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Red Blood Cell Membrane Physiology In Atorvastatin Treated Rats As Evaluated By Osmofragility Test And Possible Protective Role of Coenzyme Q 10

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Abstract
Background: *Erythrocyte Osmotic fragility (EOF) is defined as the degree of hemolysis that occurs when RBC are exposed to osmotic stress. EOF depends upon various factors including the RBC cell membrane composition, integrity, cell size, and shape and surface-volume ratios.*
Objectives of Study: *The present study was conducted to determine the red blood cell membrane physiology in atorvastatin treated rats as evaluated by erythrocyte osmofragility test &, evaluate possible protective role of Co-enzyme Q 10 against atorvastatin induced erythrocyte cell membrane injury.*
Subjects And Methods:*An experimental study was conducted at Animal house, Isra University Hyderabad and Department of Animal Husbandry and Veterinary Sciences Sindh Agriculture University Tando Jam. 50 albino Wistar rats was selected randomly according to inclusion and exclusion criteria. The rats were randomly divided into 5 groups namely A (controls), and experimental groups B, C, D and E. Atorvastatin and Co enzyme Q 10 were given for 6 weeks duration. Erythrocyte osmotic fragility test was performed with Na Cl solutions of difference osmotic concentrations e.g. as 0.1 N NaCl solution, 0.2 N NaCl solution, and so on. Data was analyzed on SPSS version 21.0 (IBM, incorporation, USA). Continuous variable weight was analyzed using students t-test. % Hemolysis was presented as graphs in Microsoft excel sheet. Statistical significance was taken at p≤ 0.05.*
Results: E*xperimental rats showed >90% hemolysis at NaCl concentrations of 0.45% and > 95% hemolysis at 0.35% and 0.30% NaCl concentrations. Hemolysis due to osmofragility was noted in atorvastatin treated animals- Groups B and C. Hemolysis was reduced in atorvastatin groups D and E which were treated concomitantly with Coenzyme Q 10. The oral use of CoQ 10 showed a decrease in osmofragility in atorvastatin treated animals. Hemolysis was noted more in all experimental rats compared to controls and hemolysis showed differences in low and high dose atorvastatin treated rats.*
Conclusion*: The present study reports increased osmofragility of red blood cells with atorvastatin. Concomitant Co-enzyme Q 10 administration reduced the osmofragility of red blood cells.*
Keywords: *Erythrocyte Osmotic fragility Atorvastatin Coenzyme Q10 Rats*

I. Introduction

Red blood cells (RBCs) are also known as erythrocytes. RBCs show red hue due an iron containing chrome known as the *hemoglobin (Hb)*. RBC is a biconcave disc shaped bag loaded with oxygen carrying pigment called the *hemoglobin (Hb)*. RBC shows no nucleus, and none of cell organelles; this maximizes its Hb carrying capacity (**1, 2**). RBC is flattened donut like biconcave in shape, with depression in the center. RBC is 7.8 µm in diameter, 2.5 µm thick at periphery and about 1µm or less in the central depressed part. It reveals that the Hb is concentrated very close to the membrane, this eases the O_2binding. A remarkable change in shape is a feature of RBC, especially when it passes through capillaries. RBC may cross capillaries smaller than the diameter of itself by squeezing and deforming it shape as flat i.e., RBC is a bag that may be deformed in almost any shape. Deforming its shape is because of high flexibility of its cell membrane. And this membrane flexibility is because of typical arrangement of cytoskeleton proteins; the spectrin, Ankyrin, protein 4.1, etc. Average of life span of RBC is 120 days (**1, 2**). RBC contains enzymes of anaerobic glycolysis. ATP provision is the function of anaerobic glycolysis. ATP is necessary for normal functioning of RBC cell membrane (**1, 2**).

