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Study of Incubation Conditions for Erythrocytes Osmotic Fragility Testing in Dromedary Camel (*Camelus dromedarius*)

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Abstract: *The values of the erythrocytes osmotic fragility (EOF) test are easily influenced by physiological and environmental conditions surounding during blood collection, and red blood cells (RBCs) preparation and incubation. Therefore, the aim of our study was to determine the influence of anticoagulant (EDTA and heparin), temperature, pH, storage time, age, sex, season, hydrogen peroxide and vitamins C and E, on EOF in dromedary camel. The results showed an increased EOF by EDTA, storage time, cold, heat, acidity and hydrogen peroxide. In addition, this EOF was influenced by age, sex, season and vitamins. As conclusion, the quality of the OF test may easily be influenced by several biological, environmental and technical factors which must be determined and mentioned in each analysis of EOF profile in camel. These factors induced damage to RBCs cell membrane and may disturb the results of other blood analyses such as measurements of various hematological and biochemical parameters in camel.*

Keywords: *Osmotic fragility, EDTA, Heparin, Temperature, pH, Storage time, Age, Sex, Season, Camel.*
Abreviations: *EDTA: Ethylene Diamine Tetra acetic Acid, EOF: Erythrocyte Osmotic Fragility H_2O_2: Hydrogen Peroxide, H%: Percent Hemolysis, OF: Osmotic Fragility, OS: Oxidant Stress PBS: phosphate buffered saline, RBCs : Red Blood Cells, ROS : Reactive Oxygen Species.*

1. INTRODUCTION

Hemolysis is usually recognized by free hemoglobin in the red blood cell (RBC)-suspending media. The erythrocyte osmotic fragility (EOF) test is used to determine the extent of red blood cells (RBCs) hemolysis produced by osmotic stress. This extent is dependent on cell volume, surface area, and functional integrity of cell membranes. Since it can be followed by changes in absorbance at 540 nm, the EOF test was frequently applied to the diagnosis of hemolytic diseases and oxidant alterations leading to destruction of erythrocytes [1,2,3]. For example, road transportation to slaughterhouses associated to heat stress had been demonstrated to cause increase of hemolysis in pig [3], goat [4] and camel [5]. Nonetheless, the technical quality of this test is easily influenced by environmental changes. In fact, hemolysis has been shown to be affected by technical conditions of the assay and several factors, including age [6], sex [7], temperature, pH [8], season [9], transportation [10,11], inappropriate storage conditions, defects in the RBC membrane and abnormality in the blood [12]. These factors have been suggested to affect the EOF test and must be strictly standardized to obtain reproducible results. Therefore, we have undertaken a study in order to determine the osmotic fragility (OF) values of RBCs under several conditions in camel. We have assessed the effects of anticoagulant (EDTA and heparin), storage time, temperature, pH, hydrogen peroxide, vitamins, sex, age and season on EOF in this species.

2. MATERIALS AND METHODS

2.1. Animals

To investigate the effect of all factors studied here on OF of erythrocytes, 66 male and 6 female camels (3 to 10 years of age, average weight of 340.24±69.98kg) from Casablanca Municipality slaughterhouse were used as indicated in Table 1. These camels were clinically healthy and feed deprived overnight.

Table1. *Experimental Design*

Parameters tested	Tubes used for blood sampling	Number and sex of camels	Period of year
Anticoagulant	EDTA Heparin	8 males 8 males	October October
Storage time, Temperature, pH	Heparin	7 males	October
Hydrogen peroxide, Vitamins C and E	Heparin	7 males	November
Sex	Heparin Heparin	6 males 6 females	March - April March - April
Age	Heparin Heparin	7 young males 7 old males	December-January December- January
Season	Heparin	8 males in summer 8 males in autumn	June - July August - September

2.2. Blood Sampling

Blood samples were collected by jugular vein puncture from each camel between 07h and 08h in a tubes with heparin and/or ethylene diamine tetra acetic acid–dipotassium (EDTA) (Table 1). Plasma was separated by centrifugation at 750g for 15min and eliminated, then red blood cells (RBCs) suspensions were prepared for the OF tests.

