# Neutrophil Infiltration of Liver in Rats Chronically Fed Ethanol or Control Diet and Subjected to Hemorrhagic Shock

Hemorrhagic shock causes a series of inflammatory events. One of these events involves the activation of polymorphonuclear granulocytic neutrophils (PMNs). Alcoholic patients fair worse after severe trauma and resuscitation. It has been hypothesized that this occurs because some of their organs, particularly the liver, are already damaged or their organs are primed for worse damage due to excessive consumption of ethanol. In this experiment it has been shown that although there is not a significant difference in PMN infiltration between control fed sham and shock rats and ethanol fed sham and shock rats there appears to be a correlation between the number of neutrophils present and the percent focal liver necrosis in each animal.

## Introduction

There are five kinds of leukocytes, commonly called white blood cells, involved in the defense mechanisms of the body. These are split into two classes according to nuclear morphology: polymorphonuclear granulocytes, which have segmented nuclei and mononuclear agranulocytes, which do not. The most common polymorphonuclear granulocytes are neutrophils. Polymorphonuclear granulocytic neutrophils (PMNs) have nuclei segmented into two to five lobes connected together by thin strands of chromatin. PMNs are attracted to areas of infection and injury where, by a process called

margination, they adhere to vessel walls. They then migrate into the surrounding tissue and engulf bacteria by a process called phagocytosis. (8)

Part of a series of inflammatory events triggered by hemorrhagic shock involves the activation of PMNs. PMNs are activated by various cytokines including granulocyte colony-stimulating factor (G-CSF) and interlukin-6 (IL-6). It has been shown that these cytokines are produced in hemorrhagic shock. (3) Hemorrhagic shock activates nuclear factor (NF)-κB, a member of the Rel transcription family, which is activated in response to many inflammatory stimuli. NF- κB transcriptionally activates many pro-inflammatory genes including the genes coding for G-CSF and IL-6 mRNA. (6) The activation of PMNs subsequently leads to the enhanced adhesiveness of the PMNs to the endothelium, to the production and release of reactive oxygen intermediates, and to degranulation and protease release. (3) These events lead in turn to organ damage and decreased patient survival rates.

G-CSF is essential for the production of neutrophils and it has been shown to enhance the functional activities of mature neutrophils. G-CSF also promotes PMN survival by suppressing the apoptosis of these cells. (2) Studies have shown it to be produced by macrophages, endothelial cells, fibroblasts, and astroglia cells. It has been shown to be beneficial in inflammatory states initiated by bacterial infection. (3) Local levels of G-CSF have been shown to be detrimental to both the lungs and the liver while elevated circulating levels have been shown to be beneficial to the liver and detrimental to the lung. (5)

IL-6 is a 2 kDa multifunctional cytokine. It is an important part of the local inflammatory and immune response involved in combating infection. It signals the

activation of proteins, called STAT proteins that serve the dual functions of signal transducers and activators of transcription. (4) IL-6 binds to a receptor protein called IL-6Rα. IL-6Rα oligomerizes with and signals through gp130, a second receptor protein. (5) Recent studies have shown that IL-6 contributes to PMN recruitment by the induction of chemokines. (4) It has been shown that local levels of IL-6 present in organs results in organ damage while circulating levels of IL-6 infused during the resuscitation phase reduced shock-induced PMN infiltration in both the lung and liver. (5) It has also been shown that the local pro-inflammatory effects of IL-6 dominate over the anti-inflammatory effects of circulating IL-6. (6)

The release of reactive radicals is one cause of organ damage in hemorrhagic shock. Reactive radicals are produced after resuscitation from hemorrhagic shock. They have been implicated in a number of signal transduction pathways. Nitric Oxide (NO) is one of these radicals. It is generated catalytically by NO synthases such as inflammatory or inducible NO synthase (iNOS). It has been shown that iNOS is up regulated in both the lungs and liver during hemorrhagic shock. (1)