Enzymes of another glucose pathway, the hexose monophosphate shunt (HMP) are also present in the RBC. HMP shunt provides $NADPH_2$ which provides redox potential to neutralize free radicals. $NADPH_2$ is

related to glutathione antioxidant system to combat oxidants (**3**). RBC life span is 120 days because enzymes of 2 glucose pathways are depleted within this time period. Depletion of enzyme system, lack of ATP and $NADPH_2$ causes the senescence of RBCs which is then easily destroyed (**1-3**).RBCs carry oxygen which is very important job to perform. Oxygen binds with Hb to for oxy-Hb which is transported to the tissues. Hemoglobin is an oxygen buffer which protects RBC by minimizing the free oxygen radical formation. Coenzyme Q 10 is a lipid soluble compound found naturally in various cells and tissues. RBC normally contains many anti-oxidant compounds such as the vitamins, minerals, and also the coenzyme Q10 (Co Q 10) (ubiquinone). Co Q10 protects against oxidative stress (**4 - 6**).Erythrocyte Osmotic fragility (EOF) is defined as the degree of hemolysis that occurs when RBC are exposed to osmotic stress. EOF depends upon various factors including the RBC cell membrane composition, integrity, cell size, shape and surface-volume ratios (**7 - 9**).Osmotic fragility test (OFT) is a term used in clinical hematology. OFT is used in diagnosing and differentiating RBC membrane abnormalities. Some inherited disorder change RBC osmotic fragility. RBC may exhibit increased or decreased osmotic fragility potential. Hereditary spherocytosis and hypernatremia are associated with increased EOF. While thalassemia, low Na+ concentration, iron deficiency anemia, chronic liver disease, and sickle cell disease show a decrease in EOF (**10**).

HMG-co A reductase inhibitors are widely used to reduce blood cholesterol. Atorvastatin is one of widely used HMG-co A reductase inhibitors. HMG-co A reductase inhibitors are reported to reduce the coenzyme Q10 (CoQ10) blood levels. Reduced CoQ 10 makes RBC prone to excessive oxidative damage and makes RBC vulnerable to hemolysis (**4 - 6**). CoQ 10 is richly concentrated in the brain, heart, kidneys and liver (**11, 12**). CoQ 10 helps mitochondria to extract energy and is a powerful antioxidant compound (**13**).Currently, use of HMG-co A reductase inhibitors (Atorvastatin), in routine clinical practice, is very common in Pakistan and many patients are receiving drug as part of cardiac drug therapy. RBCs are the most abundant circulating blood cells, which being an innocent bystander might be harmed by Atorvastatin, but nothing is known about this. On the part of clinical practitioners, the issue is highly overlooked and never thought of as Atorvastatin drug might be causing the hemolysis. Therefore, the present study will be conducted to explore the effects of Atorvastatin on the erythrocyte osmotic fragility in experimental animal model. The present research aims to explore the effects of these drugs on RBC membrane physiology as evaluated by osmofragility testing and possible protective role of Co-enzyme Q 10.

