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Research Article

Evaluation of surface properties of erythrocyte membranes in liver diseases

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ABSTRACT

Background: The physicochemical properties of Red Blood Cell membranes (RBC) are altered in liver diseases. Langmuir monolayers offer an excellent model system to study biological membrane surface properties. The aim of this study was to evaluate surface properties of erythrocyte membranes in liver diseases.
Methods: Sixty-one patients with various liver diseases and fifteen controls were enrolled. Surface properties of RBC membrane were evaluated using Langmuir monolayers. Surface pressure area isotherms were recorded at body temperature using RBC membrane lipid extract. Student's t-test and Analysis of variance tests were performed.
Results: Mean maximum surface pressure and hysteresis area were significantly higher in cirrhotic and non-cirrhotic liver disease groups compared to controls. Within cirrhotics, mean maximum surface pressure and lift off area was significantly lower in the Child C group as compared to the Child A, B and A-B groups. The mean hysteresis area was significantly lower in the Child C group as compared to the Child B and A-B groups.
Conclusion: The results of our study confirmed high rigidity of RBC membrane in mild and moderate liver cirrhosis and high fluidity in severe liver cirrhosis. This study may pave the way to the development of a surface activity based biophysical tool for therapeutic implication in liver diseases.

Keywords: Liver cirrhosis, Erythrocyte membrane, Langmuir-Blodgett technique, Surface properties, Surface pressure area isotherms

INTRODUCTION

Cirrhosis is a general term for end-stage liver disease. It represents the final common histologic pathway for a wide variety of chronic liver diseases. Cirrhosis is defined as the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury.[1] According to the National Institutes of Health, Cirrhosis is the 12th leading cause of death by disease and according to the WHO data published in 2011 liver diseases deaths in India reached 208,185 or 2.31% of total deaths.[2] Pathological alterations in the structure or functions of RBC (Red Blood Cell) membranes are involved in the etiology of liver diseases including cirrhosis. Patients with severe liver disease may have spur cell anemia with Red Blood Cells (RBCs) that have characteristic morphological abnormalities, hemolytic anemia and altered membrane lipid composition.[3] RBC is a complex structure composed of a lipid bilayer supported by a scaffolding of cytoskeletal proteins. Lipid composition determines the structure, function and integrity of RBC membranes.[4] In patients with liver disease, abnormalities in the composition of the plasma lipoproteins are associated with corresponding changes in the erythrocyte membrane lipid composition and accompanying changes of their

morphology.[5,6] These changes cause alterations in membrane fluidity and function with potential pathophysiological consequences. The normal RBC membranes are enriched in cholesterol, phospholipids. The main alteration noticed in liver disease is increased cholesterol and the phospholipid compositions and cholesterol-phospholipid (C-P) ratio are also altered.[7,8] These changes in membrane lipid composition can affect the structure, morphology and integrity of erythrocytes.[9] Changes in hepatocytes are reflected by RBC changes in liver diseases. Hence the structure and functions of RBC membranes could be used to understand pathogenesis and prognosis of liver diseases.

The membrane function of different cells is not only dependent on the lipid composition but also on the architecture (packing and phases) of the lipid membranes. Modifications of the lipid composition and the asymmetry of the bilayer have been shown to affect the overall shape of the erythrocyte, architecture and also the cell's deformability. Changes in lipid profiles of membrane also alter fluidity, which in turn affects permeability of membrane.[10] Changes in the shape, mechanical characteristics or the integrity of the erythrocyte have severe implications on the functionality and viability of the cell, as can be seen in several dysfunctional states of the erythrocyte in diseased states.[11] Langmuir monolayer is a technique used to study the surface properties of cell membranes as model monolayers. These surface properties of the membranes can give information regarding overall phase behaviour and the packing of the lipids. Till now, various studies have been conducted to see alteration in membrane lipid composition and fluidity in liver diseases. However, there is paucity of data regarding surface activity and interfacial tensiometric properties of RBC membranes in liver diseases.

The aim of this study was to evaluate and compare the surface properties using surface pressure area isotherms of erythrocyte membranes in healthy and liver disease patients and to correlate these surface parameters with RBC membrane lipid profiles and with severity of liver disease.

In India this is perhaps the first study where Langmuir Blodgett technique was used to evaluate RBC membrane surface activity using model monolayer in liver diseases.

METHODS

This study was conducted in department of biochemistry and clinical nutrition, Seth G. S. medical college and KEM hospital, Mumbai, in collaboration with department of biosciences and bioengineering, Indian Institute of Technology (IIT), Mumbai.

This study was approved by the ethics committee of Seth G. S. medical college.

