RESEARCH ARTICLE

A STUDY OF REGENERATIVE ABILITY OF LIVER AFTER REPETITIVE HEAT STRESS INDUCED LIVER INJURY

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ABSTRACT

Background: It is well known that heat stress produces liver injury. It is also known that liver regenerate after injury. This way, there should be almost no or minimal alteration in the size of the liver after regeneration.

Aims & Objective: In the present study, after heat induced liver injury, the ability of liver to regenerate itself was assessed. Liver injury was assessed by biochemical [Serum Glutamic Pyruvate Transaminase (SGPT); Serum Glutamic Oxaloacetic Transaminase (SGOT); and Alkaline Phosphatase (ALKP)], morphological, and morphometric changes. On the other hand, Liver regeneration was evaluated by morphological and morphometric observations of male adult albino rats (Wistar strain).

Material and Methods: The experimental animals were subjected to repetitive heat stress for 4 hours daily, at 37 ± 0.5 ˚C in a Biological Oxygen Demand (BOD) incubator (relative humidity 65 - 82%) for 2 and 5 consecutive days. Biochemical assessment was done on blood collected from left ventricle of beating heart of rats. Morphometric and morphological studies were conducted under light microscope on paraffin sections (H&E) of liver from control and experimental animals. The morphometric analysis was done by intersection – point counting method, using simple square lattice test system. Relative Liver Wet Weight of all animals was calculated.

Results: Progressively degenerative changes in morphological observations (disruption of cell plates in liver lobules and Kupffer cell hyperplasia), progressively increased statistical significance of morphometric (numerical density of Kupffer cells on area - Nak), and biochemical parameters informed that increasing liver damage was present with increased repetition of heat exposures in 2 and 5 days heat exposed experimental albino rats. With this liver degeneration, Relative Liver Wet Weight of all the experimental animals should have been decreased but it was not, reiterating about well-known fact of regenerative ability of liver. Along with progressive changes of heat induced liver injury, progressively increasing regenerative changes were also evident on morphological (binucleate cells and anisocytosis) observations supported by statistically significant morphometric (volume density of hepatocytes – Vvh and numerical density of hepatocytes - Nvh) parameters in experimental animals.

Conclusion: The above findings suggested that the regenerative ability of liver progressively increased with progressively increasing liver injury.

Key-Words: Regenerative Ability; Anisocytosis; Binucleate Cells; Liver Injury; Heat Exposure; Albino Rats

Introduction

The liver's ability to regenerate itself has been known for thousands of years. There is a story of a Greek titan Prometheus who steals fire from the Zeus for mankind. As a punishment for this action, Prometheus was bound to a rock, where each day an eagle was sent to feed on his liver. His liver regenerated itself during the night and next day it was again fed by the eagle. This process continued for long till Prometheus was made free.[1] This story of Prometheus indicates about regenerative ability of liver. There are a number of studies in which liver has been told to re-grow after liver injury.[2-4]

It is well known that heat stress produces liver injury as confirmed by various studies.[2,4-11] As per these studies, heat stress causes increased blood flow to skin leading to reflex vasodilatation in the cutaneous vascular bed and vasoconstriction in the hepato-splanchnic vascular zone. This vasoconstriction shifts the blood from hepato-splanchnic vascular zone to cutaneous vascular zone [4] to overcome the effect of heat stress. This hepato-splanchnic vasoconstriction causes hepato-splanchnic hypoxia leading to hepatocellular damage. This damage leads to rise in the levels of Serum Glutamic Pyruvate Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), Alkaline Phosphatase (ALKP), Lactic Acid Dehydrogenase (LDH), and Creatine Phosphokinase (CPK), as shown in various human[7-9,11] and animal[2,4] studies.

If there is liver damage & necrosis after liver injury, as per the general notion, the size of liver should decrease. But, as liver has very good regenerative ability it should re-grow and replenish itself. Thus, there should be no or minimal alteration in the size and weight of liver.

In the present study, it was tried to evaluate and to quantify the regenerative ability of the liver of albino rats who suffered repetitive heat stress induced liver injury.