2.3. Preparation of Erythrocytes Suspensions

Erythrocytes were isolated by centrifugation of blood for 15min at 1500xg. The plasma and buffy coat were carefully removed using a micropipette. The cells pellet was washed three times with 310mOsm isotonic phosphate buffered saline (PBS) solution (NaCl 6g/L, HNa_2PO_4 3.20 g/L, H_2NaPO_4, $2H_2O$ 0.33 g/L, pH=7.4) and centrifuged at 1000xg for 10min. Finally, washed cells were suspended in an equal volume of isotonic PBS and were used to test the effects of anticoagulant, T°, storage time, sex, age and season. On erythrocytes suspensions prepared in the same way but washed with isotonic PBS at various pH, the effect of pH on H% was anlyzed. However, the RBCs suspensions prepared with a proportion of 5% in isotonic PBS pH 7.4 (v/v), were used to test the effect of H_20_2 on H% and the protection of vitamins C and E against H_20_2 induced H%. These constituted the RBCs suspensions, which were stored at 4°C for 24h until further analysis [13]. Our tests were performed on the erythrocyte suspensions prepared from heparinized tubes.

2.4. Effect of Anticoagulant

To analyse the effect of the anticoagulant on erythrocytes hemolysis, OF was tested at 38°C on RBCs suspensions prepared from blood wich was collected in a tubes with heparin and EDTA.

2.5. Effect of Storage Time

The RBCs suspensions were stored at 4°C for 10 days and their OF was tested at each period of 24h during storage. All tests were performed at 38°C.

2.6. Effect of Incubation Temperature

The effect of temperature was analyzed on erythrocytes OF after incubation of RBCs suspensions in PBS isotonic solutions (pH 7.4) at 4°C, 18°C, 38°C, 42°C, 45°C and 50°C for 30mn. Before the tests, all solutions and tubes were placed at the test temperature.

2.7. Effect of pH

The effect of pH on erythrocytes OF was performed at 38°C on RBCs which were washed with PBS isotonic solutions with various pH (6.5; 7.4 and 8.5) during preparation of erythrocyte suspensions. Buffering capacity is based on the couple dihydrogenophos-phate/hydrogenophosphate (pKa=6.8), which is also one of three major mechanisms for maintaining blood pH ($HPO_4^{2-}+H^+=H_2PO^{4-}$). PBS solution at pH 7.4 contained NaCl (6g/L or 102mM), anhydrous Na_2HPO_4 (3.20g/L or 22mM) and NaH_2P0_4, $2H_2O$ crystallized (0.33g/L or 2mM). At pH 6.5 the solution contained NaCl (6g/L or 102mM), anhydrous Na_2HPO_4 (30mM) and NaH_2P0_4, $2H_2O$ crystallized (14mM). Whereas at pH 8.5 the solution contained NaCl (6g/L or 102mM), anhydrous Na_2HPO_4 (1mM) and NaH_2P0_4, $2H_2O$ crystallized (43mM). Recall that the pH=pKa+log (NaH_2P0_4/Na_2HPO_4).

2.8. Age, Sex And Season

To study the effect of age, sex and season on the OF of RBCs prepared from the heparinized blood, the test was performed at 38°C respectively on young and old camels, males and females camels and during summer and autumn seasons.

2.9. Osmotic Fragility Test

The OF of erythrocytes subjected to different conditions (anticoagulant, storage time, temperature and pH) was determined by the procedure described by Oyewale [14] and Oladele et al. [9]. Briefly, sodium chloride (NaCl) stock solution (pH 7.4) was prepared in volumes of 500mL for each of the sample in concentration, ranging from 0.1% to 0.9%. A set of 10 test tubes was used and each tube contained 10mL of the corresponding NaCl concentration from the stock solution. The test tubes were then labelled with corresponding concentrations and arranged serially in a rack of 10 tubes. A 100µl aliquot of washed RBCs suspensions were added to each of the 10 test tubes in a set. The contents of the test tubes were gently mixed by inverting the test tubes five times and allowing them to stand at room temperature for 30min. Thereafter, the contents of the test tubes were centrifuged at 1500×g for 15min. The supernatant was then transferred into a glass cup and measure data wave length of 540nm using a spectrophotometer by reading the absorbance.

