### Chapter 6

**EFFECT OF ETHANOL ON RBC MEMBRANE STABILITY**

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### 6.1A Introduction

An inherent property of cells is their ability to maintain the structural and functional integrity of their membranes under changing environmental conditions. Simple components of biological systems, such as cells, biomolecules and artificial membranes were used in *in vitro* studies since they allow the reduction of the biological variables and more precisely helps in defining and controlling the exposure parameters, compared with *in vivo* exposure. Fish erythrocytes were used to study adaptive responses to ethanol induced changes at the membrane level within short time spans as these cells are nucleated and express many functions as that of somatic cells. Unlike anucleated mammalian RBCs the nucleated RBCs of lower vertebrates preserve both nucleus and mitochondria and can provide an attractive “stripped down” model to study the effect of organic pollutants on cellular compartments (Dey et al., 1993). *In vitro* studies would contribute to clarify the fundamental mechanisms of biological effects brought about by ethanol induced toxicity. By using *in vitro* studies, it was shown that ethanol brings about chaotropic effects on membrane and cytoskeleton proteins denaturation. It is a well known
denaturating agent that can promote the exposure of polar groups in the protein unfolding processes. For this reason it has been stated that, incorporation of ethanol to the membrane environment is associated with the promotion of exposure of polar groups resulting in a membrane denaturation. Furthermore, there is evidence stating that alcohol causes membrane deformity, and brings about modification in the osmotic fragility of different cell types (Sozmen et al., 1994). Freshly drawn blood samples mixed with an anticoagulant were used for erythrocyte membrane analysis. Erythrocyte membrane is often used as a model membrane in investigating the structure and the functions of the biological membranes as well as in studying the influence of different physical and chemical factors on the membranes. Incubation of normal erythrocytes with ethanol facilitates hemolysis and increases the percentage of cells that were hemolysed at maximal rate. There is an especially great need for studies on the impact of these compounds on the properties of blood and other tissues which results in their distribution to other sites in the body. The present study describes the damaging effects of ethanol and their metabolites on the erythrocyte membrane. Cunha et al. (2007) has stated that erythrocytes suffer lysis depending upon the concentrations of ethanol. Previous reports are not available on the effect of ethanol on erythrocyte membrane of the fresh water teleost, *O. mossambicus*. Therefore this study was designed to investigate the effect of ethanol on the stability of erythrocyte membrane. The direct study of ethanol on membrane *in vitro* can show the basic aspects of its effects on cellular metabolism. This study was designed to investigate the alterations in the membrane stability after *in vitro* exposure of fish erythrocytes to different concentrations of ethanol.

### 6.1B Materials and Methods

Collection, maintenance, acclimatization of fish and determination of LC₅₀, was the same as that described in chapter 1B, Section 1.2B.1 to 1.2B.5.

#### 6.1B1 In-vitro studies.

For conducting RBC membrane stability studies in *in-vitro* conditions, *O. mossambicus* of 10 ± 2 g were taken. Fishes were sacrificed by a blow to the head and blood was drawn from the common cardinal vein using 1ml sterile plastic
insulin syringe (Smith et al., 1952) (26mm gauge size) containing sodium citrate as an anticoagulant (5mg/ml) (1:5 dilution) (Oser, 1976).

6.1B1.1 Isolation of red blood cells

Erythrocytes were used within one hour of collection, and with each experiment lasting no longer than 4 hour. Erythrocytes were isolated by centrifuging at 3000 rpm for 5 minutes. After removal of plasma and buffy coat, the red blood cells were further washed three times with three volumes in the same isotonic medium and were concentrated by centrifugation under the same conditions. In order to study the effect of ethanol on erythrocyte membrane, 0.5 ml of ethanol was directly added in the tubes in which the final ethanol concentrations in the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively. Hemolysis was determined by measuring the absorbance of hemoglobin in the supernatant at 540 nm. Hemolysis was expressed by measuring the hemoglobin concentration. Erythrocyte membrane was often used as a model membrane in investigating the structure and the functions of the biological membranes as well as in studying the influence of ethanol on the membranes.

