Introduction

The authors discuss the value of percentage of pitted erythrocytes as a marker of functional hyposplenism. They assess the percentage of pitted erythrocyte count among the different liver disease groups and compared them with age matched controls. Alcoholic cirrhosis and Hepatitis-C group have the highest percentage of pitted RBCs count signifying hyposplenism. To our knowledge this is the first time an association of Hepatitis-C with functional hyposplenism is uncovered.

Objectives:
To assess the splenic function in a variety of liver diseases and to investigate the relationship between pitted erythrocyte counts and liver disease. The association of hypersplenism with viral Hepatitis-C has not been previously reported.

Subjects, Methods and Results:
A total of 152 subjects were divided into 4-groups, 67 Controls (CONT), 38 hepatitis C (HEPC), 23 alcoholic cirrhosis (ALC), and 24 miscellaneous group (MISC). Pitted erythrocyte count was measured in a fresh venous blood with 3% glutaraldehyde solution at PH of 7.4. A total of 1,000 consecutive RBCs were examined per film per subject and the results were expressed as percentage. Hyposplenism is present when the mean value of the percentage of pitted erythrocyte count >6.68 (mean ± 2SD of the normal controls). Various biochemical and hematological parameters were compared in the four groups. The results were analyzed by the ANOVA. The overall model was significant. Pairwise comparisons were made with Tukey adjustment. Pearson Correlation Coefficient was done between continuous variables.

Results:
All subjects of ALC and HEPC groups and 3/24(12.5%) of the MISC groups were found to have hyposplenism. None of the subjects of the control group had hyposplenism. There was no significant difference in the mean values of the pitted RBCs between MISC vs CONT (p>0.06, 95% CI for the difference, -0.482 to 1.925). However, a significant difference was found between ALC vs CONT and HEPC vs CONT (p=0.0001, and p=0.0001, 95% CI, 6.63 to 8.16 and 7.79 to 9.18 respectively. A significant difference was also found between ALC vs MISC and HEPC vs MISC (p=0.0001, 95% CI, 5.26 -7.12 & p=0.0001, 95% CI, 6.33-8.24) respectively, but no significant difference was demonstrated between the percentage of the pitted RBCs count and the biochemical and hematological parameters in the various groups. However, a significant difference in the mean values in many of the other parameters studied between HEPC vs MISC (AP, ALT, GGT, and INR) and between ALC vs HEPC (AP, AST, ALT, GGT, MCV, IgG, and IgM) were found.

Conclusion:
Hepatitis-C and Alcoholic cirrhosis is associated with hyposplenism. The association of hyposplenism with viral Hepatitis-C has not previously been reported. It is possible that the mechanism of splenic involvement in alcoholics and Hepatitis-C patients is different and further studies are warranted. There was no association of splenic function and biochemical and hematological parameters in various types of liver diseases.
Keywords: Alcoholic cirrhosis; Hepatitis C, Hyposplenism; Pitted erythrocyte count

Introduction

In 1913, Eppinger proposed the word “hyposplenism” to describe the condition that develops after extirpation of the spleen [1]. It has since been accepted as a term covering all states of impaired splenic function. Hyposplenism was firmly established by Woolley et al [2] recognized that the splenic function may be impaired even when the spleen is present [3]. Hyposplenism is recognized in association with several diseases, including celiac disease, sickle cell anemia [4], inflammatory bowel disease (IBD) [5-13], and dermatitis herpetiform. The functional capacity of the spleen does not always correlate well with its anatomic size. Assessment of splenic function has been done using [10].

- Peripheral blood smear examination with standard light microscopy, which may reveal changes that are suggestive of impaired splenic function. However, none of these changes is specific, sensitive, or qualitative.
- Isotope studies using damaged RBCs scintillation scanning of the spleen.

Hyposplenism is characterized by red blood cells with inclusions (Howell-Jolly bodies, Heinz bodies and Pappenheimer bodies), target cells and acanthocytosis. Howell-Jolley bodies have been considered the most specific feature for hyposplenism. They appear within hours after removal of the spleen and remain a permanent feature of hyposplenism [3,4]. Heinz bodies on the other hand are usually seen as several small round bodies around the cell margins. They are also found in association with hemoglobinopathies and hemolytic crises secondary to drug exposure in patients with erythrocyte enzyme deficiency [11].

Pappenheimer bodies and recently increased pitted erythrocytes count are well known recognized features of hyposplenism [3,4,11,13]. Neither target cells nor acanthocytes are specific for hyposplenism and the former are found also in association with obstructive liver disease, hemoglobinopathies, thalassemia, iron deficiency anemia, alcoholic liver disease and some malabsorption states [12].