In the lung, hemorrhagic shock causes PMN infiltration, pulmonary edema, and hypoxia. The major cellular site of G-CSF mRNA production in the lung was found to be in bronchoepithelial cells. (3) IL-6 is also produced locally in the lung during hemorrhagic shock. (4) PMNs adhere to the vascular endothelium and then migrate into lung interstitium and alveolar spaces. This causes endothelial and epithelial cell damage through the production of reactive oxygen intermediates and through the release of lysosomal proteases. (2) Local levels of G-CSF and IL-6 promote PMN infiltration and lung damage. Circulating levels of G-CSF have been shown to be detrimental while

circulating levels of IL-6 have been shown to be beneficial. The local, damaging effects of IL-6 are more dominant. (5)

In the liver, hemorrhagic shock causes PMN infiltration and focal liver necrosis. A study by Camargo et .al demonstrated that the local production of IL-6 by the liver may have a protective effect on liver tissue following a direct ischemia-reperfusion insult (i.e. insult to the liver itself). It also raised the possibility that local IL-6 production in the event of direct liver injury may have a predominantly anti-inflammatory effect. (6) Local levels of G-CSF and IL-6 promote PMN infiltration and necrosis while elevated circulating levels of these cytokines exert a protective effect. The local effects are more dominant. Circulating levels of G-CSF do not affect PMN infiltration but they do greatly reduce necrosis. It appears that the protective effects of elevated levels of circulating G-CSF and IL-6 are independent of their effects on PMN recruitment. (5)

It has been observed that alcoholic patients have worse survival rates than nonalcoholic patients after suffering severe injury and resuscitation. It is thought that this is due to the fact that some of their organs are already damaged due to excessive consumption of ethanol. If this is true than there should be more PMN recruitment and damage in the organs of alcoholic patients versus nonalcoholic patients. This would be particularly true in the case of the liver. It is a well-documented fact that excessive consumption of ethanol causes liver damage.

In this experiment, the level of PMN infiltration in the livers of ethanol fed rats subjected to hemorrhagic shock was compared to the level in the livers of rats fed with a normal liquid diet and to sham controls from each group. The livers were sectioned into slides that were stained with MPO to show the PMNs. The PMNs were than counted. It

was shown that there was not a significant difference between the numbers of neutrophils present in control fed rat livers versus the number of neutrophils present in ethanol fed rat livers. However, there dose appear to be a correlation between the number of neutrophils and the percent focal liver necrosis in each liver.

## Materials and Methods

The rats were shipped to the lab from a rat breeder. Once the rats arrived, they were fed lab chow for a few days so they could become accustomed to their new environment. The rats were than split into two groups. The control group was fed a liquid diet containing no ethanol. The experimental group received 30% of its daily caloric intake from ethanol. Each group was than split again into a sham group and a shock group. The sham rats were anesthetized for the same period of time as the shock group but nothing else was done to them. The mean arterial pressure of the rats in the shock group was dropped from 80 to 40 mm Hg for 2.5 h then resuscitated back to baseline with fluid. The rats were than sacrificed 4 h after the start of resuscitation.

Once the experiment was complete, various organs were harvested from the rats including the lungs, liver, heart, and intestine. A small sample of each organ was taken and placed in a sample container in formalin. The rest of the organ mass was frozen. The right lung was frozen while the left lung was filled with formalin and placed with the other samples in the formalin container.

Once all of the rats had been operated on and their organs had been harvested, the organs were separated. All of the liver samples were placed together, all of the heart samples were placed together, etc. These were stored in formalin until they were needed.

When the organ samples were ready to be processed, they were placed in PBS and sent to the tissue processing facility downstairs. Once processed, they were embedded in paraffin blocks and cut into five-micron thick sections on a microtome. The sections were collected on slides, which were than baked to remove excess paraffin.

Samples were labeled as follows. Each animal was given a letter and number designation. The letter marks the group the rat originally came from (i.e. the group the rat was shipped in with). The number is just the number the rat is given. For example, B3 would indicate the third rat in group B. The letters went form B to E and there was also an extra (EX) group. Once the rats were split into the control and ethanol groups they were given either a C or E designation. For example, EB3 would be ethanol rat B3 and CC2 would be control rat C2. Lastly, HS designates shock rats while SH designates sham animals. For example, EB3 HS would be ethanol rat B3 hemorrhagic shock.