6.1B2 Estimation of RBC membrane stability (In vitro condition)

Reagents Needed
1. Tri sodium citrate (500 mg%)
2. 154 mM Isotonic NaCl in 10 mM Sodium phosphate buffer, pH 7.4
3. 85.47 mM Hypotonic NaCl in 10 mM Sodium phosphate buffer, pH 7.4

Procedure

Stock RBC suspensions were prepared after washing the cells thrice with isotonic saline. Different volumes of the suspension were mixed with distilled water of known volume to hemolyse the cells. It was then centrifuged at 1000g for 5 minutes. The absorbance of the supernatant was read at 540 nm against distilled water as blank. The dilution giving a suitable absorbance for 100% hemolysis was selected. Also a suitable volume of blood giving an absorbance for 100% hemolysis was noted.
The experiment was carried out with each of the three concentrations of ethanol as described below.

1. To 0.1 ml of the stock RBC suspension in a centrifuge tube, 5 ml of isotonic saline was added and incubated for 30 minutes at room temperature. It was then centrifuged at 1000g for 5 minutes. The absorbance of the supernatant was read at 540 nm. This gave the absorbance of the “blank” (B).

2. To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of distilled water was added and incubated for 30 minutes at room temperature. To this 0.5 ml of ethanol stock solution was added (in which the final concentration of ethanol in each of the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively). It was then centrifuged at 1000g for 5 minutes and the absorbance of the supernatant was read at 540 nm. This gave the absorbance corresponding to 100% hemolysis (H).

3. To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of hypotonic saline was added and incubated for 30 minutes at room temperature. To this 0.5 ml of the ethanol stock solution was added (in which the final concentration of ethanol in each of the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively). It was then centrifuged at 1000g for 5 minutes and the absorbance of the supernatant was read at 540 nm. This gave the absorbance of the control (C).

4. To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of hypotonic saline followed by 0.5 ml of the ethanol stock solution (such that the final concentration of ethanol in each of the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively) was added. It was then incubated for 30 minutes at room temperature. It was then centrifuged at 1000g for 5 minutes and the absorbance of the supernatant obtained was read at 540 nm. This gave the absorbance corresponding to the “test” (T).

6.1C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l of ethanol on erythrocyte membrane stability in *O. mossambicus* was given in Table 6.1.1 to 6.1.4 and in Figure 6.1.2.
The results obtained were analyzed statistically using One-Way ANOVA of the raw data, followed by Dunnett’s method. *In-vitro* studies conducted on the RBC membrane stability revealed that ethanol had a labilising effect on the erythrocyte membrane. 2.6 g/l exhibited maximum percentage of hemolysis (82.51%) followed by 1.3 g/l and 0.65 g/l which gave percentage hemolysis of (71.29%) and (54.84%) respectively.

**Table 6.1.1 Percentage hemolysis in *O. mossambicus* on exposure to different concentrations of ethanol (*In-vitro* conditions)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>0.65 g/l</td>
<td>54.84 ± 1.9265</td>
</tr>
<tr>
<td>1.3 g/l</td>
<td>71.29 ± 3.1025</td>
</tr>
<tr>
<td>2.6 g/l</td>
<td>82.51 ± 2.4627</td>
</tr>
</tbody>
</table>

Average of six values in each group ± SD of six observations

**Figure 6.1.2 Percentage hemolysis in *O. mossambicus* on exposure to different concentrations of ethanol (*In-vitro* conditions)**

RBC membrane exhibited an increase in hemolysis (Table 6.1.1 and Figure 6.1.2) in *in-vitro* conditions, and was found to be depended upon dosage. This was confirmed by using ANOVA (Table 6.1.3) and the result is depicted below.
Statistical analysis by using One Way- ANOVA revealed that there was an overall significant change ($P<0.001$) between concentrations in the RBC membrane stability levels during \textit{in-vitro} conditions.