Materials and Methods

Animals: 18 Adult, male albino rats of Wistar strain, weighing 125–175 grams (5 -8 weeks of age) were used, following approval of Animal Ethical Committee of HIMS, Dehradun. The animals were housed in polypropylene cages (43cm X 29cm X 15cm) with a wire mesh top and a hygienic bed of rice husk under standard laboratory conditions with ad libitum access to water and freshly cooked food (a mixture gm/kg of porridge – 630, ground nut cake – 100, milk powder – 100, black gram – 100, salt – 10, and fish meal – 60). Each cage contained one to two animals. The room temperature was kept within thermo-neutral zone for albino rats at 25 – 30 ˚C (relative humidity 65 - 85%), with the availability of normal day light. The experiment was conducted from October to January.

Experimental Protocol: The animals were divided into two groups, control having 6 animals and experimental with 12 animals. The experimental animals were subdivided into 2 subgroups of 6 animals each for heat exposure of 2 and 5 consecutive days. All the experimental animals were exposed to moderately high environmental temperatures (37 ± 0.5 ^{0}C) in a Biological Oxygen Demand (BOD) incubator (relative humidity 65-82%) for 4 hours daily (11:00 am to 03:00 pm). Animals were kept fasting for 3-5 hours prior to heat exposure. During the heat exposure period, only one animal was housed in each cage with liberty of movement and with ad libitum supply of water but no food. Following heat exposure, the animals were restored to room temperature with ad libitum supply of food as well as water. Simultaneously, the control animals were also fasted and provided with ad libitum supply of water just like the experimental animals, but kept at normal room temperature (25 ± 3 ^{0}C). Body weight of each experimental and control animal was measured daily in the morning.

Within one hour of last heat exposure, experimental as well as control animals were anaesthetized by intra-peritoneal injection of a mixture of Ketamine (50 mg/Kg body weight) and Xylazine (6.8 mg/kg body weight).[12] The thoracic cavity of anaesthetized animals was opened by midline incision and pericardial membrane cut. A fresh and sterilized Intracath canula (18 bore) was inserted into the left ventricle of beating heart and free flowing blood was collected in 5 mL AKUret™ serum gel tube with gel and clot activator for serum separation. Immediately afterwards, midline incision was extended further and abdomen was opened. The entire liver was removed in a petri-dish containing cold formalinized saline (10%). The animals were then sacrificed by exsanguination.

Evaluation of Relative Liver Wet Weight (RLWW): The weight of the wet liver was measured immediately after removal. Later on, RLWW[4] was evaluated by formula:

$$RLWW = \frac{Weight\ of\ Wet\ Liver}{Body\ Weight} X\ 100$$

Morphological Observations: Thin slices (less than 3 mm) were cut from the liver lobe. The liver slices were fixed in cold formalinized saline for 24-48 hrs. After fixation, the tissue pieces were dehydrated in graded ethanol and embedded in paraffin. Three to five micrometer thick paraffin sections were cut, and stained with hematoxylin–eosin and special stain reticulin for examination under the light microscope.[13]

Morphometric Observations: The morphometric observations on the liver sections were done by the intersection-point counting method, using simple square lattice test system A 100, having the quadratic test lines of spacing 5 mm which is equivalent to a distance of 0.125 mm in the actual specimen, considering X 40 final magnification.[14-17]

Numerical density of hepatocytes (Nvh): It tells about the number of hepatocytes in unit volume of liver.

Volume density of hepatocytes (Vvh): It tells about the volume fraction of liver tissue occupied by the hepatocytes.

Numerical density of Kupffer cells on the area (Nak): It expresses the number of Kupffer cells per unit area (per cm^2).

Biochemical Observations: Collected blood was used for Estimation of serum SGPT, SGOT, & ALKP on a semi-automatic RA-50 analyzer using the diagnostic reagent kit by DiaSys International. Along with these observations, to assess the heat stress, WBGT (Wet bulb globe temperature) index was calculated for animal room and for BOD incubator for five consecutive days of the heat exposure[18-21] as in other studies done by same author[2,22].

Data Analysis: The data was analyzed by using both quantitative and qualitative techniques. Analysis of quantitative data was done by using the Student's unpaired (independent) t-test and one way analysis of varience (ANOVA). All the data are expressed as mean ± SEM. The program "GraphPad Instat 3.06" was used for this analysis. Qualitative analysis was used for the morphological changes of the liver. The qualitative information was used to support the quantitative findings.