Hemolysis in each tube was expressed as a percentage, taking as 100% the maximum value of absorbance of distilled water. The percent hemolysis was calculated according to Faulkner and King [15] as follows:

Percent hemolysis (H%)=(Optical density of test/Optical density of distilled water)x100.

H% curve was obtained by plotting percent hemolysis against the saline concentrations.

2.10. Effect of Hydrogen Peroxide

One hundred µL of RBCs suspensions (5% in PBS pH 7.4) were mixed with 4.9mL of H_2O_2 or distilled water or PBS solution. H_2O_2 was used at 0.5mM; 2mM and 8mM and prepared with the PBS solution (Table 2).

Table2. *Effect of Hydrogen Peroxide on Hemolysis*

RBCs (5%)	PBS pH 7.4	H_2O_2 0.5; 2; 8mM	H_20
100µl	4.9ml	0	0
100µl	0	4.9ml	0
100µl	0	0	4.9ml

RBCs: *red blood cells suspensions, PBS: phosphate buffered saline.*

All mixtures were incubated at 38°C for 60mn. After centrifugation at 1500rpm for 10min, the absorbance of the supernatant was measured at 540nm to determine the rate of induced H_20_2 hemolysis as follows:

H% = [DO ((RBCs+H_20_2)–DO (RBCs+PBS))/DO (RBCs+H_20)] x100.

Hemolysis in isotonic medium without H_20_2 was:

H% = DO (RBCs+PBS))/DO (RBCs+H_20)] x100.

2.11.Protection of Vitamins C and E against H202 Induced Hemolysis

Suspensions of RBCs (5% in PBS pH 7.4) were incubated at 38°C for 30min, with vitamin C (20; 40; 60mM) or vitamin E (0.5; 1; 2mM) or both (respectively 60mM and 2mM). The vitamins solutions were prepared in PBS pH 7.4. To the mixture H_20_2 (2mM) was added. After incubation at 38°C for 60min, the mixture was centrifuged at 1500rpm for 10min and the optical density of the supernatant was analyzed at 540 nm (Table 3).

Hemolysis in isotonic medium without H_20_2 nor vitamins was:

H% = DO (RBCs+PBS))/DO (RBCs+H_20)] x100,

H_20_2 induced hemolysis in absence of vitamins was:

H% = [DO ((RBCs+H_20_2)–DO (RBCs+PBS))/DO (RBCs+H_20)] x100

and H_20_2 induced hemolysis of erythrocytes pre-treated with vitamins C and/or E was calculated as follows:

H% = [DO ((RBCs+Vitamins+H_20_2)–DO (RBCs+PBS))/DO (RBCs+H_20)]x100.

Table3. *Protection of Erythrocytes by Vitamins C and E against Hydrogen Peroxide Induced Hemolysis*

RBCs (5%)	PBS pH 7.4	Vit C 20;40;60mM	Vit E 0.5;1;2mM	Vit C (60mM) + Vit E (2mM)	PBS pH 7.4	H_2O	H_2O_2 (2mM)
100µl	100µl	0	0	0	4.8ml	0	0
100µl	100µl	0	0	0	0	0	4.8ml
100µl	100µl	0	0	0	0	4.8ml	0
100µl	0	100µl	0	0	0	0	4.8ml
100µl	0	0	100µl	0	0	0	4.8ml
100µl	0	0	0	100µl+100µl	0	0	4.7ml

RBCs: *red blood cells suspensions, PBS: phosphate buffered saline.*

2.12. Statistical analysis

The data were expressed in SI units and analyzed by the Mann-Whitney U test for comparison between situations or groups. All values were expressed as mean and standard error (SE), and $P<0.05$ was seen as statistically significant.

