Effect of hepatic iron concentration reduction on hepatic fibrosis and damage in rats with cholestatic liver disease

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AIM: To assess the effect of iron reduction after phlebotomy in rats with "normal" hepatic iron concentration (HIC) on the progression of hepatic fibrosis, as a result of bile duct ligation (BDL).

METHODS: Rats underwent phlebotomy before or after sham operation or BDL. Animals undergone only BDL or sham operation served as controls. Two weeks after surgery, indices of hepatic damage and fibrosis were evaluated.

RESULTS: Phlebotomy lowered HIC. Phlebotomy after BDL was associated with body weight increase, lower hepatic weight, less portal hypertension, less periportal necrosis, less portal inflammation, lower hepatic activity index score and higher albumin levels. On the other hand, phlebotomy before BDL was associated with body weight decrease and hepatic activity index score increase. Phlebotomy after sham operation was not associated with any hepatic or systemic adverse effects.

CONCLUSION: Reduction of HIC after induction of liver damage may have beneficial effects in BDL rats. However, iron deficiency could induce impairment of liver function and may make the liver more susceptible to insults like BDL.

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Key words: Iron; Phlebotomy; Bile duct ligation; Hepatic activity index; Rat.
with interferon, HIC does not influence the response to therapy\textsuperscript{[32]}. However, it is not yet known whether phlebotomy may improve the sustained virological response or histological score in these patients. Patients with chronic cholestatic liver disease do not usually have hepatic iron overload\textsuperscript{[30]}. Moreover, no information exists as to the effect of iron reduction in subjects with cholestatic liver diseases with no increase (“normal levels”) of HIC.

The aim of the present work was to assess the effect of iron reduction after phlebotomy on the HIC in rats with “normal” iron stores and to evaluate whether reduction of HIC may decrease the hepatic damage and slow the progression of hepatic fibrosis induced by bile duct ligation (BDL).

**MATERIALS AND METHODS**

Forty male Sprague Dawley rats (Harlan, Israel), weighing 180-223 g, were studied. Rats were housed in regular cages situated in an animal room at 24°C with a 12-h light-dark cycle. Rats were maintained on standard rat chow (Kofflok, Tel Aviv, Israel) and with free access to tap water. The Ethics Review Committee for Animal Experimentation of the Hebrew University Hadassah Medical School approved all the animal studies described

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**Table 1** Weights and phlebotomy volume in different groups of rats (mean ±SE)

| Subgroup                  | n | Weight gain (% from baseline) | Mean phlebotomy Volume (mL) | Liver weight (% of total body weight) | Splenic weight (% of total body weight) |
|---------------------------|---|-------------------------------|-----------------------------|-------------------------------------|----------------------------------------|
| Sham only                 | 5 | 15.03 ± 18.70\textsuperscript{a} | 0 ± 0                      | 3.97 ± 0.16\textsuperscript{c}     | 0.41 ± 0.07\textsuperscript{c}        |
| Sham and phlebotomy later | 4 | 9.4 ± 7.0                     | 0.70 ± 0.13                | 3.62 ± 0.32                         | 0.37 ± 0.04                            |
| Phlebotomy and sham later | 6 | -5.7 ± 17.3                   | 0.86 ± 0.18                | 3.47 ± 0.19                         | 0.38 ± 0.03                            |
| BDL only                  | 9 | -4.99 ± 3.90                  | 0 ± 0                      | 6.89 ± 1.29                         | 0.66 ± 0.10                            |
| BDL and phlebotomy later  | 8 | 8.01 ± 3.1\textsuperscript{c} | 0.71 ± 0.16                | 5.97 ± 0.45                         | 0.56 ± 0.07                            |
| Phlebotomy and BDL later  | 8 | -3.29 ± 20.1                  | 0.80 ± 0.15                | 7.02 ± 1.68                         | 0.69 ± 0.13                            |

\(\text{Mean phlebotomy volume for one phlebotomy session per rat; } ^{a}P \leq 0.01 \text{ vs BDL only.}\)

