The impact of small doses of LPS on NASH in high sucrose and high fat diet induced rats

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Abstract. – OBJECTIVE: We investigated the impact of small doses of lipopolysaccharide (LPS) on the development of NASH in the context of a high sucrose and high fat diet in rats.

MATERIALS AND METHODS: Male Wistar rats were randomly divided into groups fed a synthetic diet (n=8), a regular diet (n=8), a synthetic diet + LPS (n=8) or saline (n=8) and a regular diet + LPS (n=8) or saline (n=8). The LPS (or saline) was administered from the 6th week on (0.5 mg/kg) by subcutaneous injection every two days under the same conditions with free access to water and food. At the end of the 9th week the animals were euthanized and the liver tissue dissected for analysis. Hematoxylin and eosin (HE) and Von Gieson’s (VG) staining was performed on parafin embedded sections to observe the pathological changes of the liver, the degree of fibrosis, and infiltrative lymphocytes were counted in the liver tissue.

RESULTS: We quantitatively measured the levels of LPS in the plasma of rats, ALT activity, and TNF-α. We found that the synthetic diet + LPS group showed severe steatosis, and was associated with bridging necrosis and mild fibrosis when compared to the group fed a Synthetic diet + saline. In addition, the amount of infiltrative lymphocytes and the level of plasma ALT and TNF-α in the synthetic diet + LPS group were significantly increased. The difference observed were statistically significant (p < 0.05).

CONCLUSIONS: Small doses of LPS promote the development of NASH induced by a high sucrose and high fat.

Key Words: LPS, High sucrose, High fat, Non-alcoholic steatohepatitis (NASH), Fibrosis.

Introduction

Improvements in people’s socioeconomic status has lead to changes in diet and lifestyle leading to a global increase in non-alcoholic steatohepatitis (NASH), a major cause of chronic liver disease.

With a rising yearly incidence and an earlier onset, NASH has become the leading cause of chronic liver disease in developed countries and areas of affluence¹,². This trend is extending to China, where only chronic viral hepatitis surpasses NASH¹. Globally the incidence of NASH is 17% to 33%⁴ with the average incidence in the US population being 20%⁵,⁶. Currently, NASH is no longer defined by static and benign lesions, as some cases of NASH can develop into hepato-fibrosis, cirrhosis and hepato-carcinoma⁷ raising its visibility in the research and medical community. Its pathogenesis is not well understood, but under the two hit hypothesis⁸ factors leading to the fatty degeneration of liver cells⁹ leading to hepatic steatosis as the first hit translate into steatohepatitis with lipopolysaccharide participating in the second hit⁴.

There is a continuous release of endotoxin (LPS) produced by Gram negative bacteria in the host intestine with no consequence to the individual and in the event that a small amount of endotoxin leaks into the portal vein, it is cleared by the Kupffer cells after transit through the liver, therefore, never entering circulatory system. There is no intestinal endotoxin in the peripheral blood¹¹.

To generate intestinal endotoxin, we fed rats a high fat and sucrose diet, which increases both the propagation of intestinal bacteria and intestinal permeability or alters the sensitivity permeability¹²,¹³, thereby, decreasing phagocytosis by the Kupffer cells. This results in an elevated LPS plasma level in rats and the formation of intestinal endotoxin¹⁴.

In this study, we investigate the role that LPS plays in NASH in the “two-hit” theory.

Materials and Methods

Animals and Treatments

48 male Wistar rats (provided by the Experimental Animal Center of Shanxi Medical University, China), weighing from 200 g to 250 g were used. All these rats were fed normally for 3 days.
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when used for the experiment. They were divided randomly into 4 groups: synthetic diet + saline group (n=8), synthetic diet + LPS group (n=8), regular diet + LPS group (n=8), and regular diet + saline group (n=8). In addition, we set up a 5 week synthetic diet group (n=8) and regular diet group (n=8) to observe the long term impact on the rats’ liver as a result of a high sucrose and high fat diet. The synthetic diet consists of 70% ordinary feed, 20% of lard, 10% sucrose, 1% cholesterol, and 0.25% cholic acid. On the 6th week, LPS (0.5 mg/kg) is added by subcutaneous injection into the animals every second day under the same experimental conditions. Control animals were fed the same diet, with saline instead of LPS and treated in the same manner. All animals had free access to food and water. At the end of the 9th week the animals were euthanized by intraperitoneal injection with a 1% solution of sodium pentobarbital (0.3 ml/kg). We collected the abdominal aortic blood under sterile and pyrogen-free conditions, measuring the levels of plasma endotoxin (ET), alanine amino transferase (ALT) and tumor necrosis factor-α (TNF-α) after centrifugation at 3500 rpm. We excised the left hepatic lobe and fixed it in a 10% solution of formalin, paraffin-embedded, and sliced at 5 µm. Hematoxylin-and-eosin-stained (HE) and Van Gieson (VG) staining were used in order to observe the pathological changes of the liver tissue under light microscopy; the remaining liver tissue was stored at –70°C. At the end of the 5th week the rats fed the synthetic diet and regular diet were processed in the same manner.