3. RESULTS

3.1. Effect of Anticoagulant

As showed in Figure 1, the use of EDTA as an anticoagulant increased significantly (0.05) the OF of RBCs as compared with heparin. Thus, 0.4%, 0.3% and 0.2% NaCl induced respectively 12±2%, 21±2% and 98±4% hemolysis of erythrocytes isolated in EDTA, but induced respectively 6±2%, 10±2% and 80±4% hemolysis of erythrocytes isolated in heparin (Fig. 1).

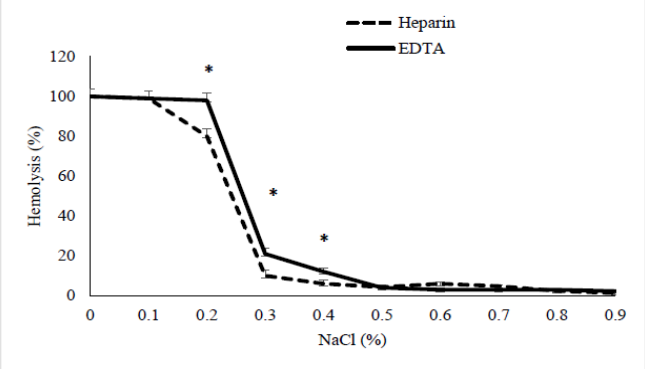

Figure1. *The effect of anticoagulant on the osmotic fragility of erythrocytes prepared from blood collected in EDTA or heparin from dromedary camel (Mean±SE, n= 8 males, *P<0.05, comparison between EDTA and heparin).*

3.2. Effect of Storage Time

During the first five days of RBCs storage at 4°C, the test of OF didn't show any significant variation of H% in hypotonic NaCl solutions (Fig. 2). However, when compared to the first day, H% measured at the 7th, 8th and 10th days of storage, showed a significant ($P<0.05$) shift of the curves to the right for 0.4%, 0.3% and 0.2% NaCl, indicating an decreased osmotic resistance of erythrocytes (Fig. 2). For example, in 0.3% NaCl, H% at days 1, 7, 8 and 10 was respectively 14±4%, 22±5%, 24±5% and 26±5% (Fig. 2).

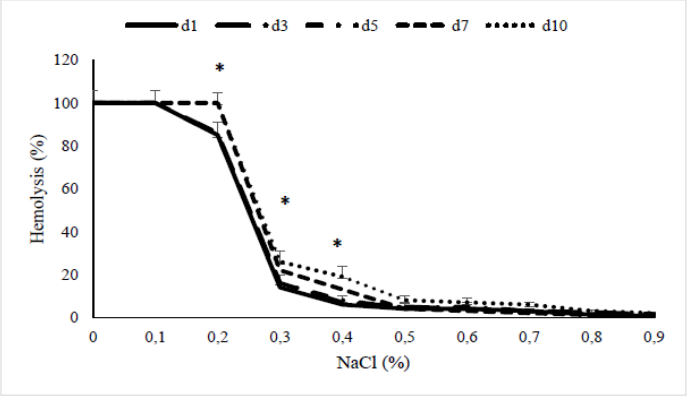

Figure2. *The effect of storage time of erythrocytes at 4°C on their osmotic fragility. The erythrocytes were isolated from blood collected in heparin from dromedary camels (Mean±SE, n= 7 males, *P<0.05, comparison to day 1).*

3.3. Effect of Temperature

The OF curves of erythrocytes incubated in hypotonic salt solutions at 18°C, 40°C and 42°C didn't show any significant variation when compared to that analyzed at 37°C. However, at 4°C and 50°C, H% becamed significantly (P<0.05) very higher than that observed at 37°C (Fig. 3). Thus, in 0.3% NaCl at 4°C, 37°C and 50°C, H% was respectively 54±3, 28±3 and 58±4 (Fig. 3).