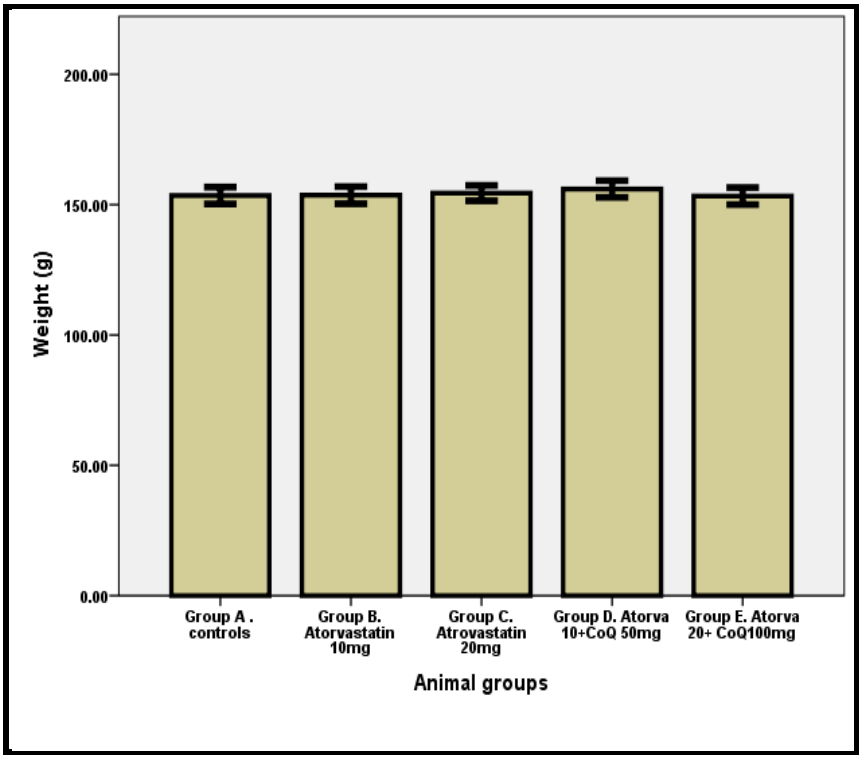
II. Methodology

The study was conducted in department of animal husbandry and veterinary sciences Sindh agriculture university Tando jam, in collaboration with Isra University Hyderabad. It was an experimental animal study, from March 2016 to November 2016 50 Albino Wistar Rats were randomly selected according to inclusion and exclusion criteria. Rats were fed on chow to both controls and experimental groups, having a scientifically approved composition as per instructions of veterinary experts. The chow was given as raw food. The rats were randomly divided into 5 groups namely A, B, C, D and E. **Group A (n=10)**: Control rats – receive 0.9% normal saline as placebo, **Group B (n=10)**: Atorvastatin 10 mg per os daily, **Group C (n=10)**: Atorvastatin20 mg per os daily, **Group D (n=10)**: Atorvastatin 10 mg + Co-enzyme Q 10 50 mg per os daily. **Group E (n=10)**: Atorvastatin 20 mg + Co-enzyme Q 10 100 mg per os daily. Atorvastatin and Co enzyme Q 10 were given for 6 weeks duration. Data was analyzed on SPSS version 21.0 (IBM, incorporation, USA). Continuous variable weight was analyzed using students t-test. % Hemolysis was presented as graphs in Microsoft excel sheet. Statistical significance was taken at $p \leq 0.05$.

III. Results

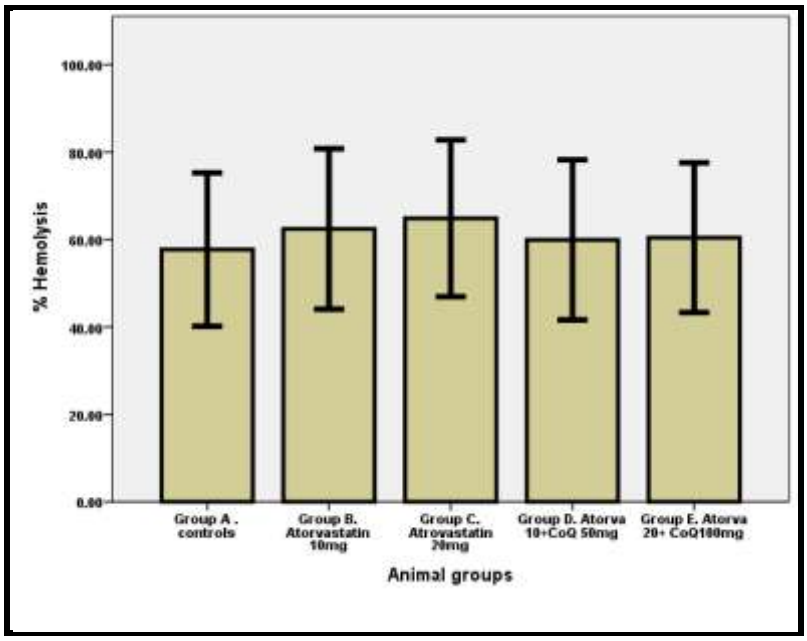
50 rats were selected for the present experimental study. Controls and experimental animals were studied according to study protocol. The rats were randomly divided into 5 groups namely A, B, C, D and E. Group A (n=10): Control rats – receive 0.9% normal saline as placebo, Experimental Groups; Group B (n=10): Atorvastatin 10 mg per os daily, Group C (n=10): Atorvastatin 20 mg per os daily, Group D (n=10): Atorvastatin 10 mg + Co-enzyme Q 10 50 mg per os daily, Group E (n=10): Atorvastatin 20 mg + Co-enzyme Q 10 100 mg per os daily. Atorvastatin and Co enzyme Q 10 were given for 6 weeks duration. Weight of rats of controls and experimental rats is summarized in table 1 and graph 1. Rats were weight matched as indicated by F value and non-significant p value % hemolysis was calculated in experimental rats compared to controls. % hemolysis results of controls and experimental groups are shown in table 2 and graphs 2-3. Red blood cells of majority of experimental rats showed >90% hemolysis at NaCl concentrations of 0.45% and > 95% hemolysis at 0.35% and 0.30% NaCl concentrations. As shown in graph IV-2, % hemolysis was noticed significantly in atorvastatin treated animals- Groups B and C. % hemolysis was reduced in atorvastatin groups D and E which were treated concomitantly with Coenzyme Q 10. The oral use of CoQ 10 showed a decrease in osmofragility in atorvastatin treated animals.

