

## Immunohistochemical and Biochemical Study of validity of Major Salivary Glands on Liver Regeneration after Partial Hepatectomy in Rats

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**Abstract: Background:** Major salivary glands, namely; parotid, submandibular and sublingual glands, play vital role in human welfare. **Aim of study:** To investigate the possible effects of the removal or existence of all major salivary glands on rat liver regeneration following partial hepatectomy (PH). **Material and Methods:** Thirty male albino rats were randomly allocated into two groups;; Group-I (comprised of ten rats) served as sham control group and Group-II (comprised of twenty rats) served as experimental group and were further subdivided randomly into two equal subgroups; IIA and IIB, which underwent either removal or survival of all their major salivary glands respectively. After two weeks recovery period, all rats of the experimental subgroups endured comparable PH. Then, half of the animals of both groups were euthanized after one week of PH, whereas the other half was sacrificed after two weeks. Before euthanization, blood samples were taken for serum alanine transaminase (ALT) analysis to evaluate liver function. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA), as a marker of cellular proliferation, was done for the remaining liver tissue. **Results:** Our quantitative ALT and PCNA analyses advocated that lack of all major salivary glands interfered with hepatic function and delayed its regeneration. **Conclusion:** Salivary glands are essential for efficient liver regeneration.

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**Keywords:** Partial hepatectomy, major salivary glands, liver.

### 1. Introduction

Generally, saliva has a considerable healing power as it contain several immunologically active substances such as immunoglobulin, antibacterial factors, and variable growth promoting factors that could possibly coordinate both normal and regenerative processes (**Brand and Veerman 2013**). Theoretically, salivary growth promoting factors together with other salivary immunologically active components could contribute to local as well as remote wound healing, possibly through their putative endocrine pathway. Locally, saliva enhanced healing of extraction wound conceivably via its lysozyme, immunoglobulins, lactoferrin, peroxidase and other factors that are normally present in saliva (**Bodner et al. 1991**). In the meantime, extraction wound in sialoadenectomized rats showed a more intense and prolonged inflammatory process followed by a slower remodeling rate of granulation tissue. Also, the healing power of saliva was depicted on curing of cutaneous wound in rats, since licking enhanced healing of ventral cutaneous wound, compared to dorsal non-licked wound, as rats licked ventral ones easily (**Abbasian et al. 2010**).

Liver regeneration is orchestrated by multiple signaling molecules, cytokines, hormones and growth

factors, which activate hepatocytes to restore liver histology and function (**Kang et al. 2012**). Noteworthy, the imperative role of salivary glands and its epidermal growth factor (EGF) in liver regeneration after PH was recognized (**Jones et al. 1995**). Unexpectedly, the potential role of submandibular salivary gland (SMG) in liver regeneration was attributed to alteration in its mineral content, such as zinc, iron, magnesium and calcium, upon PH. Thus, it was suggested that these minerals could influence the enzyme activities, signal transduction, the production or release of transcription factors involved in initiation and control of liver regeneration (**Milin et al. 2005**).

The aim of the current study was to compare alterations of ALT and liver PCNA expression in PH rats, one and two weeks of PH, upon removal or existence of all major salivary gland.

### 2. Materials and methods

#### Animals

Thirty adult male albino rats, with an average body weight of 200-250 grams, were used. All rats were allowed to equilibrate in standard conditioned animal houses under controlled temperature, a 12-hour alternating light/dark cycle, fed standard diet and tap water throughout the experimental period.

### Ethical approval statement

Approval for this study was obtained from faculty of dentistry, Tanta University Research Committee. All experimental animal care and handling were done in accordance with the guidelines and instruction for the responsible use of animals in research as a part of scientific research esthetics recommendation of the Ethics Committee of Faculty of Dentistry, Tanta University.