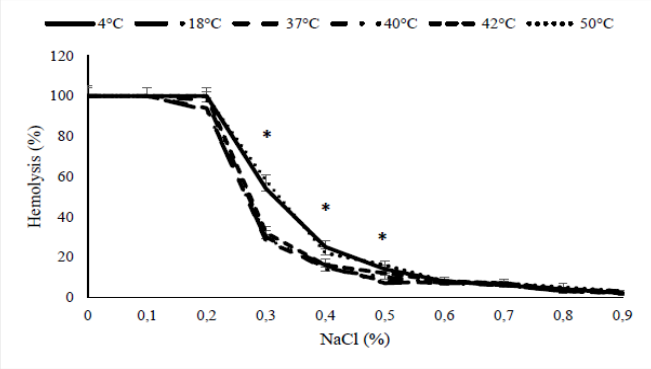

Figure3. *The effect of temperature on osmotic fragility of erythrocytes isolated from blood collected in heparin from dromedary camels (Mean±SE, n= 7 males, *P<0.05, comparison to 37°C).*

3.4. Effect of pH

Comparatively to physiological pH 7.4, H% tested in 0.3% and 0.4% NaCl showed a significant (0.05) variation with low value when pH=8.5 and high value when pH=6.5 (Fig. 4). For example, in 0.3% NaCl, H% at pH 8.5, 7.4 and 6.5, H% was respectively 17±3%, 28±4% and 48±4% (Fig. 4).

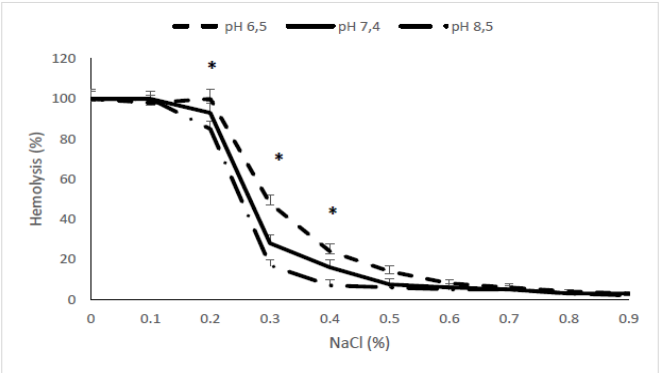

Figure4. *The effect of pH on the osmotic fragility of erythrocytes isolated from blood collected in heparin from dromedary camel (Mean±SE, n= 7 males, *P<0.05, comparison to pH 7.4).*

3.5. Effect of sex

The results presented in Figure 5 showed a shift of the H% curve of RBCs isolated from female camels to the left, indicating a significant (P<0.05) increased osmotic resistance of erythrocytes by comparison to male camels. Thus, in 0.4%, 0.3% and 0.2% NaCl, H% in males was higher than that observed in females (respectively 14±3% vs 7±2%, 25±3% vs 11±2% and 98±4% vs 84±4%, P<0.05) (Fig. 5).

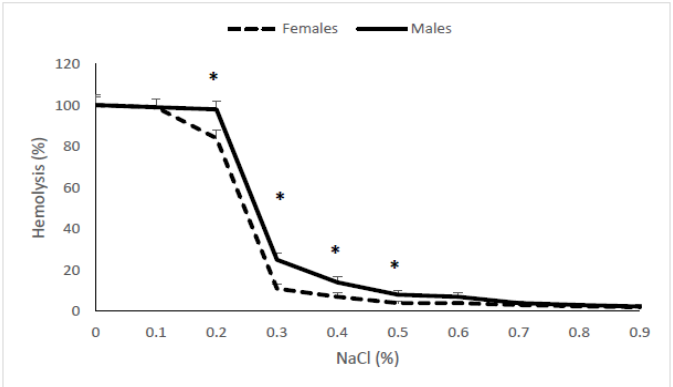

Figure5. *The effect of sex on the osmotic fragility of erythrocytes isolated from blood collected in heparin from dromedary camel (Mean±SE, n= 6 males and 6 females, *P<0.05, comparison between males and females).*

3.6. Effect of Age

Our results showed an increased osmotic resistance of erythrocytes in young male camels by comparison to that analyzed in old ones (Fig. 6). The hypotonic salt solutions 0.5, 0.4% and 0.3% NaCl induced H% significantly (P<0.05) higher in old camels than that observed in young ones (respectively 12±2% vs 7±1%, 17±3% vs 11±3% and 25±3% vs 16±3%) (Fig. 6).

