hyperemia, mitosis H/E X400(SHAM KC 1).

Caspase-3 activity in the liver

The caspase-3 activity in the liver tissue was found to be significantly higher in group 2 compared with group 1, p<0.001. GLP 1 treatment significantly decreased the caspase-3 activity in the liver tissue compared with

group 2, p<0.001. In group 3, caspase-3 activity was found to be decreased significantly compared with group 2, p<0.001.

DISCUSSION

Hepatic regeneration is an important physiological process at early period, for major liver resections in particular. Regeneration response is typically dependent on proliferation of acinar structure of residual liver tissue. Many factors take a part in this process. It is known that Hepatocyte growth factor, TNF-α, IL-6, Epidermal growth factor (EGF), TGF- α, Norepinephrine, Insulin, gender hormones, Fibroblast growth factor (FBF), Vascular endothelial growth factor (VEGF), retinoic acid, thyroid hormones, growth hormone and many medications have positive effects on liver regeneration (6).

Liver is first transit point of insulin generated at pancreatic tissue. It was reported that liver atrophy develops at cases where direction of portal vein flow is changed with portacaval shunt (12). Development of atrophy was prevented by administering insulin directly to liver tissue at rats to which experimental portocaval shut was applied (13). Insulin has great impact on liver regeneration but direct mitogen impact was not determined (12).

Even though effects of GLP-1 analogues on liver regeneration are not known, their effects on various systems were researched. These researches concentrate particularly treatment for diabetes. The study by Vered aviv et al emphasizes that GLP-1 receptors could not be determined at liver cells but it is effective on metabolism of liver (14, 15, 16).

The study conducted by Daniel J. Cuthbertson et al determined that quantity of liver fattening is reduced at persons treated with GLP-1. In a similar study, Yong Ook Kim et al reported that GLP-1 reduces liver glucose output, oxidative stress and liver insulin resistance and liver fattening (3).

Efficiency of GLP-1 analogues was intensively studied also after pancreas islet cell and pancreas transplant and positive impact on glucose metabolism was clearly revealed (17, 18).

There are question marks regarding reliability of long term use of GLP-1 analogues and impact of cancer. A study reported that use of GLP-1 may trigger development of medullary carcinoma of thyroid and multiple endocrine neoplasia Type-2 but it has preventive effects on development of breast and colon cancer. (19, 20)

In the study we found that GLP-1 analogues increase ratio of mitosis of liver cell at early stage after resection in particular (Day 14) and during bio-chemical measurements ratio of Bax BCL-2 was found higher at experimental group in comparison with control group and statistically significant.

In the clinic, especially after major liver resection, some patients may present liver deficiency symptoms. This occurs especially at 3-5% patients with previously known chronic liver disease or undergoes intensive chemotherapy within post-operative 48-72 hours. (21) The International Study Group of Liver Surgery recently developed a consensus definition for post-hepatectomy liver failure namely 'the impaired ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which are characterized by an increased international normalized ratio and concomitant hyperbilirubinemia on or after postoperative day 5 (22). This arises clinically with acid existence, INR elevation, hyperbilirubinemia, encephalopathy and recovers with support therapy (23). In addition to scarcity of residual liver tissue after liver resection, may factors like bleeding, sepsis, ischemia, venous thrombosis may cause this. Other than support therapies, antioxidant N-acetylcysteine may be used to reduce free oxygen radicals that form as a result of ischemia reperfusion damage that occur at liver (24,25).

The study determined that GLP 1 analogue increases liver tissue quantity at early period. The study we conducted is a pilot study on this subject; it encouraged us for studies that would be conducted at cellular level later.

In conclusion, GLP-1 analogues increase early period regeneration after liver resection; they make positive contribution to tissue quantity of liver. These effects do not last in the long run. We are of the opinion that in clinical practice early period effects of GLP 1 analogues may be benefited from. For this purpose, cellular and clinical studies with a broad range of case series are needed.

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