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Full Length Research Paper

Analyses of the effect of para- phenylenediamine *Takaout roumia* on the osmotic stability of human erythrocytes

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This work analysed the effects of para- phenylenediamine (p-PD), a component in permanent hair dyes products on the osmotic stability of human's erythrocytes. Hemolysis was monitored by measurement of absorbance at 412 nm following addition of erythrocytes to NaCl solutions of varying concentration. Absorbance was fitted to sigmoid regression curves given by Boltzmann equation, and hemolysis was characterized by the NaCl concentration leading to lyses of 50% of cells (H_{50}), and by the intensity (H) and the amplitude of the lyses (dS). The parameters were determined in the absence and the presence of different doses of p-PD and at different times from 30 min to 2 h. The low doses of *p*-PD used in this study (5 µg/ml) protected human erythrocytes against hypotonic shock as evidenced by a decrease of H and H50 values compared to the control solution ($p<0.05$) for 2 h. However, the higher doses of *p*-PD used (2.5 and 3 mg/ml) enhanced hemolysis, since their H_{50} values were higher than in the control group ($p<0.05$). In conclusion, it seems that the p-PD has simultaneously protector and haemolytic effects depending on the concentration used and the time of exposure.

Key words: Erythrocytes, hematotoxicity, membrane stability, *p*-phenylenediamine.

INTRODUCTION

Para- phenylenediamine (*p*-PD) is one of the key primary precursors of the oxidative hair dyes used as dark colours in permanent hair dyes products. Known for its high toxicity and hit availability with low prices, p-PD is an attractive poisoning agent for abortion or suicide attempts requiring hospitalisation in Morocco (Saito et al., 1990; Stambouli et al., 2004). The first acute p-PD intoxication case in Morocco occurred in 1980 and was reported by Arditti (Arditti el al., 1980). Then several others reports of deliberate or accidental p-PD poisoning in humans were reported (Bourquia et al., 1988; Squali et al., 1991; Zeggwagh et al., 1996; Kerkeb et al., 1998). Symptoms

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Abbreviations: ***p*-PD,** Para-phenylenediamine; **H_{50},** the NaCl concentration leading to lyses of 50% of erythrocytes; **H,** the intensity of the lyses; **dS,** the amplitude of the lyses.

of poisoning with *p*-PD are stereotyped and time of onset is on average two hours (Chugh et al., 1982; El Ansary et al., 1983; Bourquia et al., 1988). A cervico-facial edema and laryngeal-pharyngeal origin of the asphyxia syndrome was first observed requiring intubation or tracheotomy in emergency (El Ansary et al., 1983; Baud et al., 1984; Bourquia et al., 1988 ; Yagi et al., 1991; Lifshits et al., 1993; Adnet et al., 1994 ; Ashraf et al., 1994).

This cellular oedema settled early is probably the result of a change in membrane permeability (Mascres and Jasmin, 1975). Then a muscle lyses broadcasts from the 24th hour by affecting all skeletal muscles (Averbukh et al., 1989; Saito et al., 1990; Motaouakkil et al., 2006). Finally, by nephotoxicity derivatives of p-PD are characterized by oliguria issue with very dark urine (Bourquia et al., 1988; Averbukh et al., 1989; Saito et al. 1990; Yagi et al., 1991; Lifshits et al., 1993; Boles et al., 1994).

The distribution kinetics of ^{3}H labeled *p*-PD was studied after intravenous and percutaneous administration in rabbits and mice (Rehani et al., 1981). Blood concentrations rose steadily for the first 24 h. Thirty five hours after application, 0.13% of the applied radioactivity was detected per ml of blood. There is no data on the effect of *p*-PD on human's biological membranes.

Cell membrane, in order to function effectively, must combine properties of fluidity and stability (Cribier et al., 1993) to allow signalling and transport. Membrane stability represents the capacity of this biological complex to maintain its structure under different conditions such as heat, hypotonicity, pH extremes presence of solutes (Timasheff, 1998), oxidative stress (Van-Ginkel and Sevanian, 1994) and medicinal crude extracts (De Freitas et al., 2008; Roselli et al., 2007, Chaudhuri et al., 2007). The effect may be positive through restoration of fluidity (Penn-Silva et al., 2007) or may also be negative through denaturation of membranes.

Erythrocytes represent a good model for the study of membrane stability since their lyses releases the protein hemoglobin which can be readily measured spectrophotometrically. In response to variation on solvent tonicity, the osmotic stability of erythrocytes correlates with their resistance to lyses and can be determined by measurement of hemoglobin release after a fixed period of incubation of blood in solutions with decreasing concentrations of NaCl (Jain, 1973).

Evaluation of membrane stability during exposure to toxic products may be routinely considered in their evaluation, since the consumption and the increasing popularity of these products is increasing globally by the use of henna tattoos and natural products of hair dyes and could thus constitute a serious health problem (Motaouakkil et al., 2006; Redlick and DeKoven, 2007; Mc Fadden et al., 2007)

In this study, we investigated the parameter that need to be evaluated to determine the effect of the *p*-PD on the erythrocyte membrane stability.

MATERIALS AND METHODS

Preparation of para-phenylenediamine

The stock solution of *p*-PD (100 mg/ml) was dissolved in sterile distilled water and different concentrations of p-PD (5, 2.5, and 3 mg/ml) were prepared in 1 ml of different concentrations of NaCl solutions (1.8, -2.25, -2.70, -3.15, -3.60, -4.05, -4.50, -4.95, -5.4, -5.85, -6.30, -6.75, -7.20, -7.65 and 8.1 g/l) (Parpart et al., 1946 revised).