Subjects

Fifty one patients (Age-30 to 65 years and both sex) of liver cirrhosis with various etiologies and 10 patients of non-cirrhotic liver diseases (Total 61 patients of liver disease) were enrolled from liver clinic at Seth G. S. medical college and KEM hospital, Mumbai, during June 2013 to January 2014. The diagnosis of liver disease was based upon clinical features, liver function tests, International Normalized Ratio (INR), ultrasonography, upper gastrointestinal endoscopy and liver biopsy wherever feasible. Patients suffering from concomitant diseases, which can alter the lipid profiles such as diabetes mellitus, cancer, acute pancreatitis, recent parenteral nutrition and acute gastrointestinal bleeding, renal failure and patients who were on glucose or lipid lowering drugs were excluded. Child Pugh scores were calculated for liver cirrhotic patients as an index for the extent of liver damage. It was used for categorization of liver cirrhosis patients into mild, moderate and severe liver cirrhosis.[12] Out of 51 liver cirrhotic patients, 15 had mild, 18 had moderate and 18 had severe liver cirrhosis. 15 ages matched healthy subjects were enrolled as control group.

Blood samples and clinical tests

Blood sample from patients and controls (10 ml) was taken in heparin and plain evacuated tubes by venepuncture after 12 hour fasting and after taking well informed consent. Blood samples were processed for several biochemical parameters which included - serum AST, ALT, ALP, total protein, albumin, bilirubin (Total and direct), haemoglobin, prothrombin time, total cholesterol and triglycerides. These analytic procedures were done using standard kits in the central clinical biochemical laboratory, Seth G.S. medical college, Mumbai. RBC membrane ghost was prepared using method of Stack and Kant, 1974.[13] RBC membrane lipid extraction was carried using method of Rose and Oklander, 1965.[14] Aliquots of lipid extract were taken for RBC membrane cholesterol and total phospholipids estimation using the methods of Memon L et al. 2003 and Rouser G et al., 1979 respectively.[15,16]

All the surface activity studies of monolayers were conducted in a computer controlled Langmuir–Blodgett trough (KSV mini trough model, KSV instruments Ltd., Finland) in the department of biosciences and bioengineering, IIT, Mumbai. The Teflon coated trough is equipped with two Derlin barriers that can be moved inward and outward at the same speed during compression and expansion cycles. The entire trough is surrounded by a water jacket, providing temperature control. The temperature of the sub phase in the Langmuir-Blodgett trough was maintained at 37 ± 1°C. Wilhelmy plate made of platinum was used to sense the change in surface pressure during the experiment. It was roughened each time before the start of an experiment to ensure complete wetting of the plate. The trough was

cleaned with deionized water, methanol, acetone and deionized water (again), in sequence several times. The surface of the sub-phase was cleaned with the help of an aspirator. Cleanliness was confirmed by a zero reading of surface pressure. Highly pure deionized water (resistivity of 18.2 MΩ/cm) was used as the sub-phase for all surface activity experiments.

RBC membrane lipid extract in chloroform/isopropanol organic solvent of all patients and controls was used for monolayer study. 50 µl of sample (concentration corresponding to 110 A^{o2}/molecule, 19.6 ml of lipid extract corresponding to 1 ml pack RBCs) was spread as tiny drops on the surface of the sub-phase using a Hamilton syringe. 30 minute wait time was given for the evaporation of the organic solvents. The plots of surface pressure versus area change are called isotherms. The surface pressure-area isotherms were recorded by continuous compression and expansion for three cycles (1 cycle = 1 compression + 1 expansion) with a barrier speed of 50 mm/min. The maximum relative area change during compression was 75%. From the surface pressure area isotherms obtained, the following parameters were calculated - 1) Maximum surface pressure which is the maximum value of surface pressure obtained during the compression stage of the cycle, and it represents the maximum packing possible of the lipids of the monolayer. 2) The hysteresis area, which is indicative of energy trapped in a monolayer is the difference between the free energy of compression and free energy of expansion, and was calculated from the area under the corresponding surface pressure area isotherms. 3) Lift-off area which indicates interaction of molecules in the monolayer was obtained by extrapolating the area at which an increase in surface pressure from the baseline value was observed to the area cm^2 axis. All the parameters were calculated from the first compression cycle of the isotherms.[17]

Statistical analysis

Data were analysed using SPSS version 21. All the parameters were expressed as mean and Standard Deviation (SD). To compare a continuous variable between groups, the Student's unpaired t-test was performed. Analysis of quantitative data between a qualitative variable with more than two subgroups was done using one-way ANOVA. Tukey's Post Hoc test was then used for observations between individual groups of patients if P value of ANOVA was statistically significant (P <0.05).

RESULTS

In our study 61 liver disease patients and 15 controls were included. Out of 61 patients, 41 (67.2%) were male and 20 (32.8%) were female. The most common age group affected by liver diseases was 41-50 years in both male (43.9%) and female (30%).

Out of the 61 liver disease patients, 51 (83.6%) were cirrhotics and 10 (16.4%) were non cirrhotics. According to Child Pugh criteria, out of the 51 cirrhosis patients, 15 (29.4%) patients belonged to Child-Pugh class A, 18 (35.3%) patients belonged to Child-Pugh class B and 18 (35.3%) patients belonged to Child-Pugh class C.

In our study, the most common cause of cirrhosis was alcoholic (51%) and in non-cirrhotic liver diseases, 60% of the patients were of Budd-Chiari syndrome (Figure 1A & 1B).