### Experimental procedures

Rats were divided randomly into two groups; Group-I (comprised of 10 rats) served as sham control group, whereas Group-II was used as an experimental group. Group II was subdivided into two subgroup (IIA, IIB). Subgroup IIA (ablation-subgroup) consisted of ten rats that were subjected to complete ablation of their major salivary glands. Then, after a recovery period of two weeks, they were subjected to PH. Subgroup-IIB (all glands-subgroup), in which ten animals had all their major salivary glands intact, though they were subjected to PH.

### Sialoadenectomy

In anaesthetized rat, the cervical region was sterilized and a mid line-ventral incision was made to expose the salivary gland that were ligated and excised (Jonjic 2001; Vacas *et al.* 2008)

### Partial hepatectomy:

Under general anesthesia, the abdominal wall was sterilized using a surgical scrub and a transverse incision was made in the midline of the abdominal wall, rat underwent one third partial hepatectomy with resection of the median liver lobe after ligation of its vascular pedicle (Milin *et al.* 2005).

### Serum Alanine transaminase (ALT) Analysis

Before their euthanization on the 7th and 14th days of PH, The rats were anesthetized by inhalation of 5% isoflurane until muscular tonus relaxed. Blood samples were collected from their lateral tail vein and placed in chilled non-heparinized tubes, centrifuged at 3000 rpm for 10 min at 4°C. The serum was frozen at -20°C for biochemical measurements. Serum levels of ALT were measured by a spectrophotometer (Udoh *et al.* 2011).

### Euthanasia and sample collections

At the end of the experimental periods rats will be euthanized, together with its control counterpart, with an overdose of pentobarbital sodium then confirmed with cervical dislocation. Liver of each animal will be dissected carefully, immediately fixed with 4% buffered formalin solution and prepared for immunohistochemical examinations. Immunohistochemical staining with PCNA was performed in order to examine cellular proliferation in liver (Akyol *et al.* 1999).

### Immunohistochemical staining of PCNA

Immunohistochemical labeling was performed using the Avidin–Biotin–Complex (ABC) method. Representative sections were deparaffinized in xylene and re-hydrated through a descending series of ethanol concentrations. The sections were washed with TBS (20 mM Tris- HCl, 150 mM NaCl, pH 7.4). Then they were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> in dH<sub>2</sub>O at room temperature (30 min) to inhibit endogenous peroxidase. Antigen retrieval was performed according to the manufacture instructions. Slides were placed in 100 µl blocking solution (Abcam), for 30 minutes at room temperature. Then incubated with primary monoclonal antibody (anti- PCNA primary antibody, Clone PC10, Santa Cruz, Sc 56, USA at dilution 1:300) at 4°C overnight. Sections were washed in 1X Phosphate buffered saline (PBS) and then incubated with secondary biotinylated antibody (anti-mouse) (in blocking buffer for 1 hour at room temperature in a humidified chamber. To perform peroxidase visualization; sections were incubated in ABC solution for 1 hour at room temperature. Color reaction was then developed by adding DAB solution (0.5 mg/ml DAB and 0.1% H<sub>2</sub>O) onto the sections. When color reaction was satisfactory, it was stopped by rinsing with H<sub>2</sub>O for 5-10 minutes, and then sections were counterstained with hematoxylin for 2 minutes. Sections were gradually dehydrated and mounted with coverslips. Immunohistochemical staining was assessed using light microscope.

### Immunohistochemical Evaluation

Quantitative analysis of the number of PCNA positive cells of liver were done by the aid of an image analyzer computer system using the software Leica Qwin 500. Five representative areas of each stained slide were observed under high power (X 400) with the light microscope. The numbers of the brown-stained PCNA positive nuclei were counted regardless of their staining intensity and their mean values were calculated. Counting was performed by two different uninformed observers, without being informed about the specific group. Then, the mean values of all groups were tabulated and statistically analyzed.

### Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS version 23). Independent t-test and one way ANOVA were used to compare quantitative data, P-value <0.05 was considered significant difference (\*) and P-value <0.001 was considered highly significant difference (\*\*).