### 6.1.3 Multiple Comparison Test

A subsequent comparison between different concentrations of ethanol was done by Dunnett’s method and the results were depicted in the Table 6.1.3

<table>
<thead>
<tr>
<th>Groups</th>
<th>RBC (\textit{in-vitro})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dunnett 0.65g/l Vs 1.3g/l</td>
<td>0.000*</td>
</tr>
<tr>
<td>Dunnett 0.65g/l Vs 2.6g/l</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

The values are significant at $a=P<0.001$.

In the case of RBC membrane stability studies in \textit{in-vitro} conditions significant difference ($P<0.001$) was observed at 1.3 g/l and 2.6 g/l when being compared with 0.65g/l.

### 6.1D Discussion

The result from this experiment indicated that erythrocyte membrane was maximally damaged and has spilled its haemoglobin content depending upon the concentration of ethanol. The levels of released hemoglobin serves as an indicator of hemolysis, caused by increased membrane fragility. Fragility is referred to as a
membrane associated phenomenon which reflects the susceptibility of red cells to lysis. This in turn explains that RBC membrane has become more fragile when treated with ethanol. Also the change in cation concentration brings about increased fragility. This results in the increased leakage of cations from the cells resulting in swelling of the cell, thus ultimately leading to lysis. Niranjan and Krishnakantha. (2000) observed similar increase in the osmotic fragility of erythrocytes in rats fed with oxidized ghee, supports the present finding. The present data confirm that, ethanol brings about an increased disintegration of erythrocytes. An increase in the RBC hemolysis was observed when O. mossambicus was subjected to different concentrations of ethanol in in-vitro conditions. In the present study as the concentration of ethanol increased hemolysis of RBCs also increased (Table 6.1.1 and Figure 6.1.2). Chi and Wu (1991) observed similar state of increased rate of hemolysis of red blood cells when mediated by ethanol. The hemolysis rate increased depending upon the increased concentration of ethanol. Changes in the ion transport and in the osmotic fragility are considered as indicators of alterations in the erythrocyte membrane (Baranski et al., 1974; Kovacs et al., 1997). Similar observations in hemolysis were made by Cleary et al. (1982) on rabbit erythrocytes subjected to micro waves in in-vitro condition. Another reason for the labilising effect is the membrane damage brought about by the direct effect of lipid peroxidation products. The observations cited by Das and Vasudevan (2005) supported the present finding. Erythrocytes are prone to oxidative damage due to presence of polyunsaturated fatty acids, heme, iron and oxygen (Kameda et al., 1985). Red blood cell membrane being rich in polyunsaturated fatty acids are very susceptible to free radical mediated peroxidation. Lipid peroxidation mediated by free oxygen radicals, is believed to be an important cause of destruction and damage to cell membranes, since polyunsaturated fatty acids of the cellular membranes are degraded by this process with consequent disruption of membrane integrity. From these results it can be concluded that RBC hemolysis and lipid peroxidation by ethanol can be one of the molecular mechanisms involved in ethanol induced toxicity studies (Armutcu et.al., 2005). Thus, the elevated lipid peroxide concentration in the erythrocytes probably reflects a higher production of peroxyl radicals, which ultimately leads to the peroxidation of PUFA in these cells. This in
turn leads to increased fragility of the cell membranes which ultimately brings about lysis of the cells (Muduuli et al., 1982). The present investigation points out that ethanol brings about maximum damage to the RBCs when subjected to different concentrations of ethanol in *in-vitro* conditions.
6.2 IN-VIVO RBC MEMBRANE STABILITY STUDIES