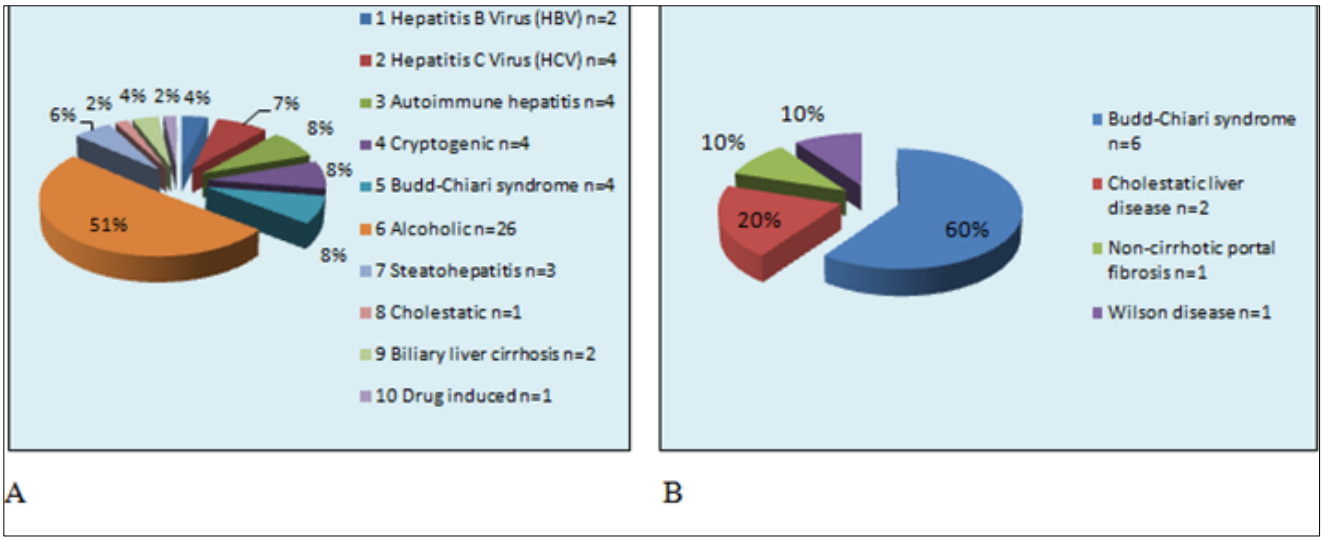

Figure 1: A] Causes of cirrhosis (N=51) and B] non-cirrhotic liver diseases (N=10) in study patients.

Biochemical parameters in control and liver cirrhosis

A comparison of biochemical parameters of serum and RBC membrane between control and cirrhotic group is shown in Table 1. Serum total cholesterol, total protein, albumin, haemoglobin and RBC membrane total phospholipids were significantly lower in cirrhotic group compared to control group. Serum AST, ALT, AST/ALT ratio, ALP, bilirubin - total and direct, RBC membrane cholesterol, RBC membrane cholesterol to phospholipid ratio were significantly higher in cirrhotic group as compared to the control group. No significant difference in serum triglycerides could be observed in cirrhotics in comparison to control group.

Biochemical parameters in control and non-cirrhotic liver disease group

In non-cirrhotic liver disease group, AST, ALT, Total cholesterol, haemoglobin, RBC membrane cholesterol, RBC membrane cholesterol to phospholipid ratio were significantly increased compared to control group. Serum total protein and albumin were significantly decreased in non-cirrhotic liver disease group compared to control group whereas no statistically significant differences were found between the groups in the other assessed biochemical parameters (Table 1).

Table 1: Comparison of biochemical parameters in cirrhosis and non-cirrhotic liver diseases with control.

Parameters	Control (n=15) Mean ± SD	Cirrhosis (n=51)		Non-cirrhotic liver diseases (n=10)	
		Mean ± SD	P value	Mean ± SD	P value
AST (mg/dl)	21.53 ± 4.74	58.70 ± 32.29	0.00001***	54.70 ± 61.99	0.048*
ALT (mg/dl)	14.60 ± 4.18	31.4 ± 29.44	0.032*	36.10 ± 37.44	0.036*
AST/ALT Ratio	1.52 ± 0.326	2.84 ± 2.08	0.018*	1.57 ± 0.39	0.746^{NS}
ALP (mg/dl)	91.53 ± 25.18	133.13 ± 76.75	0.044*	153.10 ± 173.51	0.185^{NS}
Total Protein (g/dl)	7.41 ± 0.44	6.80 ± 0.89	0.014*	6.87 ± 0.58	0.014*
Albumin (g/dl)	4.07 ± 0.27	3.11 ± 0.70	0.0001***	3.17 ± 0.44	0.019*
Bilirubin total (mg/dl)	1.10 ± 0.138	2.42 ± 2.50	0.047*	2.75 ± 3.47	0.077^{NS}
Bilirubin direct (mg/dl)	0.51 ± 0.130	1.33 ± 1.55	0.048*	1.64 ± 2.28	0.067^{NS}
Haemoglobin gm%	13.22 ± 0.81	10.33 ± 2.22	0.0001***	10.69 ± 1.52	0.0001***
INR	1.1607 ± 0.16	1.45 ± 0.42	0.011*	1.29 ± 0.25	0.121^{NS}
Serum cholesterol (mg/dl)	202.46 ± 19.75	107.18 ± 33.82	0.0001***	148.90 ± 27.74	0.0001***
Serum Triglycerides (mg/dl)	111.46 ± 19.75	102.64 ± 26.42	0.236^{NS}	116.90 ± 19.03	0.501^{NS}
RBC membrane cholesterol (mg/ml pack RBC)	1.11 ± 0.22	1.35 ± 0.40	0.036*	1.46 ± 0.329	0.004**
RBC membrane total phospholipid (mg/ml pack RBC)	2.34 ± 0.302	1.93 ± 0.36	0.0001***	2.18 ± 1.00	0.564^{NS}
Membrane C/P ratio	0.47 ± 0.080	0.72 ± 0.24	0.0002***	0.75 ± 0.352	0.007**