### 3. Results

#### First: Effect of removal of all major salivary glands prior to PH on ALT level, one and two weeks of PH

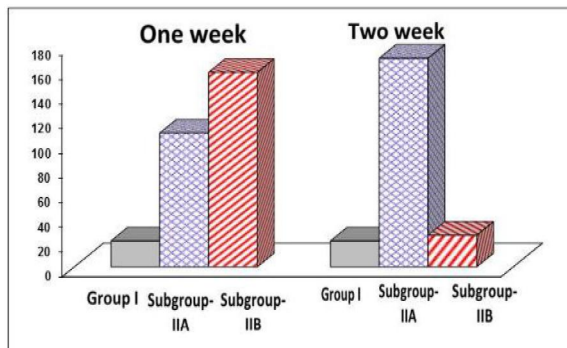
One week of PH, the mean ALT levels of PH rats that underwent whole ablation of all their major

salivary glands, prior to PH (subgroup-IIA) depicted highly significant difference from that of control rats. In the meantime, the average ALT levels of PH rats with intact major salivary glands (subgroup-IIB) revealed significant difference from both those of the control group and subgroup-IIA.

On the other hand, two weeks of PH, the mean ALT levels of subgroup-IIA disclosed significant difference from that of the control group and highly significant difference from that of subgroup-IIB. However, this later subgroup (subgroup-IIB) showed insignificant difference from that of the control group (Table 1 & Fig. 1).

**Table 1: Mean ALT levels of control group, subgroup- IIA and subgroup-IIB, one and two weeks of PH.**

Duration	Group	Mean	±	S.D	Range	t. test	p-value	Post hoc test
1 <sup>st</sup> week	Group I (control )	40.80	±	3.56	35   44	13.748	0.001*	Group I & ablation- ** Group I & all gland-* Ablation & all gland *
	(PH-ablation -subgroup)							
	(Subgroup-IIA)	79.00	±	16.30	47   89			
2 <sup>nd</sup> week	(PH-all glands- subgroup)	56.20	±	11.73	38   68	240.125	0.001*	Group I & ablation-* Group I & all gland- <sup>NT</sup> Ablation- & all gland- **
	(Subgroup-IIB)							
	Group I (control)	40.80	±	3.56	35   44			
2 <sup>nd</sup> week	(PH-ablation -subgroup)					240.125	0.001*	Group I & ablation-* Group I & all gland- <sup>NT</sup> Ablation- & all gland- **
	(Subgroup-IIA)	51.50	±	3.11	47   54			
	(PH-all glands- subgroup)	41.40	±	4.56	38   49			
2 <sup>nd</sup> week	(Subgroup-IIB)					240.125	0.001*	Group I & ablation-* Group I & all gland- <sup>NT</sup> Ablation- & all gland- **



**Figure (1): Histogram illustrates ALT levels of control group, subgroup- IIA and subgroup-IIB, one and two weeks of PH.**

**Second: Effect of removal of all major salivary glands prior to PH on liver PCNA, one and two weeks of PH**

One and two weeks of PH, the mean liver PCNA of PH rats that underwent ablation of all their salivary glands (subgroup-IIA) depicted highly significant difference from both PH rats of all glands- subgroup (subgroup-IIB) and control rats. On the other hand, one week of PH, liver PCNA of all glands- subgroup (subgroup-IIB) revealed highly significant difference from that of the control group. However, this significant difference disappeared and became insignificant after two weeks of PH (Table 2 & Fig. 2, 3).

**Table 2: Mean liver PCNA in control group, subgroup-IIA and subgroup- IIB, one and two weeks of PH.**

Duration	Group	Mean	±	S.D	Range	t. test	p-value	Post hoc test
1 <sup>st</sup> week	Group I (control )	21.00	±	7.87	12   30	98.390	0.001*	Group I& Ablation- subgroup** Group I& All gland- subgroup** Ablation& All gland- subgroup**
	PH-ablation- Subgroup ( subgroup-IIA)	108.60	±	16.95	80   125			
	PH-all glands- subgroup (Subgroup-IIB)	157.80	±	19.56	135   180			
2 <sup>nd</sup> week	Group I (control )	21.00	±	7.87	12   30	204.491	0.001*	Group I& Ablation- subgroup** Group I& All gland- subgroup <sup>NT</sup> Ablation& All gland- subgroup **
	PH- ablation- subgroup (subgroup- IIA)	169.33	±	21.34	130   186			
	PH-all glands- subgroup (Subgroup-IIB)	25.67	±	7.76	15   38			