Table 1. Weight of controls and experimental rats (n=50)				
	Mean	**SD**	**F-value**	**P-value**
Group A. controls	153.50	5.59	1.56	0.68
Group B. Atorvastatin 10mg	153.64	5.70		
Group C. Atorvastatin 20mg	154.42	5.10		
Group D. Atorva 10+CoQ 50mg	156.00	5.54		
Group E. Atorva 20+ CoQ100mg	153.28	5.64		

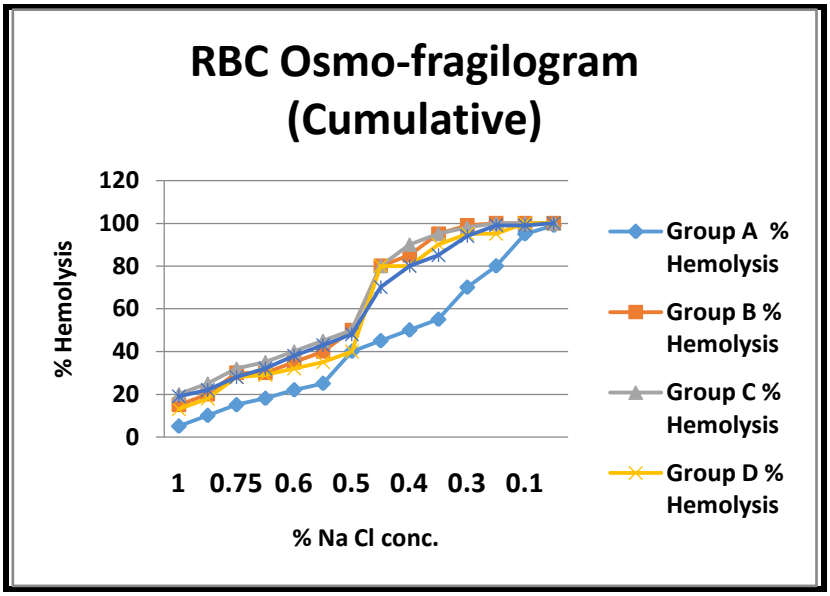


Graph 1. Weight of controls and experimental rats (n=50)