Thirty-three rats survived the procedure and their organs were harvested. Six were control fed sham rats, nine were control fed shock rats, nine were ethanol fed sham rats, and nine were ethanol fed shock rats. Twenty-two groups of slides were freshly cut. Previously cut slides were used for the other eleven samples. At least three slides were cut for each of the twenty-two freshly cut samples. One set was set aside for staining with myeloperoxidase (MPO) antibodies, one set was H & E stained, and the rest were stored as extras.

Twenty slides were H & E stained. This was done for two reasons. First, to make sure the samples were cut well. Second, to compare the two types of staining. H & E staining is done in following manner. The slides are first placed in xylene to remove any paraffin not eliminated by the baking. Next, the slides are taken through a series of ethanol and finally water washes to prepare them for staining. They are then placed in the hematoxylin stain for five minuets. They are than washed thoroughly until most of the excess stain is removed from the slides. They are briefly dipped in acid alcohol and than washed again. Next, they are placed in ammonia water to bring the stain color in the tissue back. They are washed in a series of ethanol washes and than briefly dipped in eosin. After this comes another series of ethanol washes and lastly xylene again. Once the slides have dried, cover slips are placed on them.

A complete set of thirty-three slides was MPO stained. Myeloperoxidase (MPO) is an enzyme only produced in readily detectable amounts by neutrophils in the tissue. A monoclonal antibody against MPO was applied to the tissue sections, washed off, and followed by an enzyme-conjugated antibody against the MPO monoclonal antibody. After washing, a substrate for the enzyme was layered over the slide. When the enzyme cleaved the substrate, a brown-colored molecule was produced. The PMNs show up in the tissue as cells containing dark brown spots.

Each slide was than examined using a light microscope. Excel and Primer were used to analyze the data. Each slide was analyzed in this way. Twenty different fields were observed on each slide at 200x power. The number of PMNs in each field was counted. These numbers were recorded on an Excel data sheet. Using Primer, the mean and standard deviation were than calculated for each animal. The mean, standard

deviation, and the standard error of the mean were than calculated for each group using the previously calculated means, also on Primer. Two different proportion tests were than done to determine if differences in the data were statistically significant. One test was done completely by the computer using Primer. The second test was more manual. The proportion data was calculated in Excel and than compared in Primer. The next thing done was a T-test. A Man-Whitney rank sum test was preformed because the data was not in a normal distribution pattern.

Several steps were taken to determine if there was a correlation between the number of neutrophils and percent focal liver necrosis (determined in another experiment). A graph was plotted with percent focal liver necrosis on the x-axis and mean MPO+ cells per 200x field on the y-axis. The data for each point was plotted and a line of best fit was drawn through the data. Primer was then used to calculate r and p. As r approaches 1 and p approaches 0, the correlation is more significant.

Several steps were taken to avoid bias in the data collecting. The slides were marked with general numbers AA to ZZ and A1 to A7. This way, it is not known whether the slide is from a control or ethanol group or from a sham or shock group. Also, the choosing of fields was random. A random "path" was taken depending on the shape of the tissue sample. This random "path" served two purposes. It significantly decreased the likelihood of an overlap and it eliminated bias.

## Results

The H & E stained slides were observed under the microscope and comparisons were made. The liver samples from both sham groups appeared normal while necrotic areas could be seen on the samples from both shock groups (fig. 1). In previous experiments it has been observed that necrosis commonly occurs around central veins (fig. 2). However, it is difficult to clearly determine structures in rat liver samples.

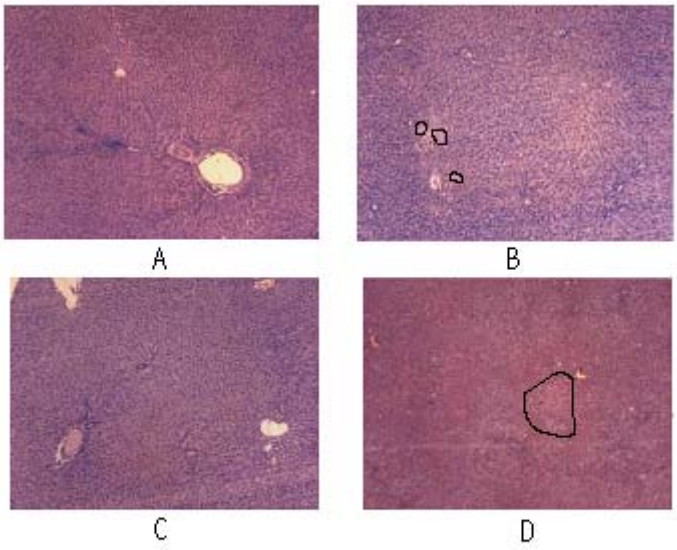


Fig. 1. H&E stained liver samples. Circled areas are necrotic tissue. (A=control sham, B=control shock, C=ethanol sham, D= ethanol shock)