Results

Study of Degenerative Changes

Morphological Observations: There was disruption of liver cell plates at some sites and mild Kupffer cell hyperplasia in two days heat exposed experimental animals. While in five days heat exposed experimental animals there was single cell necrosis along with small foci of necrosis disrupting cell plates in lobules, moderate Kupffer cell hyperplasia along with ballooning degeneration and sinusoidal compression (as shown in Figures 2 and 3 in comparison to controls in Figure 1). These findings indicated that with increasing repetition of heat exposure there was increased severity of degeneration in the liver parenchyma.

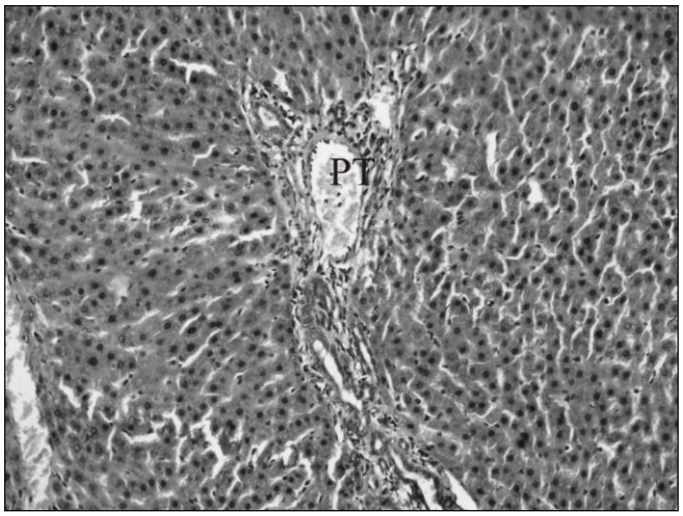

Figure-1: Photomicrograph of liver of control animal showing single cell thick liver cell plate, normal sinusoids, and portal triad (PT). Mild to moderate mononuclear infiltration in portal area. (HE. 400X)

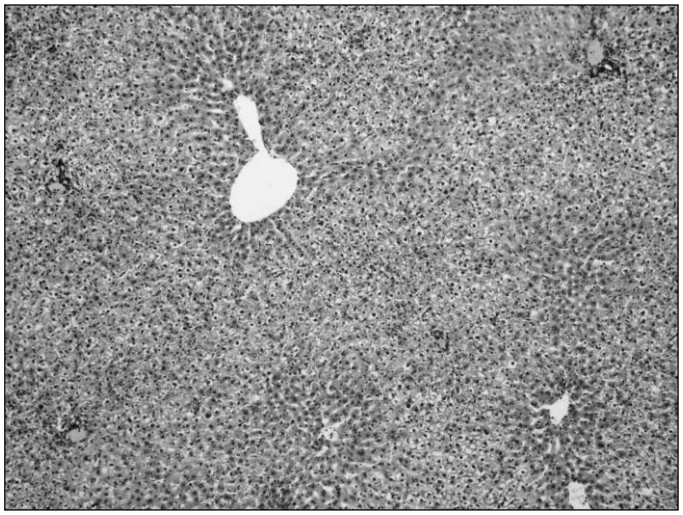

Figure-2: Photomicrograph of liver of experimental animal (05 Days Exposure) showing foci of necrosis, mainly in zone 2, disrupting liver cell plate. Hepatocytes show ballooning degeneration. (HE. 100X)