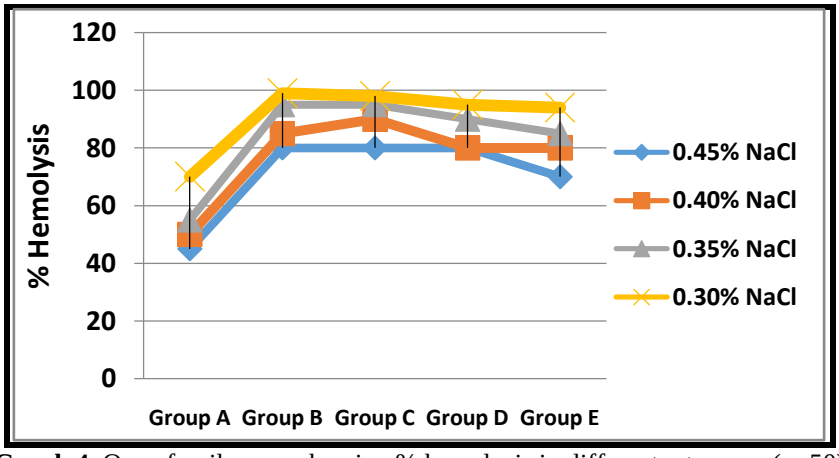
Table 2. Osmotic fragility in different animal groups showing % hemolysis (n=50)					
	Group A	**Group B**	**Group C**	**Group D**	**Group E**
% NaCl	% Hemolysis	% Hemolysis	% Hemolysis	% Hemolysis	% Hemolysis
1	5	15	20	13	19
0.85	10	20	25	18	22
0.75	15	30	32	28	28
0.65	18	30	35	29	32
0.6	22	35	40	32	38
0.55	25	40	45	35	43
0.5	40	50	50	40	48
0.45	45	80	80	80	70
0.4	50	85	90	80	80
0.35	55	95	95	90	85
0.3	70	99	98	95	94
0.2	80	100	100	95	99
0.1	95	100	100	100	99
0	99	100	100	100	100



Graph 3. % hemolysis in controls and experimental rats (n=50)



Graph 3. Osmofragilogram showing % hemolysis different rat groups (n=50)



Graph 4. Osmofragilogram showing % hemolysis in different rat groups(n=50)

IV. Discussion

To the best of our knowledge, the present study is the first research which reports the effects of cholesterol lowering drug atorvastatin on the red blood cell osmofragility and possible protective effects of Co enzyme Q 10 in experimental rat model. The present study reports increased osmofragility with atorvastatin use and a reduction was noted by concomitant use of CoQ10.Mechanical fragility (MF) is defined as the degree of RBC hemolysis when exposed to mechanical stress. Standardized methods are not available to examine the MF.

EMF may be used in diagnostic tests, calibrations to compare hemolysis by blood devices (**18**) or estimating sub clinical sub lethal non hemolysing cell damage as during dialysis (**19**) or intra-operative auto-transfusion (**20**). EMF may help in estimating RBC damage as may occur in stored blood in blood banks and blood transfusions (21) Hemolysis susceptibility form causes other than as mentioned above are not uncommon; for example hemolysis caused by free radicals. RBC may be tested for cell deformability, cell morphology and cell dimensions. Deformability measures the contortion produced by a controlled applied force. Other RBC properties include adhesion and aggregation. RBC properties of adhesion, aggregation and cell deformability are collectively termed as *RBC flow properties*. (23, 24) The findings of present study are incomparable to any previous study as it is the first time being reported. Uydu et al 2012 **(24)** conducted study on the effects of atorvastatin drug therapy on rheological characteristics of erythrocyte membrane, serum lipid profile and oxidative status in patients with dyslipidemia. Uydu et al **(24)** evaluated 44 patients with dyslipidemia. 10 mg of atorvastatin was given orally daily for 12 weeks. Effects on the lipid profile, Na+/K+-ATPase activity, oxidative markers and EOF. A significant change was observed in EOF values in mixed type dyslipidemia patients. The findings significant change in EOF is a comparable finding to our present work; however, the details of EOF by Uydu et al are not mentioned in detail. Osmofragility has been reported by the Zahediasl **(25)** in experimentally induced hyperthyroid rats from University of Brussels, Belgium. However, findings of above study were inconclusive and not comparable to our present study. A recent case report **(26)** has reported toxic epidermal necrolysis and rhabdomyolysis by Atorvastatin in human being.