***P <0.001, **P < 0.01, *P <0.05, NS = Not significant (P >0.05)

Comparison of surface activity parameters between various liver disease groups and control

The Langmuir monolayers were subjected to three compression cycles. Figure 2 represents the surface pressure-area isotherms of different groups (average of first compression cycle) and provides the overall comparison of tensiometric profile of various groups. The tensiometric parameters calculated from the isotherms were Maximum Surface Pressure (MSP), Hysteresis area and lift-off Area (from the first compression cycle).

In the mild and mild-moderate liver cirrhosis group all the three surface parameters MSP (P <0.05), hysteresis area (P <0.001), and lift-off area (P <0.05) were significantly higher compared to the control group. In moderate liver cirrhosis group MSP and Hysteresis area was significantly higher (P <0.05), (P <0.001) compared to control group whereas no significant difference was observed in lift-off area between both the groups. In cirrhotic and non-cirrhotic liver disease groups hysteresis area was significantly higher compared to control group (P <0.05), whereas no significant changes could be observed in MSP and lift-off area between cirrhotic group and control group. In severe liver cirrhosis group MSP (P <0.001) and lift-off area (P <0.001) were significantly lower as compared to control group (Table 2).

RBC membrane lipids and surface activity parameters vs. severity of liver cirrhosis

Table 3A reports mean value of RBC membrane lipids and surface activity parameters according to severity of liver cirrhosis in cirrhotic group. Further Table 3B reports group significance between individual liver cirrhosis groups. The RBC membrane cholesterol (mean ± SD) and

C/P ratio was statistically lower in the severe liver cirrhosis group (Child C group) as compared to the moderate liver cirrhosis group (Child B). The RBC total phospholipid (mean ± SD) was not statistically different between the four groups (Child A, B, A-B and C)

The maximum surface pressure and lift off area (mean ± SD) was statistically lower in the Child C group as compared to the Child A, Child B and Child A-B groups. The hysteresis area (mean ± SD) was statistically lower in the Child C group as compared to the Child B and Child A-B groups (Table-3A & B).

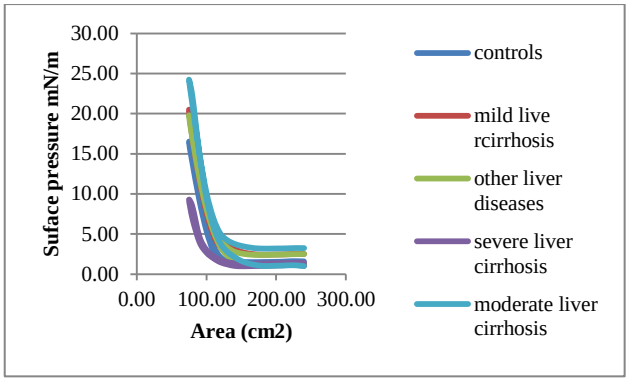

Figure 2: Average surface pressure area isotherms of different groups.

Table 2: Comparison of maximum surface pressure, hysteresis area and lift off area of diseased groups with controls.

Groups	Max surface pressure (mN/m) (Mean ± SD)	Hysteresis area (Micro joule) (Mean ± SD)	Lift off area (cm^2) (Mean ± SD)
Controls (n=15)	16.5 ± 3.33	80.26 ± 29.1	115.5 ± 7.95
Mild liver cirrhosis (n=15)	20.6 ± 5.50*	200.00 ± 159.85**	127.0 ± 18.1*
Moderate liver cirrhosis (n=18)	24.34 ± 11.6*	260.77 ± 196.39***	123.5 ± 17.7^{NS}
Mild-moderate liver cirrhosis (n=33)	22.64 ± 9.43*	233.15 ± 180.59**	125 ±17.6*
Severe liver cirrhosis (n=18)	9.48 ± 8.98**	77.55 ± 125.12^{NS}	101.5 ± 17.0**
Total cirrhosis (n=51)	18.00 ± 11.1^{NS}	178.23 ± 178.4*	112.5 ± 20.5^{NS}
Non-cirrhotic liver disease (n=10)	20.02 ± 8.09^{NS}	178.1 ±164.2*	120 ± 15.9^{NS}

***P <0.001, **P < 0.01, *P <0.05, NS = Not significant (P >0.05)

Table 3A: RBC membrane lipids and surface activity parameters in cirrhotics according to child criteria.