Hyposplenism carries a great morbidity and mortality. It is also associated with many changes in; platelets count, shape, and volume of cells [14-16], decrease levels of IgM and blunted antibody response [17,18], overwhelming infection with pneumococcus [19,20], meningococcus, Hemophilus influenza, staphylococcus aureus [12,20-22], Neisseria gonorrhea and candida albicans. Splenectomized patients are likewise at risk from Plasmodium falciparum malaria and protozoa of the genus Babesia [23-25]. Patients Splenectomized for hematological diseases are at greater risk than other patients [26] and Youngs are at particular risk, but overwhelming sepsis can similarly occur in adults. Fatal pneumococcal septicemia has been reported in patients with extensive splenosis [27] and in Splenectomized subjects who receive pneumococcal vaccine [28].

Materials and Materials

A total of 152 subjects were included in this study. The aim is to investigate the splenic function in different patterns of liver diseases. The subjects studied were classified into 4-groups, (Table-1);

- Normal controls (CON)
- Viral hepatitis C (HEPC)
- Alcoholic cirrhosis (ALC)
- Miscellaneous group (MISC)

Patients were recruited from medical outpatient, Hepatitis-C clinic, and in-patient wards at University College Hospital, Galway, Ireland.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CONT # patients</th>
<th>HEPC # patients</th>
<th>ALC # patients</th>
<th>MISC # patients</th>
</tr>
</thead>
<tbody>
<tr>
<td># patients</td>
<td>67</td>
<td>38</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>F/M</td>
<td>34/33</td>
<td>35/3</td>
<td>0/23</td>
<td>7/17</td>
</tr>
<tr>
<td>Mean age (Years)</td>
<td>47.8</td>
<td>45.7</td>
<td>52.1</td>
<td>45.2</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>(31-73)</td>
<td>(32-58)</td>
<td>(43-63)</td>
<td>(35-55)</td>
</tr>
<tr>
<td>Mean pitted RBCs±(2SD)</td>
<td>3.717(6.68%)</td>
<td>12.2(16.35%)</td>
<td>11.11(14.68)</td>
<td>4.92(7.72%)</td>
</tr>
<tr>
<td>Range of pitted RBC’s</td>
<td>All had no hyposplenism</td>
<td>1-male patient had transfusion after KT</td>
<td>(7.9-13.7%)</td>
<td>(2.3-7.8%)</td>
</tr>
<tr>
<td>Remarks</td>
<td>All had no hyposplenism</td>
<td>2-males had transfusion after MVA</td>
<td>All had hyposplenism</td>
<td>7/24</td>
</tr>
</tbody>
</table>

Table1: Biometrical parameters in the different groups.
• Normal controls: a total of 67 healthy volunteers were included in this study (hospital staffs, at University College Hospital Galway (UCHG), and Gynecology and Obstetrics clinics, Galway). The mean age was 47.8-years, age range (31-73 years). Thirty-four were females, mean age 45.4-years, age range (31-57-years); the rest were males with mean age of 50.3-years, age range (35-73-years).

• Viral Hepatitis-C group (HEPC): This group consists of 38 subjects, 35 females and 3 males, mean age 45.7-years, range (32-58-years). All female patients had received anti-D antibody on late seventies and early eighties. One of the three male patients had renal transplant with blood transfusion while the other two had blood transfusion following motor vehicle accidents.

• Alcoholic cirrhosis group (ALC): A total of 23 patients (all males) were included in this group. The mean age was 52.1-years and the age range were (43-63-years).

• Miscellaneous group (MISC): This group consists of 24 patients (7 females and 17 males, mean age 45.2-years, range 35-55-years). Seven out of 24 had chronic active hepatitis and the rest had hemochromatosis.

Methods

Pitted Erythrocytes: three drops of fresh blood were mixed with 1ml of 3% buffered glutaraldehyde solution at Ph of 7.4. A few drops of this solution were plated on a slide and a coverslip was carefully applied. The edges of the cover slip were sealed with D.P.X mountant (BDH chemicals) to prevent drying artefact. The slides were then labelled and coded so that counts were performed without knowledge of the subject status. In each case 1,000 consecutive red blood cells were examined using interference-phase microscopy at a magnification of 1,000. The results were expressed as the percentage of erythrocytes that had one or more “pit” present. All counts were performed by one observer.

Statistical Analysis: Systat software for IBM compatible computer was used for statistical analysis. A database was created consisting of variables compared and analyzed in this study (diagnosis, age, sex, % pitted RBCs, total protein, prothrombin time, activated partial thromboplastin time “APTT”, international normalization ratio “INR”, bilirubin, alkaline phosphatase, AST, ALT, GGTP, platelets, MCV, RBCs, WBCs, IgG, IgM, IgA, hepatitis A, B, C screening, and liver biopsy. Data were analyzed using the student “t” test and analysis of variance with tukey HSD correction for multiple comparison of continuous variables wherever applicable. A p-value of <0.05 is considered statistically significant.