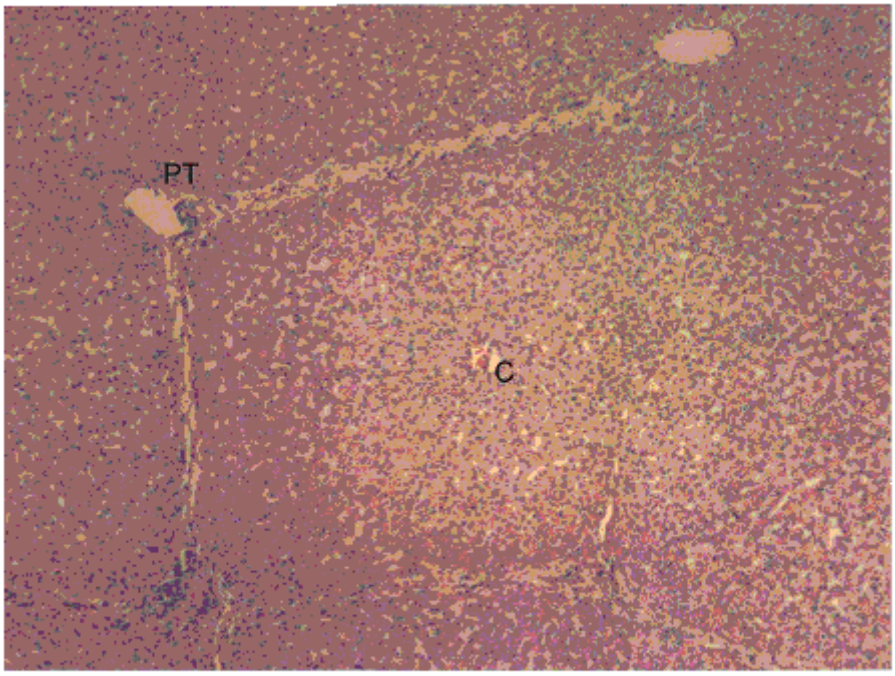


Fig. 2.Pig liver sample clearly showing a lobule. The central vein (C) and a portal triad (PT) are shown.

The MPO stained PMNs did not stain as darkly as expected. The MPO+ cells looked dark brown in color. There appeared to be more PMNs in the shock animals than in the sham animals (fig. 3). PMNs seemed to be concentrated around necrotic tissue.