Parameters	Child A (Mild liver cirrhosis)	Child B (Moderate liver cirrhosis)	Child A-B (Mild-moderate liver cirrhosis)	Child C (Severe liver cirrhosis)	F value	Significance
RBC membrane cholesterol mg/ml pack RBC)	1.28 ± 0.250 1.33	1.56 ± 0.46	1.43 ± 0.40	1.18 ± 0.40	3.40	0.022*
RBC membrane total phospholipid mg/ml pack RBC	2.08 ± 0.46	1.86 ± 0.24	1.96 ± 0.37	1.87 ± 0.34	1.32	0.274
RBC membrane C/P ratio	0.66 ± 0.24	0.84 ± 0.25	0.76 ± 0.26	0.63 ± 0.16	2.95	0.037*
Maximum surface pressure (mN/m)	20.61 ± 5.50	24.34 ± 11.66	22.64 ± 9.43	9.48 ± 8.98	9.82	0.00001***
Hysteresis area (micro joule)	200.0 ± 159.85	260.77 ± 196.39	233.15 ± 180.59	77.55 ± 125.12	4.27	0.007**
Lift off area (cm^2)	126.80 +18.02	123.55 +17.71	125 + 17.64	101.55 ± 17.06	8.47	0.00005***

All values are expressed as Mean ± SD
***P <0.001, **P < 0.01, *P <0.05, NS = Not significant (P >0.05)

Table 3B: Relationship between various parameters and severity of liver damage in cirrhotic patients (Post Hoc tests: multiple comparisons: Using Tukey HSD test).

Parameters	Group A vs. B	Group A vs. C	Group A+B vs. C	Group B vs. C
RBC membrane cholesterol mg/ml pack RBC	0.167	0.895	0.133	0.023*
RBC membrane total phospholipid mg/ml pack RBC	0.311	0.340	0.818	1.0
RBC membrane C/P ratio	0.126	0.991	0.282	0.048*
Maximum surface pressure (mN/m)	0.663	0.005*	0.00003*	0.00004*
Hysteresis area (micro joule)	0.738	0.177	0.013*	0.010*
Lift off area (cm^2)	0.952	0.001*	0.0001*	0.002*

Result is significant at <0.05 level*

DISCUSSION

While the fluid nature of biological membrane is well recognized, the term membrane fluidity is ambiguous; it refers to several aspects of the dynamic structure of membranes including a variety of molecular motions of both the lipid and protein constituents.[18] The structural and dynamic properties of membrane lipid matrix have been extensively studied using techniques such as Nuclear Magnetic Resonance (NMR), Electron Spin Resonance (ESR), Flash Photolysis (FP) etc.[19] Langmuir Blodgett (LB) technique is a well-established sensitive technique and has been verified as an excellent model system to study biological membrane properties using model monolayer.[20] Studies with monolayers can help understanding the behaviour and role of different lipids in biological membranes. Membrane physical properties depends on the amount of free cholesterol content, phospholipid composition, degree of fatty acid saturation, length of acyl chains.[19] In our study, erythrocyte plasma membrane surface properties were determined in liver diseases by Langmuir Blodgett technique using monomolecular layer of RBC membrane lipids.

Surface activity - mild and moderate liver cirrhosis

In mild liver cirrhosis group, MSP was significantly higher compared to control group. A higher value of surface pressure indicates that the lipids are closely packed and are compressed to a "rigid gel state". In mild cirrhosis group, hysteresis area was significantly higher compared to control group which indicate the formation of stable monolayer. In mild cases, lift off area was also significantly higher compared to control group. This high lift-off area in patient samples indicates less amount of fluidity in the monolayer compared to control group. So in mild liver cirrhosis, all three surface activity parameters show lower surface activity indicating high rigidity of RBC membrane lipid extract compared to control group. In moderate cases, the surface activity parameters which include MSP and hysteresis were significantly higher compared to control. When surface activity parameters of moderate liver cirrhosis were compared with mild liver cirrhosis group, the increase was not statistically significant.

It can be assumed that in mild and moderate liver cirrhosis, lipid mixture contained increased amount of rigidifying lipids in the membrane which may include saturated fatty acids, cholesterol and some phospholipids. These lipids cause the formation of tightly packed and highly ordered compressed monolayer. The present study measured RBC membrane cholesterol and total phospholipids which are known to contribute to the membrane activity. Significantly increased cholesterol and C/P ratio in mild and moderate liver cirrhotic group indicate towards increased rigidity. Cholesterol would be expected to increase membrane rigidity by increasing the lateral packing of phospholipid acyl chains. The increase of cholesterol has a condensing effect and reduces acyl chain mobility and decrease of polyunsaturated fatty acid content within the phospholipid acyl chains make the monolayer more rigid.[6,21] RBC membrane phospholipid fatty acid composition also affects the membrane fluidity. Studies have shown that in liver diseases, fatty acid composition of phospholipid is altered. The content of palmitic acid is increased and that of arachidonic and stearic acid is decreased. Thus polyunsaturated fatty acid (PUFA) content is reduced corresponding to reduce fluidity.[19,22]

Surface activities - severe liver cirrhosis

In severe liver cirrhosis, isotherms obtained have lower MSP values, less hysteresis and lift-off area compared to mild, moderate, mild plus moderate liver cirrhosis and control groups. Low values of MSP indicate that the lipids are packed into the more fluid 'liquid condensed state' on the maximum compression. Low lift-off area also indicates a low intermolecular interactions and high amount of fluidity in the lipid bilayer. Thus surface parameters indicate towards higher fluidity of RBC membrane lipid extract in severe liver cirrhosis.