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**Table 2** Liver function tests and enzymes in different groups of rats (mean ±SE)

| Subgroup                  | n | GGT (IU/L) | ALK-P (IU/L) | Bilirubin (μmol/L) | GPT (IU/L) | Albumin (G/L) |
|---------------------------|---|------------|--------------|-------------------|------------|---------------|
| Sham only                 | 5 | 7.0 ± 2.0\textsuperscript{a} | 254 ± 52      | 4.6 ± 1.8         | 53 ± 11\textsuperscript{a} | 28.0 ± 1.1\textsuperscript{a} |
| Sham and phlebotomy later | 4 | 6.0 ± 1.4  | 305 ± 58     | 4.0 ± 0.8         | 66 ± 8     | 29.2 ± 2.2    |
| Phlebotomy and sham later | 6 | 14.3 ± 10.3| 305 ± 61     | 41.7 ± 39.0       | 55 ± 20    | 28.4 ± 2.3    |
| BDL only                  | 9 | 74.0 ± 63.4| 580 ± 174    | 106.0 ± 57.0      | 115 ± 50   | 24.3 ± 2.2    |
| BDL and phlebotomy later  | 8 | 65.9 ± 17.9| 579 ± 75     | 104.0 ± 16.2      | 125 ± 22   | 29.0 ± 2.0\textsuperscript{c} |
| Phlebotomy and BDL later  | 8 | 64.3 ± 17.6| 665 ± 166    | 109.0 ± 39.0      | 144 ± 40   | 23.7 ± 2.1    |

\(\text{Mean phlebotomy volume for one phlebotomy session per rat; } ^{a}P \leq 0.007, ^{c}P \leq 0.02 \text{ vs BDL only.}\)
Table 4 Hepatic prostaglandin E2 (PGE2) generation, myeloperoxidase (MPO) activity and hydroxyproline (HP) content in different groups of rats (mean±SE)

| Subgroup                  | n  | PGE2 generation (ng/g liver) | HP content (ng/g liver) | MPO activity (units/g liver) |
|---------------------------|----|----------------------------|-------------------------|-----------------------------|
| Sham only                 | 5  | 14.8±9.9                   | 8.59±0.55               | 0.45±0.14                   |
| Sham and phlebotomy later | 4  | 14.7±5.2                   | 8.11±1.05               | 0.31±0.12                   |
| Phlebotomy and sham later | 6  | 6.9±3.2                    | 8.02±1.00               | 0.18±0.05                   |
| BDL only                  | 9  | 8.5±4.5                    | 11.00±0.97              | 0.59±0.21                   |
| BDL and phlebotomy later  | 8  | 3.9±2.1                    | 9.80±1.68               | 0.52±0.10                   |
| Phlebotomy and BDL later  | 8  | 3.4±2.3                    | 11.90±1.40              | 0.27±0.17                   |

1P ≤ 0.018 vs BDL only.

Table 5 Hepatic iron content and concentration in different groups of rats (mean±SE)

| Subgroup                  | n  | Iron concentration (μg/g liver) | Iron content (μg/total liver) |
|---------------------------|----|-------------------------------|------------------------------|
| Sham only                 | 5  | 91.8±12.9                     | 861.3±158.9                  |
| Sham and phlebotomy later | 4  | 10.1±8.8                      | 351.5±63.3                  |
| Phlebotomy and sham later | 6  | 55.0±19.7                     | 358.9±104.4                 |
| BDL only                  | 9  | 80.9±17.2                     | 1122.1±358.6                |
| BDL and phlebotomy later  | 8  | 37.4±6.8                      | 477.2±85.1                  |
| Phlebotomy and BDL later  | 8  | 49.0±29.6                     | 628.0±224.7                 |

1P ≤ 0.0001, 2P ≤ 0.012 vs BDL only; 3P ≤ 0.016 vs sham only.

graded BDL-induced liver damage was assessed by a modification of the Knodell score[35]. Each specimen was examined for the following features and scored by points of increment from 0-4, according to the severity of the findings as follows: perportal necrosis: none = 0, mild = 1, moderate = 2, marked = 3, severe = 4; portal inflammation: none = 0, mild = 1, moderate = 2, marked = 3, severe = 4; lobular necrosis: none = 0, mild = 1, moderate = 2, marked = 3, severe = 4; fibrosis: none = 0, portal expansion = 1, septal formation = 2, marked bridging fibrosis = 3, cirrhosis = 4. Hepatic activity index (HAI) was calculated for each rat by summation of the points of these four parameters. In addition, bile duct proliferation was assessed in each specimen by allocating points of increment from 0-4, according to the severity of the findings. Histological evaluation of the pathological specimens was done on a blind basis by our pathologist. Reliable correlation was seen in repeated and blinded histological evaluation of the specimens.

Determination of PGE2

One hundred and fifty mg of liver tissue was placed in preweighed tubes containing 1.0 mL of phosphate buffer (50 mmol/L, pH 7.4). The liver was minced with scissors and centrifuged in an Eppendorf centrifuge for 10 s. The pellet was resuspended in 1.0 mL of the above buffer, incubated for 1 min in a vortex mixer. Indomethacin (10 μg) was added and the tubes were centrifuged for 60 s. The supernatant was kept at -20 °C before the surgical procedure (BDL or sham operation).