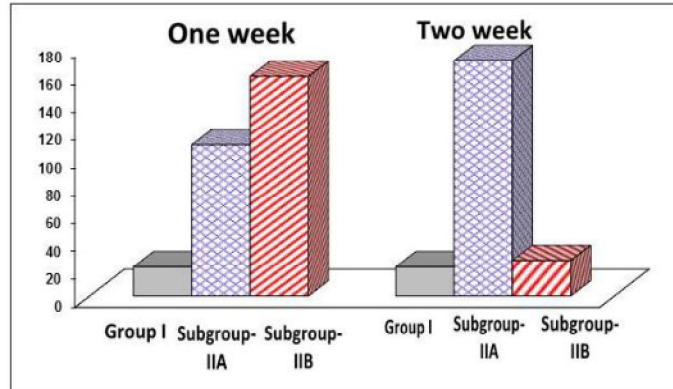


Figure (2): Histogram shows liver PCNA in control group, subgroup-IIA and subgroup-IIB, one and two weeks of PH.

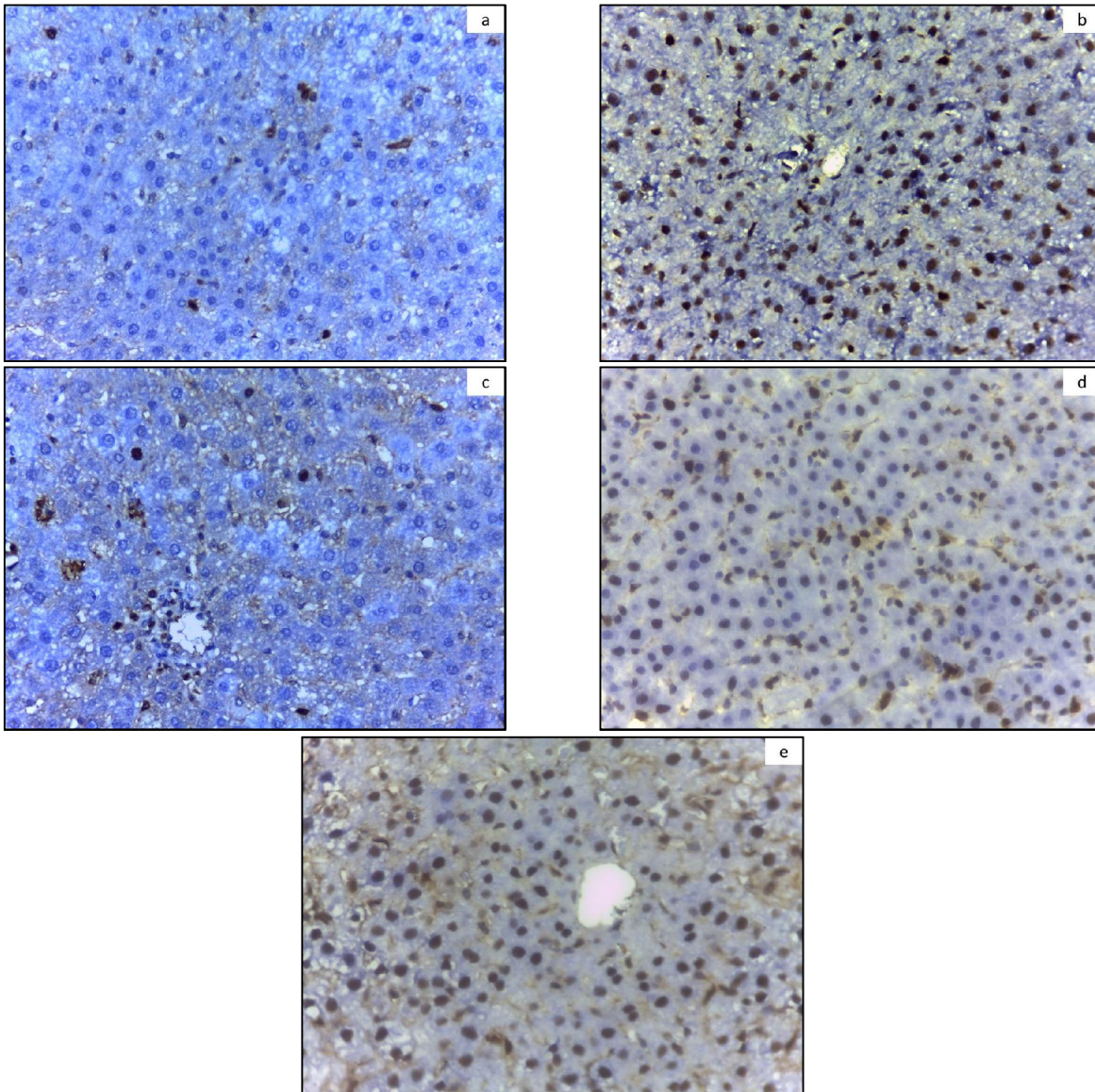


Figure (3): Immunohistochemical expression of PCNA (a) control liver (b) subgroup IIB, one week of PH. (C) subgroup IIB, two weeks of PH. (d) subgroup IIA, one week of PH. (e) subgroup IIA, two weeks of PH.

#### 4. Discussion

Throughout our two study periods, the mean ALT levels in PH rats which were subjected to complete sialoadenectomy were higher than that of PH rats that survived all their major salivary glands. This finding was in accordance with **Sánchez et al. (2007)** who depicted compromised hepatic cell function and turn over with elevated serum ALT levels after sialoadenectomy that led also to exaggeration of hepatotoxic effect of stressful stimuli. This could be attributed to inadequate production of proinflammatory cytokines such as interleukin- 6 and TNF- $\alpha$  induced by sialoadenectomy **Sánchez et al. (2008)**. In addition, by the end of the second week of PH, The elevated serum ALT after PH was declined to control level after two weeks in rats with their salivary glands indicating better liver condition. On the other hand, the ALT level still showed significant difference with control level in case of complete sialoadenectomy. This highlights the imperative role of major salivary glands in alleviation of liver damage.