It can be assumed that high membrane fluidity may be the consequence of increased lipid peroxidation in severe liver cirrhosis. Lipid peroxidation causes formation of short-chain, ionized and unsaturated fatty molecules. Lipid mixtures with higher content of unsaturated fatty acids or ionized short chain fatty acids form less ordered monolayer. Unsaturated fatty molecules have a kinked structure due to the presence of unsaturated bonds. These

kinks cause lipids to take up more area in the monolayer, and hence disrupt their ordered packing. Ionized fatty molecules repulse each other and suppress intermolecular interaction thus causing low intermolecular interactions.

Lipid peroxidation is a degenerative process that affects polyunsaturated fatty acids of membrane phospholipids under conditions of oxidative stress.[23] Lipid peroxidation causes reductive degradation of lipid acyl side chains and formation of lipid peroxidation products. Lipid peroxidation products accumulate in the bilayer and further contribute to changes in the structural organization and intermolecular packing of membrane lipid components and thermodynamics of the membrane bilayer.[23,24] It also promotes an increase in the molecular volume associated with the unsaturated region of the hydrocarbon core and induces the apparent inter-digitations of the phospholipid acyl chain terminal methyl segments.[25] There is evidence of increased RBC membrane lipid peroxidation with increased cirrhosis severity. In a study conducted by A. Geetha et al. 2007, free radical mediated oxidative stress in severe liver cirrhosis was evidenced by the elevated levels of lipid peroxides and lipid hydroperoxides in RBCs due to imbalance between the prooxidants and/ or free radicals and antioxidizing systems. In this manner lipid peroxidation of phospholipid fatty acid causes formation of short, ionized, unsaturated fatty molecules.[26]

Although, total phospholipid concentration is not affected by lipid peroxidation because primary target of lipid peroxidation is PUFA and secondary target is the cholesterol molecule. In this study no significant alterations in RBC membrane total phospholipids could be observed in severe liver cirrhosis. RBC membrane cholesterol and C/P ratio were decreased compared to moderate cirrhosis group which indicate some amount of cholesterol oxidation and increased fluidity of RBC membrane respectively.[27] As a monounsaturated lipid, cholesterol is also susceptible to peroxidation (although albeit less than polyunsaturated phospholipids) and contribute to membrane oxidative damage. Its oxidation products include epoxides, peroxides and diols.[28] Oxidized forms of cholesterol are known to alter the structural order and dynamic properties of lipids, with adverse effects on the function of biological membranes.[29] Lipid peroxidation also causes decreased uptake of cholesterol from circulation, decreases the cholesterol concentration in the membrane and increases the concentration of its peroxidation products in the membrane.[30] Altered phospholipid composition can also play a role in altering the RBC membrane fluidity. In a study conducted by James Owen et al., 1982, it was observed that phosphotidylcholine/sphingomylin ratio is increased in liver diseases. This causes increase in RBC membrane fluidity because of decrease in sphingomyelin molecules compared to phosphatidylcholine. Rigidifying nature of sphingomyelin molecules is because of its high content of saturated fatty acids, trans double bond in sphingosine chains, inter and intramolecular hydrogen bonding of free hydroxyl groups and amide linkages.[19]

Our findings are evidenced by the study conducted by Tukasz Gwoz et al., 2011. In this study, in patients of alcohol induced severe liver cirrhosis; increased fluidity was observed at the depth of 5th atom of carbon of fatty acid hydrocarbon chain, which points to modification of polar heads of phospholipids in the surface areas of cell membrane.[31] Increase of fluidity of erythrocyte membrane lipids was also observed in model studies with t-butylhydroperoxide which initiated the process of peroxidation.[32] Complete lipid composition of RBC membrane lipid extract was not analysed in our study, Nevertheless, evidence suggests that interaction of membrane cholesterol, altered phospholipid composition, altered fatty acid composition of phospholipid fatty acids and accumulation of lipid peroxidation products in RBC membrane played a role in altering membrane fluidity. And this pushed the membrane fluidity from more rigid towards more fluid state with increase in liver cirrhosis severity. On the basis of our findings, it can be suggested that surface properties can be used as a tool to distinguish between grade A, B and C of liver cirrhosis.