Two weeks after the surgical procedure rats were anesthetized with ether. The abdominal cavity was opened by midline incision, and blood was drawn from the inferior vena cava, centrifuged, aliquoted and frozen. Serum albumin, bilirubin, calcium, phosphor, liver enzymes, kidney function tests and electrolytes were measured by the dry chemistry method (Kodak, Rochester, NY, USA). Serum iron (SI) as determined by Guanidine/FerroZine method (Cobas Integra 700). Transferin was determined using the immunoturbidimetric method (Cobas Integra 700). The liver and spleen were removed and weighed. Sections from the liver of each animal included in the study were taken for histological evaluation, determination of HIC, myeloperoxidase (MPO) activity, prostaglandin E2 (PGE2) generation and hydroxyproline (HP) content.

Liver histology

Sections from the liver of each animal were fixed in phosphate-buffered formaldehyde, embedded in paraffin, and 5-μm thick sections were prepared. Sections were stained with hematoxylin and eosin for evaluation of necroinflammatory grading and Masson’s trichrome stains for fibrosis and architectural changes. Histological stains for fibrosis and architectural changes. Histological staining with hematoxylin and eosin for evaluation of phosphate-buffered formaldehyde, embedded in paraffin, Sections from the liver of each animal were fixed in 700). The liver and spleen were removed and weighed. Serum iron (SI) as determined by Guanidine/FerroZine method (Cobas Integra 700). Transferin was determined using the immunoturbidimetric method (Cobas Integra 700). The liver and spleen were removed and weighed. Sections from the liver of each animal included in the study were taken for histological evaluation, determination of HIC, myeloperoxidase (MPO) activity, prostaglandin E2 (PGE2) generation and hydroxyproline (HP) content.
taken for determination of enzyme activity according to Bradley et al.\textsuperscript{13} The correlation coefficient based on 10 standard curves was \( r = 0.98 \) and inter assay variation was \( 0 \pm 1.65\% \) (mean \( \pm SE, n = 57 \)).

**Determination of hepatic iron content**

Hepatic non-heme iron concentration was measured by the method of Torrance and Bothwell\textsuperscript{16}.

**Determination of hepatic hydroxyproline content**

Hepatic hydroxyproline content was measured by the method described by Woessner\textsuperscript{37}.

**Statistical analysis**

Data were expressed as mean \( \pm SE \). Group variance was analyzed by the Kruskal-Wallis test, followed by the Mann-Whitney test for multiple comparisons to allow pair-wise testing for significant differences between groups. \( P \leq 0.05 \) was considered statistically significant.

**RESULTS**

**Effect of bile duct ligation**

Two weeks after BDL all rats were jaundiced, had lesser weight gain, greater liver and spleen weight than SO rats (Table 1). BDL rats also had higher serum levels of liver enzymes, higher bilirubin and lower albumin levels, higher fibrosis score, higher hepatic HP content, higher hepatic activity index and higher bile duct proliferation score than SO rats (Table 2, Tables 3 and 4 and Figure 1). No change in HIC and content or SI and transferrin were observed as a result of BDL (Tables 5 and 6).

**Effect of phlebotomy**

Phlebotomy after surgical procedure (SO or BDL) resulted in a decrease of HIC and content. The change was more pronounced in BDL rats than in SO rats. Rats that were phlebotomized before the surgical procedure had higher HIC than those undergone phlebotomy after the surgical procedure (Table 5). No change in SI and transferrin was noted in any group as a result of phlebotomy (Table 6).

Phlebotomy after BDL was associated with body weight in crease, higher albumin levels and lower hepatic PGE\(_2\) generation at sacrifice compared to BDL only rats (Tables 1-3). Moreover, periportal necrosis, portal inflammation, fibrosis scores, total HAI and hepatic HP content decreased in rats that were phlebotomized after BDL, although the results did not reach statistical significance. Phlebotomy before BDL was associated

![Figure 1](https://www.wjgnet.com)

**DISCUSSION**

The role of iron in the progression of hepatic damage in various clinical and experimental conditions has usually been studied by iron loading\textsuperscript{14,16,18-20}, or by iron depletion in situations where mild to moderate iron overload is present\textsuperscript{13,24,25,30}. In the present study, we examined the effect of reduction of “normal” hepatic iron stores on liver function and histology in rats subjected to BDL. This situation may be more relevant and analogous to and may mimic more closely the common clinical conditions where most of the patients with non-hemochromatosis and non-end-stage liver diseases do not have iron overload\textsuperscript{19}.