Results

Pitted erythrocytes were counted in a fresh venous blood sample for each subject and used to assess the splenic function in patients with liver diseases and controls. The following parameters were compared in the different groups (age, sex, % pitted RBCs, total protein, albumin, prothrombin time, APTT, INR, bilirubin, alkaline phosphatase, AST, ALT, GGTP, platelets, MCV, RBCs, WBCs, IgG, IgM, IgA). The normal values are compared with the UCHG laboratory parameters.

Normal controls: The mean “pitted” erythrocyte count in the control group was 3.717 ± (2SD) = 6.68%. It did not reach significant difference between females and males (0.2-6.2% vs 1.7-6.7%). Although, no significant difference was found with age, the “pitted” count tends to be higher in the older subjects, in the control group. The upper limit of “pitted” erythrocyte counts for normal group was taken from the mean value ± 2SD, this value being 6.68%. No control value exceeded this figure. Therefore, any subject with liver disease with a “pitted” erythrocyte count of less than 6.68% was said to have normal splenic function. Hyposplenism is considered in any subject with mean pitted erythrocyte count of more than 6.68%.

Miscellaneous (MISC): Three patients in this group were in the hyposplenism range (12.5%). The mean “pitted” erythrocyte count in this group was 4.92 ± 2SD=7.72% with a range of (2.3-7.8%) and did not differ significantly from normal controls (p>0.06, 95% CI, -0.482-1.925). Within the study groups, MISC group has statistically significant lower values of pitted RBC’s than both the ALC and HEPC groups (p <0.001 & p<0.007), respectively (Table-2).

Alcoholic Cirrhosis (ALC): All patients in this group had low pit count percentage indicating hyposplenism. The mean “pitted” erythrocyte count in this group was 11.11 ± (2SD) =14.68% with a range of (7.9-13.7%) and did differ significantly from normal controls (p<0.001, 95% CI, 6.63-8.16). When compared to MISC group a highly significant difference was found between the 2 groups (p<0.001, 95% CI, 5.26-7.12), but it did not differ significantly from HEPC (p=0.07), (Table-2).

<table>
<thead>
<tr>
<th>ALC vs CONT</th>
<th>ALC vs MISC</th>
<th>ALC vs HEPC</th>
<th>HEPC vs CONT</th>
<th>HEPC vs MISC</th>
<th>HEPC vs ALC</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.07</td>
<td>P&lt;0.001</td>
<td>95% CI (7.79-9.18)</td>
<td>95% CI (6.33-8.24)</td>
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<tr>
<td>95% CI (6.63-8.16)</td>
<td>95% CI (5.26-7.12)</td>
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</table>

Table 2: Comparison between the different groups using ANOV.

Viral hepatitis C (HEPC): All subjects in this group had evidence of hyposplenism. The mean “pitted” erythrocyte count was 12.2 ± (2SD) = 16.35% with a range of (8-16.5%) and there was a significant difference from normal controls and MISC groups (p<0.001, 95% CI 7.79-9.18) and (p<0.001, 95% CI 6.33 – 8.24) respectively, but no significant difference was found from the ALC groups, (Table-2).

Others: The mean values of the following parameters (liver function tests, coagulation tests, immunoglobulins, platelets, and MCV results) are analyzed in each group separately and the statistical difference between the studied groups assessed. Normal values for all parameters are those used in the UCHG, Ireland. There was statistically significant difference in the following parameters between the ALC and the MISC groups (total protein, albumin, PT, INR, bilirubin, alkaline phosphatase, AST, GGT, MCV, platelets, IgG, and IgM). However, no significant difference in the rest of parameters studied were found.

Between the HEPC and MISC groups the following results were statistically significant (INR, bilirubin, AP, ALT, and GGT) the mean values of the rest of other parameters were not significant.

In the ALC and HEPC groups the difference between the mean values of the following results were statistically significant (total protein, albumin, INR, bilirubin, AP, AST, ALT, GGT, MCV, platelets, IgG, and IgM), the difference in the mean values of the rest of the parameters were not significant.

The percentage of the pitted erythrocyte counts were plotted against the parameters studied in the different groups and a good correlation were found only in the ALC group between the percentage of the pitted erythrocytes and the following parameters (INR, AST, ALT, platelets, IgG and IgM), no correlation between the percentage of pitted RBCs and the higher the mean values of (INR, AST, ALT, IgG and IgM and the lower the platelets count), data not presented.