6.2A Introduction

Red blood cells possess a much simple structure. It is composed of a single membrane surrounding a solution of haemoglobin (this protein forms about 95% of the intracellular protein of the red cell) (Robert et al., 2000). The erythrocyte membrane has long been served as a convenient model system employed for studying the chemical and physical properties of cell membrane due to its relative simplicity (Salil and Shyamali, 1999). Lipids are crucial structural components of biomembranes which dictate the integrity of the membranes (Hummel, 1993). Membrane lipid composition directly reflects the membrane properties (Yeagle, 1985). Cholesterol, one of the major components of the membranes, plays a central role in membrane biosynthesis, integrity and cell growth. It also regulates membrane fluidity. The functions of membrane are therefore determined by membrane composition and organization (Levin et al., 1990). Erythrocytes (RBCs) are frequently used to evaluate oxidative stress. The RBC membrane is rich in polyunsaturated fatty acids, a primary target for reactions involving free radicals, which seems to be very susceptible to lipid peroxidation (Devasena et al., 2001). Lipid peroxidation is an autocatalytic process, which ultimately results in cell death (Dsouza and Dsouza 2002). An oxidative cellular defect in the red blood cell (RBC) can accentuate oxygen radical formation and risk damage to cellular components (Flynn et al., 1985; Scott 1993). Ethanol disrupts the physical structure of cell membranes (Goldstein, 1986), and thus brings about oxidative damage. Oxidative damage alters membrane permeability and eventually lead to hemolysis (Lubin and
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Erythrocytes are exposed to continuous oxidative stress due to continuous generation of free radicals brought about by the oxidation of haemoglobin. In addition, oxidative damage causes immune recognition of RBC (Low et al., 1985).

The most commonly used method of erythrocyte ghost preparation is to hemolyse RBC in hypotonic solutions to remove hemoglobin. Even though this technique is widely used, certain important variables, namely pH and ionic strength of the hemolyzing solutions, appear partially responsible for conflicting reports on the composition and function of RBC membrane (Stubbs and Smith, 1984). While these studies have identified oxidative damage as a major determinant of RBC survival, the detailed mechanism of these damages to RBC brought about by ethanol remains largely ill-defined. Membrane lipids are susceptible to peroxidation induced damage as they are largely composed of polyunsaturated fatty acids. Peroxidative reactions involving free radicals in lipid domains results in damage to integral membrane proteins, leading to alteration of membrane dynamics and function (Sevanian and Hochstein, 1985). The accumulation of activated oxygen causes hemolysis (Hebbel, 1986; Shinar and Rachmilewitz, 1990 and Saltman, 1989). However, the chemical composition of the red blood cell (RBC) membrane may vary depending on the methods used to isolate the membrane (Dodge et al., 1963; Ponder, 1961 and Weed and Lacelle, 1969). For the present study, fish red blood cells (RBCs) has been used as a model system to delineate the effects of oxidative damage brought about by ethanol on red blood cells of O. mossambicus. The cellular membrane is a lipid bilayer essentially constituted by phospholipids, cholesterol and glycolipids. Small variations in percentage composition and molar ratio of the different classes of phospholipids and glycolipids, as modifications in the composition in fatty acid and cholesterol amount, result in changes of the physical-chemical status (with implications on membrane’s fluidity and permeability) of enzyme’s activity and/or of channels and ionic pumps constituted by intrinsic membrane proteins. Moreover, in these molecules, a difference in composition in the fatty acids can result in a greater sensibility to peroxidative stress, with a consequent increase in membrane fragility (Angela et al., 2007).
6.2 B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC\textsubscript{50}, bioassay method and experimental design was the same as that described in chapter 1, section 1.2B.1 to 1.2B.5. Isolation of red blood cells remains the same as described in 6.1.B1.1.

6.2B.1 Estimation of RBC membrane stability (\textit{In vivo} condition)

Reagents Needed

1. Tri sodium citrate – 500 mg%
2. 154 mM isotonic NaCl in 10 mM sodium phosphate buffer, pH 7.4
3. 85.47 mM hypotonic NaCl in 10 mM sodium phosphate buffer, pH 7.4

Procedure

Blood containing citrate as an anticoagulant was employed for membrane stability studies. It was then centrifuged at 4\degree C in a refrigerated centrifuge at 3000 rpm for 30 minutes. The pellet obtained contains erythrocytes. It was then washed thrice with isotonic saline solution. Different volumes of erythrocyte suspensions were prepared by mixing with distilled water. This hemolyzes the cells which were then centrifuged at 1000g for 5 minutes. The supernatant obtained was taken. It was then read at 540 nm against distilled water as blank. The RBC suspension which gives a suitable absorbance for 100% hemolysis was selected and this was used as stock. Also a suitable volume of blood giving a suitable absorbance for 100\% hemolysis was noted.