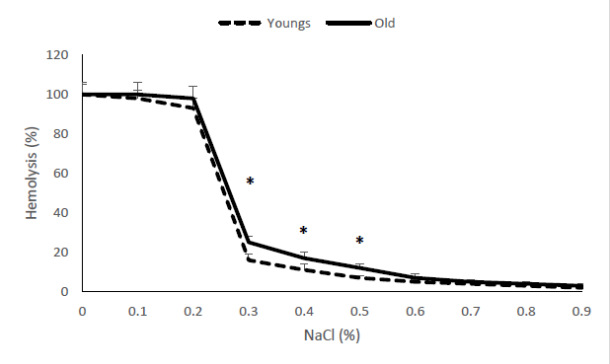

Figure6. *The effect of age on the osmotic fragility of erythrocytes isolated from blood collected in heparin from male dromedary camel (Mean±SE, n= 7 youngs and 7 olds, *P<0.05, comparison between youngs and olds).*

3.7. Effect of Season

Erythrocytes of male camels, showed a significant (P<0.05) high osmotic resistance automn when compared to that analyzed during summer. Thus, 0.4% and 0.3% NaCl solutions induced a high H% during summer when compared to that measured during autumn (respectively 15±3% vs 8±2% and 27±3% vs 13±3%, P<0.05) (Fig. 7).

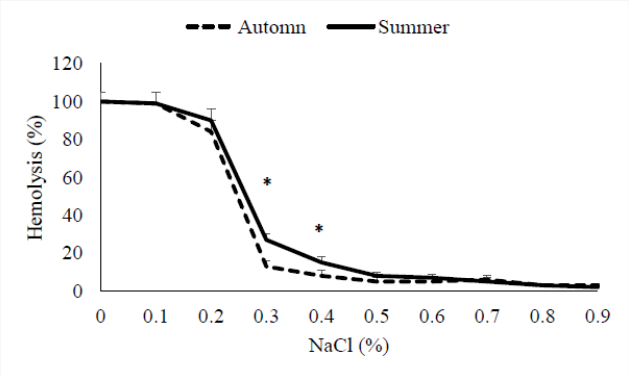

Figure7. *The effect of season on the osmotic fragility of erythrocytes isolated from blood collected in heparin from male dromedary camel (Mean±SE, n= 8 for each season, *P<0.05, comparison between summer and autumn).*

3.8. Effect of Hydrogen Peroxide

After incubation with hydrogen peroxide H% increased significantly when compared with that observed in distilled water. The H_20_2 induced hemolysis was dose-dependant, thus, at 0, 0.5, 2 and 8mM H_20_2 the H% was respectively 18±1.6%, 46±8.6, 62±7.2 and 92±7.8 (Fig. 8).

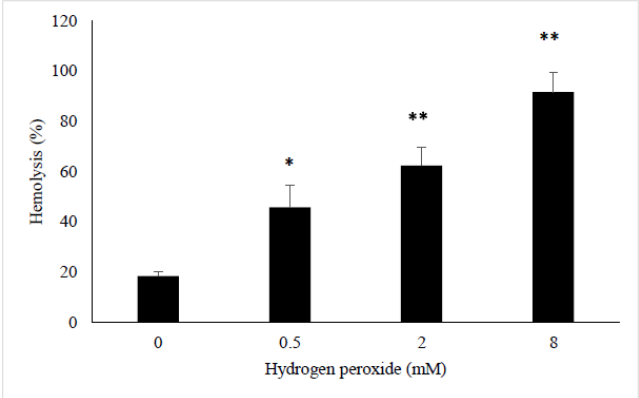

Figure8. *The effect of hydrogen peroxide on the osmotic fragility of erythrocytes prepared from blood collected in heparin from dromedary camel (Mean±SE, n= 7 males, *P<0.05, comparison to 0mM hydrogen peroxide).*

3.9. Protection of Vitamins C and E against H202 Induced Hemolysis

The preincubation of RBCs with viamins C or E reduiced significantly ($P<0.005$) the H_20_2 induced H%. The effects of these anti-oxidants were dose-dependant and becomed more significant ($P<0.001$) when the vitamins were associated (Fig. 9). H_20_2 induced H% was 5.7±0.6, 7.2±0.6 and 4.1±0.2 after preincubation respectively with viamin C (60mM), vitamin E (2mM) and both (60mM vit C+2mM vit E). However, H_20_2 induced H% without preincubation any vitamin was 72.4±6.3 (Fig. 9).