Discussion

The percentage of “pitted” erythrocytes has been used before in the assessment of splenic function in patients with different medical diseases including alcoholic liver cirrhosis and hepatitis [29,30]. It has also been used in the assessment of splenic function in many other diseases, and was found to correlate well with both clearance rates of labelled, heat-damaged erythrocytes and splenic size computed from scinti-scans in treated celiacs [31-37], but to our knowledge it hasn’t been used in comparing the splenic function in patients with different liver diseases compared to the age and sex matched normal controls.

Although pitted erythrocyte counts are reasonably accurate quantitative assessment of splenic function, however, it tends to have false negatives both in Splenectomized and celiac patients [32], but there is no evidence in favor of the existence of false positives. Therefore, pitted erythrocyte counts are reasonably accurate quantitative assessment of splenic function. The technique has the advantage of being simple, precise, and free from exposure to radiation. There is considerable variation in the number of pitted erythrocytes found in both controls and studied population in many published series [32-37].

The mean splenic weight decreases after the age of 60-years and females usually have smaller spleens than males [37], therefore, splenic functional impairment might be expected with advancing age. In a previously reported study by O’Grady [37], the effect of age on pitted erythrocyte counts was found to be higher in over 60-age group than in under 25-age group. A larger number of older subjects included in the study of O’Grady may explain the positive effects of age. Even though the number of subjects included in the present study was relatively small, a positive association of pitted erythrocyte counts with age was observed. However, sex was found to have no effect on pitted erythrocyte counts in the current study or in any previous study.

Recently, hyposplenism has been recognized to be a complication of alcoholic cirrhosis, although the mechanism is still not fully understood. There are two theories to explain the association.

- Either hyposplenism is due to the direct toxic effect of alcohol on the spleen which may cause generalized depression of the reticuloendothelial system (RES) or.
- Due to cirrhosis of the liver per se [29,30]. The authors could demonstrate a strong correlation between alcoholic cirrhosis and hyposplenism as indicated by a high pitted erythrocyte count. This confirms the observation [29,30].

Splenic function in patients with viral hepatitis C has not been assessed before. A strong correlation was found between viral hepatitis C and hyposplenism compared with age and sex matched controls. As only three of the patients with hepatitis C were males it was not possible to show an effect of gender on splenic function. Within the study groups, viral hepatitis C patients had the highest mean pitted erythrocyte count compared with both alcoholic cirrhosis and miscellaneous groups. There is still no clear explanation for the effect of the virus on the splenic function, although generalized depression of RES seems a likely possibility. This could explain the increased vulnerability of alcoholics and cirrhotic patients due to HEPC to pneumococcal and other infections [29,30]. A question arises as to whether these subjects should be vaccinated against pneumococcal pneumoniae?

This study had demonstrated a clear relationship between various liver diseases and the degree of impairment of splenic function. However, the pitted erythrocyte count cannot be used to differentiate one type of liver disease from another. There is poor correlation between the percentage of pitted RBCs and the clinical severity of the underlying disease. Although, viral Hepatitis-C patients had the highest mean pitted RBC count compared with both alcoholic cirrhosis and miscellaneous groups. There is still no clear explanation for the effect of the virus on the splenic function, although generalized depression of RES seems a likely possibility. This could explain the increased vulnerability of alcoholics and cirrhotic patients due to HEPC to pneumococcal and other infections [29,30]. A question arises as to whether these subjects should be vaccinated against pneumococcal pneumoniae?

This study had demonstrated a clear relationship between various liver diseases and the degree of impairment of splenic function. However, the pitted erythrocyte count cannot be used to differentiate one type of liver disease from another. There is poor correlation between the percentage of pitted RBCs and the clinical severity of the underlying disease. Although, viral Hepatitis-C patients had the highest mean pitted RBC count, they were all clinically healthy. These patients were attending a Hepatitis-C clinic for routine follow-up and their liver function tests, biochemical and hematological parameters were within the normal range. The alcoholic cirrhosis patients who had mean pitted RBC count lower than that of viral Hepatitis-C patients had variable abnormalities of liver function tests, biochemical and hematological parameters and clinically many of them were sick.

Using a multiple regression comparison, a statistically significant correlation was found only in alcoholic cirrhosis group between the pitted RBC count and (INR, AST, ALT, platelets, IgG, and IgM, p<0.05). There was no significant correlation between
the above-mentioned parameters and the pitted RBC count in either viral Hepatitis-C or miscellaneous groups. This positive correlation with alcoholic cirrhosis could not be explained.

Conclusion

Evidence of hyposplenism was demonstrated in all patients with viral Hepatitis-C, alcoholic cirrhosis and in approximately 12.5% of miscellaneous liver disease group. The association of hyposplenism with viral Hepatitis-C has not previously been reported. It is possible that the mechanism of splenic involvement in alcoholfics and Hepatitis-C patients is different and further detailed studies are warranted. The authors have demonstrated that impaired splenic function does not influence the biochemical and hematological parameters in various types of liver diseases.

References