To 0.1ml of the stock RBC suspension, 5 ml of isotonic saline was added. It was then incubated for 30 min at room temperature. After incubation it was centrifuged at 1000g for 5 min. The supernatant which contains haemoglobin was read at 540 nm. This serves as the ‘blank’ (B). Similarly 5 ml of distilled water was added to 0.1 ml of the stock RBC suspension taken in a centrifuge tube. It was then incubated for 30 min at room temperature. After incubation it was centrifuged at 1000g for 5 min and the absorbance of the clear supernatant was measured at
540 nm. This gave the absorbance corresponding to 100% hemolysis (H). In the third set, 4.5 ml of hypotonic saline and 0.5 ml of distilled water were added to 0.1 ml of the stock RBC suspension in a centrifuge tube. It was then incubated for 30 min at room temperature. After incubation the tubes were centrifuged at 1000g for 5 min and the absorbance of the supernatant was read at 540 nm. This gave the absorbance corresponding to the control (C). To the fourth set, 4.5 ml of hypotonic saline was added to 0.1 ml of stock RBC suspension. It was then incubated for 30 min at room temperature. After incubation 0.5 ml of distilled water was added and it was then centrifuged at 1000g for 5 min. The absorbance of the supernatant was read at 540 nm. This gave the absorbance corresponding to the test (T).

### 6.2C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l of ethanol on RBC membrane stability in *O. mossambicus* are given in Table 6.2.1 to 6.2.3 and Figure 6.2.2. The results obtained on exposure to the three sub lethal concentrations of ethanol for 21 days followed by a periodical sampling at 7 days were analyzed statistically using Two Way ANOVA of the raw data, followed by Dunnett’s method. It exhibited that as the concentration of ethanol increases, the labilisation of erythrocyte membrane also increases. Exposure to 2.6 g/l concentration of ethanol exhibited highest labilisation effect on *O. mossambicus* when being subjected for 7 days and 21 days experiment. Minimum effect of labilisation was seen in 0.65 g/l in the case of 7 days of exposure whereas on prolonged exposure, minimum effect was exhibited by 1.3 g/l.

### Table 6.2.1 Percentage hemolysis in *O. mossambicus* on exposure to different concentrations of ethanol for 7 days and 21 days (*In-vivo* conditions).

<table>
<thead>
<tr>
<th>Days of Exposure</th>
<th>Groups</th>
<th>0.65g/l</th>
<th>1.3g/l</th>
<th>2.6g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7 days</strong></td>
<td></td>
<td>36.60±</td>
<td>99.55±</td>
<td>99.92±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8958</td>
<td>0.0848</td>
<td>0.0799</td>
</tr>
<tr>
<td><strong>21 days</strong></td>
<td></td>
<td>76.59±</td>
<td>24.75±</td>
<td>96.60±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6613</td>
<td>3.8241</td>
<td>0.6665</td>
</tr>
</tbody>
</table>

Average of six values in each groups ± SD of six observations.
Values are expressed in %
Figure 6.2.2 Levels of RBC Membrane stability (In-Vivo) in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.

A significant difference (P<0.001) in RBC membrane stability was observed in *O. mossambicus* when treated with various sub lethal concentrations of ethanol with respect to control during 7 and 21 days of exposure period (Table 6.2.1 and Figure 6.2.2). This was statistically supported by employing ANOVA and the results obtained is shown below (Table 6.2.2a).
Statistical analysis done by using Two Factor ANOVA indicated that RBC membrane stability levels varied significantly between days (P<0.001). There was a significant difference (P<0.001) between concentrations. While comparing both the days as well as concentrations (Interaction) effect together, significant difference (P<0.001) was noted.