Simmons D **(27)** has reported that the Lipitor, which is atorvastatin calcium, may cause hemolytic anemia in human being, but the effect on the Osmofragility was not studied. The above report`s finding of increased hemolysis is a consistent finding but not the osmofragility of present study. Garbe et al 2011**(28)** has also reported atorvastatin induced hemolysis in human beings.CoQ10 is synthesized by similar HMG Co A pathway of cholesterol. Mevalonate is the precursor of cholesterol as well as CoQ10. As the Atorvastatin inhibits this pathway, hence it also inhibits the CoQ10 biosynthesis. CoQ10 levels fall by 40- 49% with use of statin drugs. Adenkola et al **(29)** has reported a study on the effect of ascorbic acid on erythrocyte osmotic fragility and hematological parameters in rabbit model. The study was conducted at the Agriculture University, Makurdi, Nigeria. Experimental animals were fed on ascorbic acid (200 mg/kg) dissolved in water orally. EOF was determined by the Faulkner and King (1970) method. % hemolysis observed in the control animals was found greater than the observed value obtained in the experimental animals ($P < 0.05$). Osmofragilogram of the control animals was shifted towards right. The findings of above study, although, not completely comparable, but indicate a protective role offered by Ascorbic acids against osmotic fragility. CoQ 10 exerts anti-oxidant effects similar to ascorbic acid. In present study, the CoQ 10 exerted similar reduction in % hemolysis in atorvastatin treated animals.

Alhassan et al **(30)** conducted a study on the EOF in Wistar rats treated with ascorbic acid (AA) during the hot-dry season. The study was conducted at the Department of Human Physiology, Ahmadu Bello University, Nigeria. Controls rats were given sterile water as placebo, while experimental rats were fed ascorbic acid 100 mg/kg body weight orally during the hot-dry season. Experiment lasted for 8 weeks. A significant reduction was observed in ascorbic acid treated Wistar rats compared to controls ($P < 0.05$). It was concluded that the AA stabilizes RBC membrane integrity and lowers % hemolysis in Wistar rats during hot dry season. Ambali et al **(31)** reported experimental animal study form Ahmedu Bello University, Nigeria. The Chlorpyrifos (CPF) was administered chronically in Wistar rats to study EOF. Experimental animal groups were given vitamin C and vitamin E for effects on the EOF. Ambala used 20 rats in their study which were divided into four groups, each containing five rats. Soya oil (2 ml/kg), vitamin C (100mg/kg), vitamin E (75 mg/kg) and chlorpyrifos (10.6 mg/kg) were used in 4 groups respectively. EOF testing showed increased % hemolysis in CPF rats, and EOF showed an improvement in % hemolysis in vitamin C and E treated animals. Harisa et al **(32)** has reported a study on the human erythrocyte as a potential carrier of Pravastatin, which is a HMG CoA reductase inhibitor similar to atorvastatin. It was an in vitro study conducted on human erythrocyte using electron microscope. Harisa et al **(32)** reported that the human erythrocytes were successfully loaded with pravastatin. As regards effects of Pravastatin on RBC osmofragility was not observed. Increased EOF was not observed by Harisa et al. The findings of above study are in contrast to present and previous studies.

The possible reasons of contradictory results may be; different study population, different drug agent – Pravastatin vs. Atorvastatin. Both are HMG co A reductase inhibitor, but molecular structure is different, study designs, methodology bias and moreover laboratory facilities and instrumentation. The limitations of study

include, we could not measure serum cholesterol, anti-oxidant- enzymes and non-enzymes, lipid peroxidation and oxidants such as free radicals and red blood cell membrane examination by direct electron microscopy. However, strength of study is authenticated by study design, accurate formation of Tyrode`s solution and proper protocol of interpreting the results. The observations of present study are in favor of increased % hemolysis by atorvastatin and CoQ 10 protected against atorvastatin induced osmofragility in experimental rats.

V. Conclusion

The present study reports increased osmofragility of red blood cells with atorvastatin. Concomitant Co-enzyme Q 10 administration reduced the osmofragility of red blood cells.

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