From the other hand, one week of PH, the mean liver PCNA of subgroup-IIA and subgroup-IIB were much greater than that of the control, which seemed to reflect an initial response upon PH. In the meantime, the mean liver PCNA of subgroup-IIA was much lower than that of subgroup-IIB, which clearly highlighted the imperative role of major salivary glands on hepatic regeneration. This agreed with **Lindroos et al. (1991)** who considered major salivary glands one of the main extra-hepatic sites for HGF after PH that led to obvious increase (about 17 times) in its plasma level, since the remaining liver showed negative staining. This HGF was declared to be the most irreplaceable contributor to liver regeneration (**Michalopoulos Gk 2007**). Also, this coincided with **Lambotte et al. (1997)** who indicated that sialoadenectomy before or three hours after PH decreased or at least delayed liver regeneration, in rats.

Remarkably, subgroup-IIB showed reappearance of the standard normal liver PCNA after two weeks of PH, while, subgroup IIA still revealed much greater levels than control group indicating delayed regeneration and prolonged in their regeneration time beyond the usual two weeks period, reported by others, after PH (**Michalopoulos and De Frances, 2005**). This could be attributed to alternation in the early hepatic proto-oncogene response induced by sialoadenectomy together with deficiency of humoral circulating factors normally secreted by salivary glands that enhance hepatic cell proliferation after PH as epidermal growth factor (**Jones et al. 1995**). Thus, the current analysis highlighted the role of major

salivary glands in alleviation of liver damage as well as hepatic regeneration.

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