In the present study we were able to demonstrate that it was possible to reduce HIC by phlebotomy and that such a procedure after sham operation was not associated with hepatic adverse events. Liver disease developed in the rats after two weeks of BDL was not associated with HIC increase. Phlebotomy before the surgical procedure was associated with HIC reduction but not with SI or transferrin reduction. The changes in liver histology (mainly necrosis and inflammation) following the HIC reduction indicated that hepatic iron might not have a role in the fibrosis process after BDL\textsuperscript{39}. Alternatively, we may also speculate that further reduction of HIC by more
aggressive phlebotomy would have an effect on fibrosis. Since SI and transferrin levels did not change following phlebotomy, we assume that the phlebotomy process performed in our rats was not sufficient to alter the course of the fibrosis process. Although no change was observed in fibrosis score, phlebotomized rats after BDL exhibited favorable effects. These rats had body weight increase, higher albumin levels and lower hepatic necroinflammatory activity. Iron deficiency induced by iron chelation may reduce the severity of inflammation in joints and in the gastrointestinal tract.

HIC was only mildly reduced in the rats undergone phlebotomy before the surgical procedure compared to the BDL only rats. Only the total amount of iron per whole liver was reduced significantly. This could be due to the ability of rats to absorb iron during the time interval between the last phlebotomy session and the sacrifice of the rats (18 d). This may indicate that phlebotomy has a transient effect and that continuous phlebotomy sessions over time should be done in order to sustain a beneficial effect. In cirrhotic subjects an increased iron absorption due to increased duodenal expression of iron transporter, divalent metal transporter 1 has been reported. However this phenomenon does not exist in rats with cholestatic liver disease.

Phlebotomy had a favorable effect on the rats after BDL, but a definite negative effect before BDL. The latter rats lost weight and had a worse HAI (a worse score in periportal necrosis and portal inflammation) as compared to the other BDL groups. A similar trend of changes was observed in the rats subjected to phlebotomy before sham operation. Iron deficiency may affect various organs including eccentric cardiac hypertrophy in rats and various hepatic enzyme functions. Anemic patients with early cirrhosis may have worse hemodynamic parameters (lower systemic vascular resistance, increased cardiac index and hepatic venous pressure gradient) than non-anemic control patients. Increased blood hemoglobin may attenuate splanchnic vasodilatation in portal hypertensive subjects by nitric oxide inactivation. In our study, the rats undergone phlebotomy before BDL had the highest splenic weight (a marker of portal hypertension). Based on this finding we may speculate that deterioration of hemodynamic parameters in these rats is associated with activation of the renin-angiotensin system. This system may have a role in hepatic fibrosis in BDL rats.

Hepatic PGE2 generation and MPO activity were used as adjuvant markers for hepatic inflammatory activity in our study, and decreased PGE2 generation was observed in the BDL groups undergone phlebotomy. This is unexpected as iron is known to down-regulate PGE2 generation in various tissues. Higher PGE2 levels were also expected in the BDL groups with increased hepatic inflammatory changes. This is similar to the results in our previous study of rats to BDL and colitis, in which colonic PGE2 generation was reduced as compared with a control group. One may speculate that cyclooxygenase activity or even phospholipase A2 activity may be defective in rats with experimental liver disease.

As MPO is found primarily in neutrophil granules, it is a marker of neutrophil content and influx into tissues. Our previous study in BDL rats with colitis found that mucosal inflammation is correlated with MPO activity. In the present study, however no correlation was found between the hepatic inflammatory changes and hepatic MPO activity. Decreased MPO activity in the Carrageenan model of inflammation in rats with acute cholestasis due to bile duct resection has been reported. Additional defects of neutrophil function in BDL rats, like impairment of adhesion, defective phagocytosis, increased superoxide generation and chemotaxis, and increased number of neutrophil counts, have been reported. Iron deficiency per se may also impair neutrophil function and MPO activity and cause a selective defect in the process of inflammation. Iron repletion improves some of the above functions. In this study, hepatic MPO activity was the lowest in the rats undergone phlebotomy before the surgical procedure. However there was no clear correlation between HIC, hepatic MPO activity and hepatic inflammation.

In summary, hepatic iron depletion in rats after a liver insult like BDL, may have beneficial effects. “Hepatic iron deficiency” may make the liver more vulnerable to insults such as laparotomy or BDL. Hepatic PGE2 generation and hepatic MPO activity are not reliable markers for hepatic inflammation in rats with cholestatic liver disease.

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