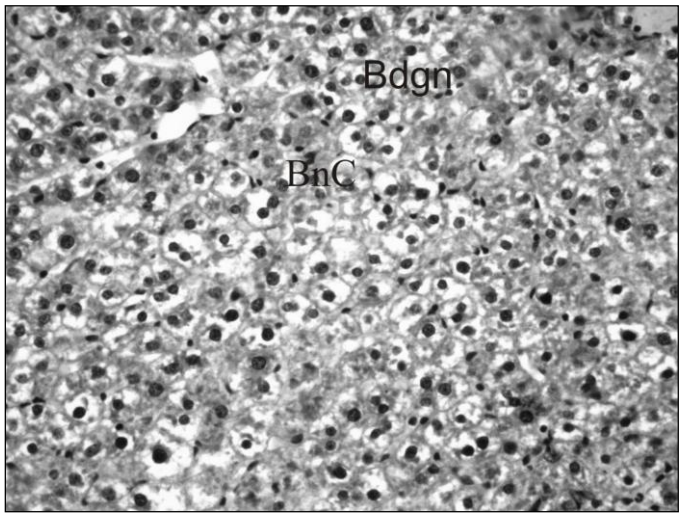

Figure-3: Photomicrograph of liver of experimental animal (05 Days exposure) showing foci of necrosis, mainly in zone 2, disrupting liver cell plate. Ballooning degeneration (Bdgn), Binucleate cells (BnC), and Kupffer cell hyperplasia are also seen. (HE. 400X)

Table-1: Effect of Repetitive Heat Stress (4 hours/day at 37 ± 0.5°C) on Numerical density of Kupffer cells on the area (Nak)

Morphometric Parameter	Controls (n = 6)	Experimental		p-value (ANOVA)
		02 Days (n = 6)	05 Days (n = 6)	
Nak (per cm^2)	4905.7 ± 9.47	5046.7 ± 38.86**	7062.0 ± 16.89***	< 0.0001

Values are expressed as Mean ± SEM. $p^{ns}>0.05$; $p^{*}<0.05$; $p^{**}<0.01$; and $p^{***}<0.001$, as compared with control values.

Table-2: Effect of Repetitive Heat Stress (4 hours/day at 37 ± 0.5°C) on Serum Levels of Liver Enzymes

Liver Enzymes (IU/L)	Controls (n = 6)	Experimental		p-value (ANOVA)
		02 Days (n = 6)	05 Days (n = 6)	
SGPT	43.8 ± 4.79	52.8 ± 2.92^{ns}	82.8 ± 2.92***	< 0.0001
SGOT	111.67±10.95	123.00±8.28^{ns}	189.67±14.38***	< 0.0005
ALKP	203.1±6.12	232.1±6.89 ^{ns}	446.3±25.53***	< 0.0001

Values are expressed as Mean ± SEM. $p^{ns}>0.05$; $p^{*}<0.05$; $p^{**}<0.01$; and $p^{***}<0.001$, as compared with control values. SGPT: Serum Glutamic Pyruvate Transaminase; SGOT: Serum Glutamic Oxaloacetic Transaminase: ALKP: Alkaline phosphatase

Table-3: Effect of Repetitive Heat Stress (4 hours/day at 37 ± 0.5°C) on Relative liver wet weight (gm)

	Controls (n = 6)	Experimental		p-value (ANOVA)
		02 Days (n = 6)	05 Days (n = 6)	
RLWW	4.85 ± 0.0567	4.81 ± 0.0826^{ns}	4.85 ± 0.0569^{ns}	>0.05

Values are expressed as Mean ± SEM. $p^{ns}>0.05$; $p^{*}<0.05$; $p^{**}<0.01$; and $p^{***}<0.001$, as compared with control values.

Table-4: Effect of Repetitive Heat Stress (4 hours/day at 37 ± 0.5°C) on Morphometric Parameters

Morphometric Parameters	Controls (n = 6)	Experimental		p-value (ANOVA)
		02 Days (n = 6)	05 Days (n = 6)	
Vvh	0.796 ±0.0042	0.802 ± 0.0024^{ns}	0.924 ± 0.0030***	< 0.0001
Nvh (millions/mL)	14.94 ± 0.126	15.10 ± 0.063^{ns}	19.02 ± 0.078***	< 0.0001

Values are expressed as Mean ± SEM. $p^{ns}>0.05$; $p^{*}<0.05$; $p^{**}<0.01$; and $p^{***}<0.001$, as compared with control values. Vvh: Volume density of hepatocytes; Nvh: Numerical density of hepatocytes

Morphometric Observations: The details of Numerical density of Kupffer cells on the area (Nak) are summarized

in Table 1.

Biochemical Observations: The details of serum levels of liver enzymes are summarized in Table 2.