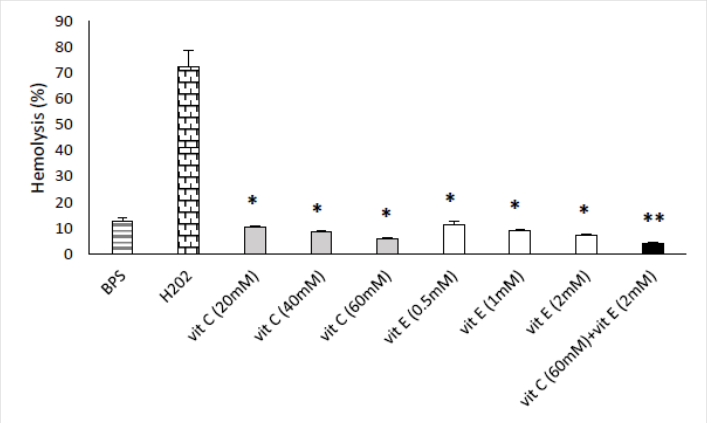

Figure9. *The protector effect of vitamins C and/or E against the hdrogen peroxide (2mM) induced hemolysis of erythrocytes isolated from blood collected in heparin from dromedary camel (Mean±SE, n= 7 males, *P<0.005, **P<0.001 comparison between presence and absence of vitamins).*

4. DISCUSSION

The EOF test is easily influenced by several environmental and physiological factors. Therefore, we have undertaken an investigation whose aim was to determine osmotic fragility reference values in camel. Our results showed a significant shift of the hemolysis curves to the left in presence of. In addition, a significant shift of hemolysis curves to the right was observed in presence of heparin and vitamins C and E, indicating a significant increased osmotic resistance of erythrocytes. These curves shifted to the right in presence of EDTA, storage time, heat, cold, acidity, sexe male, ageing and summer season, indicating a significant increased susceptibility to hypotonic lysis of RBCs. These differences were significant in NaCl concentrations of 0.3–0.4% where the changes in the extent of lysis as a consequence of changing NaCl concentrations were substantial.

The increased EOF obtained in our study when blood samples were collected in EDTA compared with heparin, was reported in man by several laboratories to result in an increased RBCs susceptibility to hypotonic lysis [16,17,18]. In hypotonic solution, vertebrate cells showed a rapid increase in cell volume followed by a slower regulatory volume decrease back to the original size [19]. This mechanism increases the efflux of intracellular osmolytes, thereby decreasing the driving force for water influx. Ca^{2+} ions may play an important role in this process for most cell types [19]. The depletion of extracellular Ca due to the chelation by EDTA may therefore be able to the decreased volume recovery of RBCs following hypotonic stress when compared to heparin. In addition, RBCs of animals bred on Ca^{2+}-defficient diet showed an increased EOF [20].

In the work reported here, hemolysis of the RBC increases with storage time. Several reports on the effects of blood storage have shown significant alteration in RBC membrane integrity and flow properties and significant increase in the levels of free hemoglobin [21,22]. The presence of leukocytes in RBC suspension may also contribute significantly to the increase in hemolysis during storage [23,24]. During storage, leukocytes break down and release a number of chemicals and enzymes such as hydrogen peroxide and proteases. Proteases released by leukocytes during storage have been reported to cause RBC lysis during storage and are detrimental to their metabolism and viability [23,25]. Storage of red cells causes a progressive increase in hemolysis [26].

Hemolysis of camel's RBCs in hypotonic salt solutions increased much more when their incubation occured at very low (4°C) or very high temperatures (50°C). In fact, it was estabilished that the temperature greatly affects membrane deformability 50,56 and, therefore, the stability of the membrane during processing. So, human RBCs are damaged if warmed to a temperature of 40°C 54.