Surface activity in cirrhotic and non-cirrhotic liver disease group

In cirrhotic and non-cirrhotic liver disease group, mean hysteresis area was significantly increased compared to control group. In cirrhotic and non-cirrhotic liver disease group, mean hysteresis area was almost equal to each other. High hysteresis area indicates toward the high stability of the monolayer which can be attributed to increased cholesterol concentration in the lipid mixture. Our results confirm the presence of high cholesterol in both the patient groups. In the cirrhotic group, RBC membrane cholesterol and C/P ratio is increased and membrane total phospholipid in decreased significantly as compared to control. In non-cirrhotic liver disease group RBC membrane cholesterol and C/P ratio is increased compared to control group. Data suggests the same pattern of surface activity in both the groups. This indicates that RBC membrane fluidity was mildly decreased in both disease groups. Increased membrane cholesterol can modulate membrane lipid order based on its stereospecific interactions with component phospholipids and contribute to increased membrane rigidity.[33] A similar finding was observed by James S. Owen et al., 1982.[19] They studied RBC membrane composition and fluidity in patients of liver diseases and found that membrane fluidity was significantly decreased in patient's erythrocytes and correlated significantly with the C/P ratio ($r = 0.88$, $P < 0.002$) Increase in membrane cholesterol, decreased phospholipids and increased C/P ratio in the disease group may be due to impaired exchange of cholesterol and phospholipids from the plasma and lipoproteins. It can also be stated that the decreased levels of phospholipids might have affected the exchange of cholesterol. In liver disease, net transfer of

cholesterol occurs from the lipoproteins to the erythrocyte membrane. This is due to cholesterol in the plasma as a consequence of secondary lecithin-cholesterol-acyl-transferase deficiency which saturates the lipoprotein surface causing shift in the equilibrium.[7] Thus membrane cholesterol level increases.

Biochemical parameters

Significantly higher levels of serum AST, ALT, ALP, AST/ALT ratio were observed in cirrhotic group as compared to control group. In non-cirrhotic liver disease group, ALP, AST/ALT ratio was not significantly different. In our study mean AST/ALT ratio in cirrhosis patients was 2.84 and 1.52 in the control group. An elevated serum AST in relation to serum ALT has been proposed as an indicator of liver damage. This can be the result of reduction in hepatic ALT content due to a deficiency in the cofactor pyridoxine-5-PO_4 and mitochondrial damage leading to release of mitochondrial AST in serum.[34] In our study no direct correlation was observed between liver enzymes and surface parameters however it can be postulated that as ALT/AST ratio increases with increased severity confirming liver damage. This alteration in hepatocytes was reflected through altered surface parameters of RBC membrane.

Serum cholesterol levels were significantly lower in liver cirrhotic group and non-cirrhotic liver disease group as compared to control group. The significant decline in the serum total cholesterol in cirrhotic patients compared with control group has been confirmed by earlier studies.[35] Mehbob, et al., 2007 studied 51 cirrhosis patients and there was significant decline in serum total cholesterol levels in patients (P-138.9, C-184.6 P = 0.030).[36] This can be attributed to reduced biosynthetic property of liver in liver diseases. As confirmed by AST/ALT ratio, gradual increase in hepatocytes damage can cause gradual decrease in serum cholesterol synthesis.

CONCLUSIONS

Model cell membranes are systems in which the lipid organization mimics the arrangement of lipids in natural cell membrane. This study provided useful insight on RBC membrane organization and lipid packing in physiological conditions and liver diseases (especially cirrhosis). Interesting evidences of the altered phase states and surface activity profiles of monolayers of RBC membrane lipid extracts in mild, moderate and severe liver cirrhosis were observed. The difference in surface activity denotes alteration in RBC membrane lipid composition, physical-chemical properties and thus their molecular packing. Significant correlation of Langmuir Blodgett surface parameters with RBC membrane cholesterol was observed in liver cirrhosis. Thus, in liver diseases differences in surface activity of membranes as measured by tensiometric parameters (MSP, hysteresis area, lift-off area) from surface pressure area isotherms is associated with altered RBC membrane properties. RBC's health status is crucial to the overall wellness of the liver disease patient. This study attempts to show a way to the development of a less-invasive biophysical tool (based on interfacial properties using tensiometric parameters) to design therapeutic strategies, track efficacy of treatment regimens on the surface properties, integrity and health status of RBCs in liver diseases. Hence Langmuir Blodgett technique may have therapeutic and prognostic implication for the benefit of liver disease patients.

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REFERENCES

1. Schuppan D, Afdhal NH. Liver cirrhosis. Lancet. 2008;371(9615):838-51.
2. World Health Rankings. India: liver disease, 2014. Available at: http://www.worldlifeexpectancy.com/india-liver-disease.
3. Cooper RA. Hemolytic syndromes and red cell membrane abnormalities in liver disease. Semin Hematol. 1980;17(2):103.
4. Eggleton CD, Popel AS. Large deformation of red blood cell ghosts in a simple shear flow. Phys Fluids. 1998;10(8):1834-45.
5. Day RC, Harry DS, McIntyre N. Plasma lipoproteins and the liver. In: Day RC, Harry DS, McIntyre N, eds. Liver and biliary disease: Pathophysiology, Diagnosis and Management. 1st ed. London: WB Saunders; 1979: 63-82.
6. Salvioli G, Rioli G, Lugli R, Salati R. Membrane lipid composition of red blood cells in liver disease: regression of spur cell anaemia after infusion of polyunsaturated phosphatidylcholine. Gut. 1978;19(9):844-50.
7. Cooper RA. Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells. J Supramol Struct. 1978;8(4):413-30.
8. Grattagliano I, Giudetti AM, Grattagliano V, Palmieri VO, Gnoni GV, Lapadula G, et al. Structural and oxidative modifications of erythrocyte ghosts in patients with primary biliary cirrhosis: relation with the disease stage and effect of bile acid treatment. Eur J Clin Invest. 2003;33(10):868-74.
9. Cooper RA, Diloy-Puray M, Lando P, Greenberg MS. An analysis of lipoproteins, bile acids, and red cell membranes associated with target cells and spur