Main Reagents

The ALT assay kit was obtained at the Nanjing Jiancheng Bioengineering Institute. The Limulus kit at the Shanghai Eva Clinical Medicine Technology Company and the TNF-α radioimmunoassay kit (by the RIA institute of Science and Technology Development Center in Chinese PLA General Hospital).

Main Testing Indexes and Methods

We measured the plasma TNF-α level by Radioimmunoassay (RIA). We tested ALT activity by the colorimetric method of Reitman and Frankel, which uses 2, 4 DNPH; and the LPS level quantitatively with Limulus reagent.

Statistical Analysis

We presented the data as “mean ± standard deviation”. When comparing groups we applied the t test or single-factor analysis of variance and the results were considered statistically significant at a p < 0.05 using SPSS15.0 (SPSS55 Inc., Chicago, IL USA).

Results

Rats Fed a Synthetic Diet Dignificantly Increase Body Size After 5 Weeks

We noted a significant increase in the body weight of rats fed the synthetic diet compared to the rats fed a regular diet, with the difference beginning at 2 weeks of treatment and becoming statistically significant at the 5th week of treatment (p < 0.05, Figure 1).

Morphological Changes of the Liver Tissue

The liver was dissected and stained by HE. We observed steatosis in the hepatic cells of rats fed the synthetic diet with small vesicant liposome accounting for the majority of the steatosis, while infiltration by inflammatory cells was not obvi-
ous (Figure 2B). In the treatment groups with LPS added to the diet and fed for 9 weeks we found that there were no significant morphological changes in the hepatic cells of regular diet + LPS group rats stained by HE (Figure 3A). There was no significant degeneration or infiltration of inflammatory cells, nor evidence of liposomal or inflammatory cell infiltration. In the group receiving the synthetic diet and LPS we found that the liposomes had fused with each other into larger liposomal vesicles, there was ballooning degeneration, the steatosis appearing more severe, and focal necrosis, bridging necrosis (Figure 3B) and mild fibrosis were visible (Figure 4B). In the synthetic diet + saline group, liver was steatosic with large vesicant liposomes accounting for the majority. There was infiltration of inflammatory cells in the liver lobule and portal with some dot necrosis (Figure 3D) and little focal necrosis was visible (Figure 4D).

We measured the levels of plasma LPS and ALT in the rats fed the synthetic diet + saline group. We found that they were significantly higher than in the normal group ($p < 0.05$). In addition the amount of hepatic lymphocytes was also significantly higher suggesting that intestinal endotoxin was present in synthetic diet + saline group and that LPS acted synergistically with high sucrose and high fat to damage the liver tissue. To corroborate this, our synthetic diet + LPS group did not show changes in the plasma LPS level but both the plasma ALT level and the

![Image](https://via.placeholder.com/150)

**Figure 2.** Histopathological change for fat deposition and lymphocytes of rat liver at 5 weeks of feeding. Representative hematoxylin-and-eosin-stained tissue sections were prepared from rats exposed to (A) regular diet and (B) synthetic diet. Magnification 100x.

![Image](https://via.placeholder.com/150)

**Figure 3.** Histopathological change in fat deposition and lymphocytes of rat liver fed LPS and a high fat and sucrose diet. Representative hematoxylin-and-eosin-stained tissue sections were prepared from rats exposed to regular diet + LPS, synthetic diet + LPS, regular diet + saline and synthetic diet + saline. Magnification 100x.
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amount of hepatic lymphocytes were significantly higher than those fed a synthetic diet + saline group and regular diet + LPS (Table I).

TNF-α is the effector of LPS, and plasma TNF-α in rats fed a synthetic diet + LPS were significantly higher (Table I) relative to the levels in the rats fed a synthetic diet + saline and regular diet + LPS (Table I, \( p < 0.05 \)).