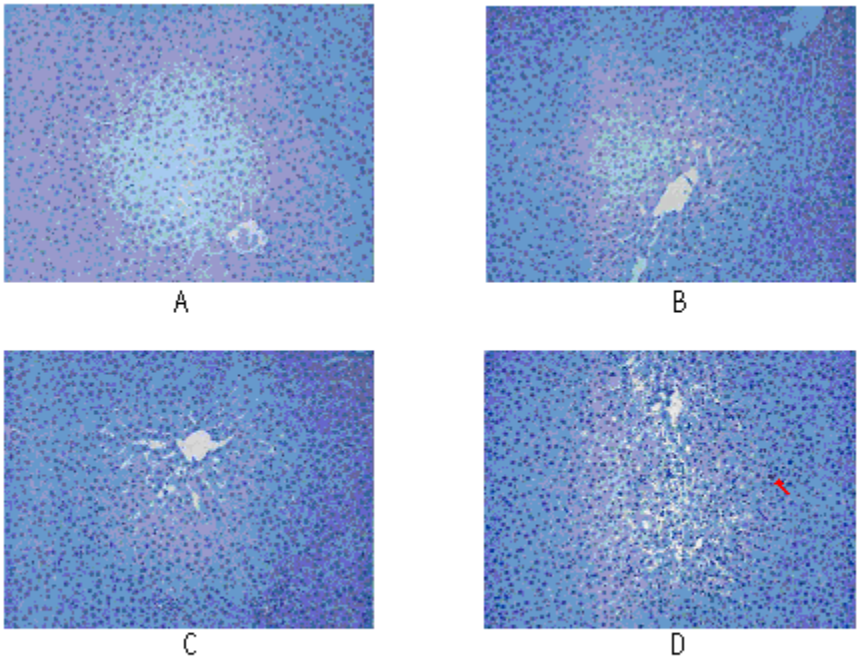


Fig. 3.Rat liver samples stained with MPO. Neutrophils stain brown(arrow). (A=conttrol sham, B=control shock, C=ethanol sham, and D=ethanol shock)

The mean MPO+ cells per 200x field per animal in each group was: 5 ±5 in the control sham group, 18 ±25 in the control shock group, 7 ±4 in the ethanol sham group, and 16 ±16 in the ethanol shock group (fig. 4). The Student-Newman-Kneuls-Test showed that none of the differences in the data were statistically significant. The proportions test showed the same results. For control shock vs. control sham, p=0.169. For control shock vs. ethanol sham, p=0.198. For control shock vs. ethanol shock, p=0.707. For ethanol shock vs. control sham, p=0.368. For ethanol shock vs. ethanol sham, p=0.461. Lastly, for ethanol sham vs. control sham, p=0.939. ($p<0.1$ for the difference to be statistically significant). The t-test showed that neither control sham vs. control shock (p=0.236, t=-1.242) nor ethanol sham vs. ethanol shock (p=0.121, t= -1.637) were statistically significant.

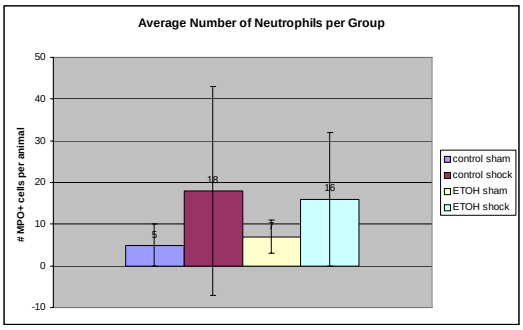


Fig. 4. Graph comparing the average number of neutrophils in each group. Error bars represent standard deviation. Note the high standard deviations in both shock groups.

The data in this experiment did not follow a normal distribution pattern (fig. 5). Therefore, a Mann-Whitney rank sum test was performed on control sham vs. control shock ($p=0.193$) and ethanol sham vs. ethanol shock ($p=0.359$). The differences were not statistically significant.

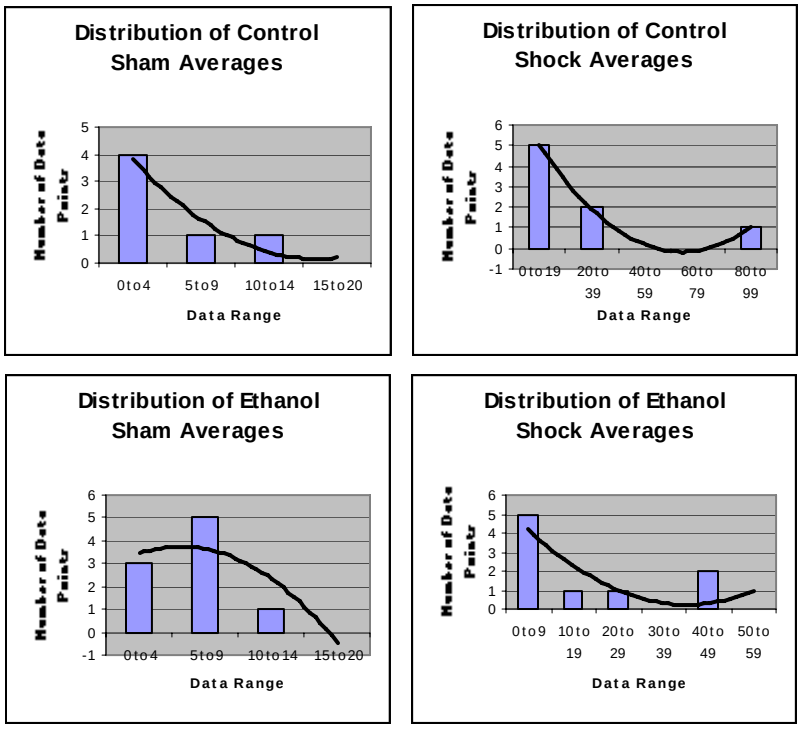


Fig. 5. Graphs showing how each group deviates from a normal distribution pattern. Each data point is one animal.