Relative Liver Wet Weight (RLWW): The study of RLWW is summarized in Table 3.

Study of Regenerative Ability

Morphological Observations: It was observed that in two days heat exposed experimental animals there was increased variation in size and staining of hepatocytes nuclei (anisocytosis) along with degenerative changes. Whereas the regenerative changes binucleate cells and anisocytosis, were diffused and much more in five times heat exposed animals. These findings indicated that there was progressively increasing regeneration of liver tissue along with progressively increasing liver injury.

Morphometric Observations: The details of Volume density of hepatocytes (Vvh) and Numerical density of Kupffer cells on the area (Nak) are summarized in Table 4.

The calculated WBGT indexes for animal room and BOD incubator respectively were 24.4 ± 0.30 and 36.8 ± 0.03°C. This change in WBGT index was statistically significant (P value is < 0.0001). These results are similar to the results reported in other studies done by the same authors.[2,22]

Discussion

Degenerative Changes

Morphological Observations: As observed in results, degenerative changes in 5 days heat exposed animals were more profound in comparison to 2 days heat exposed animals. These findings clearly indicated that on increasing repetition of heat exposure there was increased severity of degeneration in the liver parenchyma.

Morphometric Observation: Kupffer cell hyperplasia was observed as an established sign of liver injury by various researchers in their studies of severe[9,11] and moderate[2] heat stress. The statistically significant Kupffer cell hyperplasia was also observed in the current study proposing liver injury. The increased hyperplasia of Kupffer cells as revealed by Nak suggested that with repetition of heat exposure there was increased liver injury.

Biochemical Observations: As shown in Table 2, the serum levels of liver enzymes in experimental animals were elevated (more in 5 days heat exposed animals) in comparison to controls. SGPT is a liver specific enzyme. High levels of SGPT are indicative of liver injury; SGPT rises dramatically in acute liver damage.[23,24] High levels of SGOT and ALKP along with high SGPT levels are also indicative of liver injury.[23,24] Serum SGPT, SGOT, and ALKP are the most sensitive markers of liver damage because they are cytoplasmic in location and are released into the circulation after hepatocellular damage.[4,25] Just like these and various other[2,7-9,11] studies, there was elevation of serum levels of liver enzymes in the current study, indicating liver injury.

These morphological, morphometric, and biochemical observations confirmed and validated that with increased repetition of heat exposure, severity of liver injury and degeneration was progressively increased.

Relative Liver Wet Weight (RLWW)

From the above findings, it can be speculated that with increased liver degeneration, the weight of liver should decrease. To substantiate this speculation, relative liver wet weights was calculated. It was observed that after two and five days heat exposure, at 37 ± 0.5°C for 4 hours, there was no statistically significant change in relative liver wet weights of experimental animals in comparison to control animals as summarized in Table 3. Despite all these degenerative proofs, findings of Table 3 recapitulated about the fact that liver has the ability to regenerate itself after degenerative injury

Study of Regenerative Ability

Morphological Observations: These findings indicated that there was progressively increasing regeneration of liver tissue along with progressively increasing liver injury.

Morphometric Observations: Parameters Vvh and Nvh were raised to statistically significant level in 5 days heat exposed animals; while in 2 days heat exposed animals, there was no statistically significant rise. The successive increase in Vvh and Nvh revealed that there was a continued proliferation of hepatocytes with increased repetition of heat exposures.

These changes are very similar to changes observed by Sharma[4] and the same authors in their other study[2]. The morphometric findings supported by morphological findings confirmed that with increasing liver injury there was increased rate of proliferation and regeneration of liver.

While doing this study, few limitations were noticed but some of them were inadvertent such as small sample size which was the only size permitted by Animal Ethical Committee. Another limitation was not to study DNA cytometry and evaluation of hepatic growth factor but because of limited funds for the study, observations were done very selectively, judiciously, and in a justified way.

Conclusion

These morphological, morphometric, and biochemical observations confirmed and validated that with increased repetition of heat exposure, severity of liver injury and degeneration was progressively increased. The morphometric findings supported by morphological findings confirmed that with increasing liver injury there was increased rate of proliferation and regeneration of liver.

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