In a separate comparison of the levels of lymphocytes in the synthetic diet + LPS group relative to those in the synthetic diet + saline groups and those in the regular diet + LPS group were significantly higher (Table I, \( p < 0.05 \)).

Discussion

In the present study, we simulated the nutritional habits of modern people by using a high sucrose and high fat synthetic diet to induce NASH in rat models to assess the role of LPS in NASH onset. The morphological changes observed in the liver tissue indicated that 9 weeks of high sucrose and high fat diet had caused liver damage, the formation of liver steatosis, dot and focal necrosis, and infiltration of a large quantity of inflammatory cells, of which lymphocytes accounted for the main part. The increasing plasma ALT level in-

Table I. Summary of LPS, ALT and TNF-α levels in the plasma, and lymphocytes infiltration in liver tissue.

<table>
<thead>
<tr>
<th></th>
<th>ALT</th>
<th>TNF-α</th>
<th>LPS</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular diet + saline</td>
<td>32.55 ± 4.07Δ</td>
<td>0.29 ± 0.09Δ</td>
<td>0.20 ± 0.05Δ</td>
<td>0</td>
</tr>
<tr>
<td>Regular diet + LPS</td>
<td>30.17 ± 6.63</td>
<td>0.32 ± 0.10</td>
<td>0.19 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>Synthetic diet + saline</td>
<td>118.67 ± 5.89</td>
<td>0.65 ± 0.13</td>
<td>0.52 ± 0.21</td>
<td>30.07 ± 6.11</td>
</tr>
<tr>
<td>Synthetic diet + LPS</td>
<td>193.06 ± 37.58*Δ</td>
<td>1.12 ± 0.08*Δ</td>
<td>0.55 ± 0.16*Δ</td>
<td>65.29 ± 10.68*Δ</td>
</tr>
</tbody>
</table>

*\( p < 0.05 \) vs regular diet + LPS, \( p < 0.05 \) vs synthetic diet + saline.
duced liver damage and following 9 weeks of a high sucrose and high fat diet higher plasma LPS levels were recorded relative to control groups. We also found that circulating LPS levels increased following a high fat diet\textsuperscript{12}, suggesting that intestinal endotoxin developed in the synthetic diet + saline group and suggesting that LPS had contributed to the formation and development of NASH. Therefore, in order to examine the direct effects of LPS, we set up a synthetic diet + LPS group, which simulates the features of small levels but persistent levels of intestinal endotoxin. We observed a significant increase in the generation of TNF-\(\alpha\) in the high sucrose and high fat synthetic diet, exacerbating liver damage and mild fibrosis. In support of this, there is clinical data showing that NASH patients have overgrowth of bacteria in the small intestine and higher TNF-\(\alpha\) levels\textsuperscript{15} when compared to normal groups. In a study by Joseph et al\textsuperscript{16}, liver damage is mediated by TNF-\(\alpha\) while others propose that TNF-\(\alpha\) itself induces hepatic death in NASH mice models\textsuperscript{17}. In the liver, TNF-\(\alpha\) is released by LPS after activating Kupffer cells\textsuperscript{18}. If the secretion of TNF-\(\alpha\) is inhibited, then the liver damage can be correspondingly reduced\textsuperscript{9}. Based on the above information, LPS has probably aggravated the development of NASH further in high sucrose and high fat synthetic diet induced rats by increasing the secretion of TNF-\(\alpha\).

Here, we found that LPS itself, in the absence of existing liver damage, did not significantly change the function of the liver suggesting that small doses of LPS do not have an effect on the liver in normal rats. However, the same dose of LPS may lead to severe consequences in rats with mild lesions. We set up a synthetic diet consisting of high fat and high sucrose for 5 weeks, in the presence of LPS. The experimental results showed that after only 5 weeks of high sucrose combined with a high fat synthetic diet could cause mild steatosis of liver cells in rats (Figure 2B), while the body weight of rats increased significantly (the difference reached was statistically significant). This increase in body weight may increase the sensitivity to small doses of LPS.\textsuperscript{19} The study was funded by the Shanxi Natural Science Foundation (2013011054-2); Shanxi Medical Technology Innovation Fund (1201107); 331-based medical technology cultivated Fund (201215).

### Conclusions

LPS promotes the development of NASH by increasing the secretion of TNF-\(\alpha\).

### Acknowledgements

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### Conflict of interest

The Authors declare that they have no conflict of interests.

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