A liner regression and correlation was done to determine if there was a correlation between percent focal liver necrosis and the number of PMNs. The graph is shown in fig. 6. The linear regression coefficient, r=0.499 and p=0.009. There is a strong correlation between the two measurements. Focal liver necrosis was previously determined in the lab for each rat by Ms. Edith Hardison

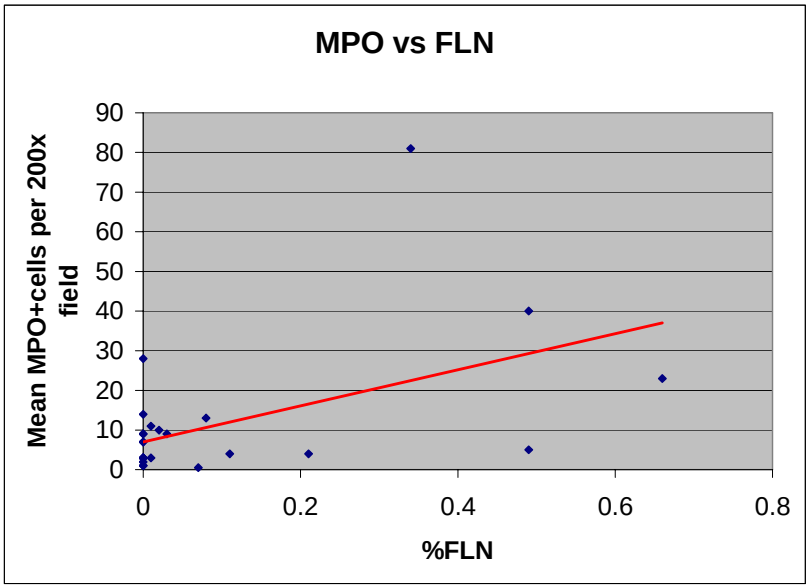


Fig. 6. Graph showing the correlation between percent focal liver necrosis and mean MPO+ cells per 200x field. Each point represents one animal.

## Discussion

The strong correlation between percent hepatic liver necrosis and number of PMNs seems to show that the differences between the sham and shock values are significant. The strong correlation supports this because the values for focal liver necrosis in sham and shock animals were statistically significant.

However, the difference between the sham and shock values of both groups was not statistically significant. This was primarily due to the high standard deviations in both shock values. These high standard deviations were due to two main factors. First, every rat is different and reacts differently to the procedure. Some shock rats are more affected than others. The fact that some rats die during the procedure lends support to this point. Second, each slide contains a very small sample of the rat's liver. As the liver is not uniformly damaged by hemorrhagic shock, it is not surprising that some samples have many PMNs while others have few. Both of these factors lead to a high variation of results and thus a high standard deviation.

It was surprising that there was almost no difference between the control shock and ethanol shock values and that the control shock value was slightly larger (though this difference was not significant). It was particularly surprising because percent focal liver necrosis was significantly greater in ethanol shock animals. The differences between each rat and the nonuniform distribution of liver damage cannot account for this lack of difference in PMN infiltration values. Perhaps ethanol negatively affects PMN recruitment in some way. It may interfere with some aspect of the signaling pathway or do something to the PMNs themselves. This would explain why there is a higher degree of focal liver necrosis in ethanol shock rats vs. control shock rats but not a higher number of PMNs.

Several pieces of information can be gained from this experiment. First, that there is a correlation between percent focal liver necrosis and number of PMNs. Second, the amount of PMN infiltration in rats subjected to hemorrhagic shock varies widely. Lastly, that ethanol may negatively affect PMN recruitment. This information along with other

data could support the hypothesis that alcoholic patients fair worse after severe trauma and resuscitation because some of their organs are already damaged due to excessive consumption of ethanol or their organs are primed to become more damaged following a shock/trauma.

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