cells in patients with liver disease. J Clin Invest. 1972;51(12):3182.
10. Bennett-Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, et al. Evolution of adverse changes in stored RBCs. Proceedings Natl Acad Sci. 2007;104(43):17063-8.
11. Girasole M, Pompeo G, Cricenti A, Congiu-Castellano A, Andreola F, Serafino A, et al. Roughness of the plasma membrane as an independent morphological parameter to study RBCs: a quantitative atomic force microscopy investigation. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2007;1768(5):1268-76.
12. Child CG, Turcotte JG. Major problems in clinical surgery. In: Child CG, Turcotte JG, eds. The Liver and Portal Hypertension. 1st ed. New York: WB Saunders; 1964.
13. Steck TL, Kant JA. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol. 1974;21:172-93.
14. Rose HG, Oklander M. Improved procedure for the extraction of lipids from human erythrocytes. J Lipid Res. 1965;6(3):428-31.
15. Memon L, Spasojević-Kalimanovska V, Jović P, Spasić S, Bogavac-Stanojević N. Determination of cholesterol in erythrocyte membrane. Jugoslovenska Medicinska Biohemija. 2003;22(3):213-9.
16. Rouser G, Fleischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids. 1970;5(5):494-6.
17. Preetha A, Banerjee R, Huilgol N. Surface activity, lipid profiles and their implications in cervical cancer. J Cancer Res Therapeut. 2005;1(3):180.
18. Hare F, Amiell J, Lussan C. Is an average viscosity tenable in lipid bilayers and membranes? A comparison of semi-empirical equivalent viscosities given by unbound probes: a nitroxide and a fluorophore. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1979;555(3):388-408.
19. Owen JS, Bruckdorfer KR, Day RC, McIntyre N. Decreased erythrocyte membrane fluidity and altered lipid composition in human liver disease. J Lipid Res. 1982;23(1):124-32.
20. Hussain SA. Langmuir-Blodgett Films a unique tool for molecular electronics. arXiv preprint arXiv. 2009;0908:1814.
21. Papahadjopoulos D. Cholesterol and cell membrane function: a hypothesis concerning the etiology of atherosclerosis. J Theoret Biol. 1974;43(2):329-37.
22. Neerhout RC. Abnormalities of erythrocyte stromal lipids in hepatic disease. J Lab Clin Med. 1968;71(3):438-47.
23. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res. 1998;39:1529-42.
24. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978;52:302-10.
25. Mason RP, Walter MF, Mason PE. Effect of oxidative stress on membrane structure: small-angle X-ray diffraction analysis. Free Radic Biol Med. 1997;23:419-25.
26. Geetha A, Lakshmi Priya MD, Jeyachristy SA, Surendra R. Level of oxidative stress in the red blood cells of patients with liver cirrhosis. Indian J Med Res. 2007;126(3):204.
27. Punchard NA, Senturk H, Teare JP, Thompson RPH. Resistance of erythrocytes to lipid peroxidation in alcoholic patients. Gut. 1994;35:1753-6.
28. Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B: Biol. 2001;63(1):103-13.
29. Verhagen JC, Ter Braake P, Teunissen J, Van Ginkel G, Sevanian A. Physical effects of biologically formed cholesterol oxidation products on lipid membranes investigated with fluorescence depolarization spectroscopy and electron spin resonance. J Lipid Res. 1996;37(7):1488-502.
30. Jain SK, Shohet SB. Red blood cell [14C]cholesterol exchange and plasma cholesterol esterifying activity of normal and sickle cell blood. Biochem Biophys Acta. 1982;688:11-5.
31. Gwoździński Ł, Krawczyk P, Dworniak D, Kowalczyk E, Błaszczyk J. Alterations in the erythrocyte plasma membranes in patients with alcohol-induced liver cirrhosis-preliminary results. Arch Med Sci. 2011;7(1):87-91.
32. Brzeszczynska J, Gwozdzinski K. Erythrocyte membrane damage induced by t-buthyl hydroperoxide. Curr Top Biophys.1998;22:238-41.
33. Yeagle PL. Cholesterol and the cell membrane. Biochim Biophys Acta. 1985;822:267-87.
34. Torkadi PP, Apte IC, Bhute AK. Biochemical evaluation of patients of alcoholic liver disease and non-alcoholic liver disease. Indian J Clin Biochem. 2014;29(1):79-83.
35. Ghadir MR, Riahin AA, Havaspour A, Nooranipour M, Habibinejad AA. The relationship between lipid profile and severity of liver damage in cirrhotic patients. Hepatitis Monthly. 2010;10(4):285.
36. Mehboob F, Ranjha FA, Masud S. Changes in serum lipid profile among patients suffering from chronic liver disease. Ann King Edward Med Univ. 2010;13(3):209-11.

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