At temperatures at or less than 43°C and even up to 45–46°C, it appears that blood heating is safe and causes hemolysis only in clinically negligible proportions [27]. According to Sharma et al. [28], thermal stress redistributes the body's nutrients including protein and energy at the expense of growth, reproduction, production and health of the animal. This enhances the occurrence of lipid peroxidation and induces changes in the fragility and antigenicity of the erythrocyte membrane [29].

The results showed that in camel, RBC hemolysis in salt hypotonic solutions, decreased when pH of incubation medium was 8.5 and increased when pH was 6.5. Variations of environment pH involves all the main structural and functional RBCs links and may result in oxidative damage of cell membrane leading to hemolysis. The principal functions of RBCs are recognised to be the transport of oxygen and CO_2, as well as the binding of acids and alkali, formed in tissues by metabolism, and their acidification can result in an elevation of potential endogenous cytotoxic metabolites [30]. Acidosis leads also to a release of metals with variable valency from metalloproteins, which participate in Fenton and Haber–Weiss reactions, stimulating $OH^{\cdot}$ and oxidant formation [31] that can result in oxidative damage to cell membranes and hemolysis [32].

Our results showed that EOF was very high in males camels than that observed in females. In fact, it has been established that erythrocytes of males are more susceptible to hemolysis than those of females in domestic fowl [33,34], cattle [35], WAD sheep [36], White Fulani cattle [37] and camel [38]. However, in contrast to these observations, other findings has enregistred a lower EOF in male compared to female. Such findings have been reported in African giant rats [39], dogs [40], turkeys [6] and goat [41]. On the other hand, in camel, the osmotic resistance increased significantly during the green season and decreased in winter [42].

The significant increase of EOF with age reported here, might be explained by metabolic alterations and a reduction of defense against OS. According to Walls et al., [43], young human cells were able to effectively protect themselves against thyroxine-peroxide induced hemolysis; however, old cells exhibited less protection. It should also be noted that the activity of G-6-PD is known to decrease with erythrocyte aging [44]. This decrease is associated with a decrease in glutathione concentration [45] and increased methemoglobin formation [46] in older cells. In addition, erythrocytes life span had been reported to decrease by 50% in older rats by comparison to younger ones [47], and their hemolysis had been suggested du to an increase of dolichol levels in their membranes [48].

The hydrogen peroxide induced hemolysis in camels, was attenuated by vitamins C and/or E. The hydrogen peroxide increased metabolic activity in the RBC wich resulted in elevation in the production of reactive oxygen species (ROS). Due to the higher level of polyunsaturated fatty acids in erythrocyte cell membrane, the peroxidation of unsaturated chain of their membrane lipids increases the susceptibility of osmotic hemolysis [49,50]. It has been well established that the EOF test can be used to evaluate lipid peroxidation or oxidant stress (OS) in pig [10], rat [51], rabbit [49] and camel [11]. ROS are highly reactive non-specific molecules derived from the metabolism of oxygen including oxygen free radicals and other species such as hydrogen peroxide and singlet oxygen [52]. In rats submetted to OS induced by high altitudes stress, supplementation with vitamins C ans E reduced membrane instability and ROS, in terms of osmotic fragility and hemolysis, and increased the activity of glutathione peroxidase in the RBCs [53]. Furthermore, Vitamin E supplementation was showed able to protect human erythrocyte membranes from OS and improve membrane fluidity and reducing hemolysis [54]. In addition, in patients with advanced cirrhosis, low vitamin E plasma levels are associted with abnormal erythrocyte membrane lipids, structural alterations and chronic hemolysis [55].

Our results taken toghether indicate that EOF in camel may be influenced by EDTA, cold, heat, acidosis, sexe male, storage time, age and ROS. These factors induced erythrocytes destruction by damage to the their cell membrane and may disturb the results of other blood analyses such as measurements of various hematological and biochemical parameters in camel. Finally, vitamins C and/or E exhibited a good protection of camel RBCs against hemolysis.

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