6.2D Discussion

The ability of organic solvent such as ethanol to destabilize the membrane as well as the membrane proteins correlates to their cytotoxicity. The present experimental data indicated that erythrocyte membrane was maximally damaged and exhibited increased rate of hemolysis when treated with ethanol. This is due to the direct effect of lipid peroxidation products. Membrane peroxidation lead to changes in membrane fluidity, permeability and also results in the enhanced rates of protein...
degradation, which will eventually lead to cell lysis. The deformity of destructed RBC and increased rate of RBC hemolysis results in the increased production of free radicals. High levels of polyunsaturated fatty acids (PUFA), continual exposure to high concentrations of oxygen or the presence of iron, a powerful transition-metal catalyst, render erythrocytes highly susceptible to peroxidative damage (Clemens and Waller, 1987). Also ethanol exhibits a denaturing effect on erythrocyte membrane which is also been associated with abnormal RBC morphology resulting in an increased susceptibility to hemolysis (Prokopieva et al., 2000; Chi et al., 1990).

In the present study an increase in RBC hemolysis (Figure 6.2.2) was observed when *O. mossambicus* was exposed to ethanol for 7 days. This increase in RBC hemolysis is due to the increased activities of serum specific enzymes. Ivanov (2001) observed increase in RBC destruction and hemolysis when treated with ethanol, supports the present finding. Another reason for the increase in RBC hemolysis is due to membrane lipid peroxidation. RBC membranes are prone to peroxidative damage because they are rich in unsaturated fatty acids and are exposed to high oxygen concentration in the blood. The findings by Niki *et al.* (1988) and Hayam *et al.* (1993) supports the above observation. Xenobiotics are oxidized to free radicals within RBCs and induce hemolysis of the RBC membrane (Armutcu *et al.*, 2005), which results in the release of hemoglobin thereby inducing a multitude of toxic effects. The findings of Everse and Hsia (1997) supports the present study. Ivanov (2001) observed similar destruction of RBCs and hemolysis on the membrane when treated with organic solvents.

RBC membranes are prone to peroxidative damage because they are rich in unsaturated fatty acids and are exposed to high oxygen concentration in the blood (Hayam *et al.*, 1993). Membrane lipid composition determines membrane fluidity. Any alteration in the concentration of cholesterol is known to affect the transport functions and activity of membrane enzyme (Stubbs, 1983). It has been suggested that cholesterol regulates the lipid mobility in the membrane in physiological situations (Chailley *et al.*, 1981). The decrease in hemolysis observed at 1.3g/l when *O. mossambicus* was exposed for 21 days indicates that RBC membrane has become rigid. This can be due to the slight increase in the cholesterol/phospholipid ratio. The
observations made by Yeagle et al. (1990) and Kuypers et al. (1996) supports the above finding. Another possible reason for rigidity can be due to the decrease in unsaturated fatty acids in the erythrocyte membrane followed by an increase in saturated fatty acids, as the saturated fatty acids residues, because of their linear hydrocarbon residues and hydrophobicity, interact very strongly with each other. Oxidative damage has been shown to change a number of RBC properties. A fall in PUFA followed by an elevation of cholesterol increases the rigidity of the phospholipid bilayer. The findings made by Dobrestov et al. (1977) supports the present observation. Lipid peroxidation is known to cause polymerization of membrane components, thus decreasing cell deformability (Pfafferott et al., 1962). Bourel et al. (1987) suggested that alcohol, or one of its metabolites, induces erythrocyte morphological alterations that correlates with some alterations in the lipid content of the erythrocyte membrane (such as increased cholesterol/ phospholipid ratio). Increased membrane rigidity and decreased RBC deformability can also be induced by oxidative cross-linking of membrane protein.

Beauge et al. (1994) observed that alcohol induces rigidification of red blood cell membrane, supports the above observation. Levin et al. (1990) have proposed that the oxidation of membrane lipids results in the formation of peroxidation degradation products (such as for e.g. Malondialdehyde, MDA) which leads to the cross linking reactions of the lipid-lipid and lipid-protein type thereby making the membrane more rigid and hence less fluid. Similarly the increase in hemolysis observed at 0.65 g/l and 2.6 g/l on prolonged exposure can be due to the decrease in Hb and PCV values which arises due to the increased fragility of the erythrocytes. Observations made by Patra et al. (2001) supports this finding.

It can be concluded that ethanol brings about maximum damage to the RBCs when subjected to different concentrations of ethanol in in-vivo conditions.