Collection of human blood samples

This work was previously approved by the local institutional Ethics Committee. Blood samples (7 ml) were collected (Regional Center for blood transfusion - Casablanca, Morocco) by intravenous puncture in evacuated tubes containing EDTA from healthy volunteers (25-35 years), selected from a pool of healthy, no history of sensitization to PPD or exposure to hair dyes/tattoo, non-smokers who had not been exposed to drugs or medicine for the previous 4 weeks.

Determination of the osmotic stability of human erythrocytes

Two duplicate sets of assay tubes containing 1 ml of on of 1.8-8.1 g/l NaCl solutions prepared in deionised water (control) or 1 ml of one of 1.8-8.1 g/l of NaCl containing 25 µl of different concentrations of *p*-PD (final concentrations: 5, 2.5 or 3 mg/ml) were incubated at room temperature for different time periods (30 min, 1 h, 1 h 30 min or 2 h) with 50 µl aliquots of blood samples. The tubes were then centrifuged at 1300 x g for 10 min.

The lyses of erythrocytes were followed by measuring absorbance of the supernatants at 412 nm. The absorbance values (A) were plotted against NaCl concentration and fitted to the sigmoid regression curve given by the Boltzmann equation (De Freitas et al., 2008)

$$\text{Hemolysis (\%)} = \frac{A_1 - A_2}{1 + e^{(S - H_{50})/dS}} + A_2$$

Where A_1 and A_2 are the mean maximal and mean minimal absorbance values of the sigmoid, respectively, S is NaCl concentration, H_{50} is the NaCl concentration that cause 50% of hemolysis and dS is the amplitude of the sigmoidal transition between A_1 and A_2.

The intensity of the hemolysis was measured by the difference between A1 and A2 (H). The percentage of hemolysis in each tube was calculated by the equation:

$$\text{Hemolysis (\%)} = \frac{A}{A_1} \times 100\%$$

Calculation and statistical analysis of the experimental data

Results are expressed as mean + SD from at least three independent experiments. Statistical analysis was performed according to student's test by one way analysis of variance. Significant difference was taken as $p<0.05$ or $p<0.001$ (Dell'Aquila et al., 2005).

The dependence of H_{50}, H and *dS* on the densities of *p*-PD was analysed by regression with $p<0.05$ indicating statistically significant relationships.

RESULTS

Determination of the parameters used in the characterisation of the osmotic stability of erythrocytes

As described above (De Freitas et al., 2008), we used a typically control curve of osmotic stability of human erythrocytes for the determination of the different parameters. This curve (Figure 1) shows how each analysed parameter (H, H_{50} and dS) was determined. The presence of erythrocytes in environments of decreasing tonic promotes the release of hemoglobin in the external environment from 3.38 g/l of NaCl at 30 min of incubation; this distribution peaked at 2 h of incubation where 100% hemolysis was observed for 3.60 g/l of NaCl.

Effect of the p-PD on the osmotic stability of erythrocytes

Under the action of various doses of *p*-PD, the erythrocyte reacts differently in solutions of decreasing tonicity. Increases membrane resistance towards osmotic forces of the external environment was observed until 2 h of incubation at concentrations of *p*-PD ranging from 5 to 2.5 mg/ml. A 100% of hemolysis is reached only for 2.25 g/L of NaCl compared to control where the overall hemolysis appeared for 3.60 g/L of NaCl.

Membrane resistance of red blood cells has been increasingly weakened leading to early hemolysis for 5.85 g/L for NaCl at 2 h of incubation in the presence of higher concentrations of *p*-PD, 3 mg/ml. However, whatever the concentration of p-PD in the external environment, hemolysis is complete after 1 h 30 min of incubation.

The Figure 2 presents the lysis transition curves for all volunteers in the presence and in the absence of different concentrations of p-PD. Values of all the analysed parameters (H, H_{50} and dS) were determined from curves in both situations. The mean of this value are shown in Table 1. The presence of the *p*-PD at 5 µg/ml in the solution produced significant ($p<0.05$) decreases in the H values in relation to the control. Compared to the control, the values of H50 were significantly lower ($p<0.05$) in the presence of the 5µg/mL p-PD, but significantly higher ($p<0.05$) in the presence of 3mg/mL PPD for 2hours. The values of dS increased significantly ($p<0.05$) in the presence of 5 and 2.5 mg/mL *p*-PD compared to the control.

DISCUSSION

Reactive action of *p*-PD on the membrane of human red blood cells was first tested in this study by analyzing the behavior of erythrocytes in hypotonic environments in the presence of *p*-PD at different concentrations (5, 2.5 and 3 mg/mL). The *p*-PD could potentially have altered osmotic stability through perturbation of any of the three parameters described in Figure 1 (H, H_{50} and dS). A stabilizing effect would be associated with an increase in dS and decreases in H_{50} and H compared to the control, whereas a destabilizing effect would be indicated by a decrease in dS and an increase in H_{50} but not in H, since the H value for the control already represents the maximum possible intensity of hemolysis.

As previously stated, exacerbation of haemolysis would not increase H, since H already represents the maximum haemolysis possible for the blood aliquot used. A direct perturbation of the spectral properties of haemoglobin could potentially have caused an increase in H, but such an increase was not observed in any of the scenarios wetested.

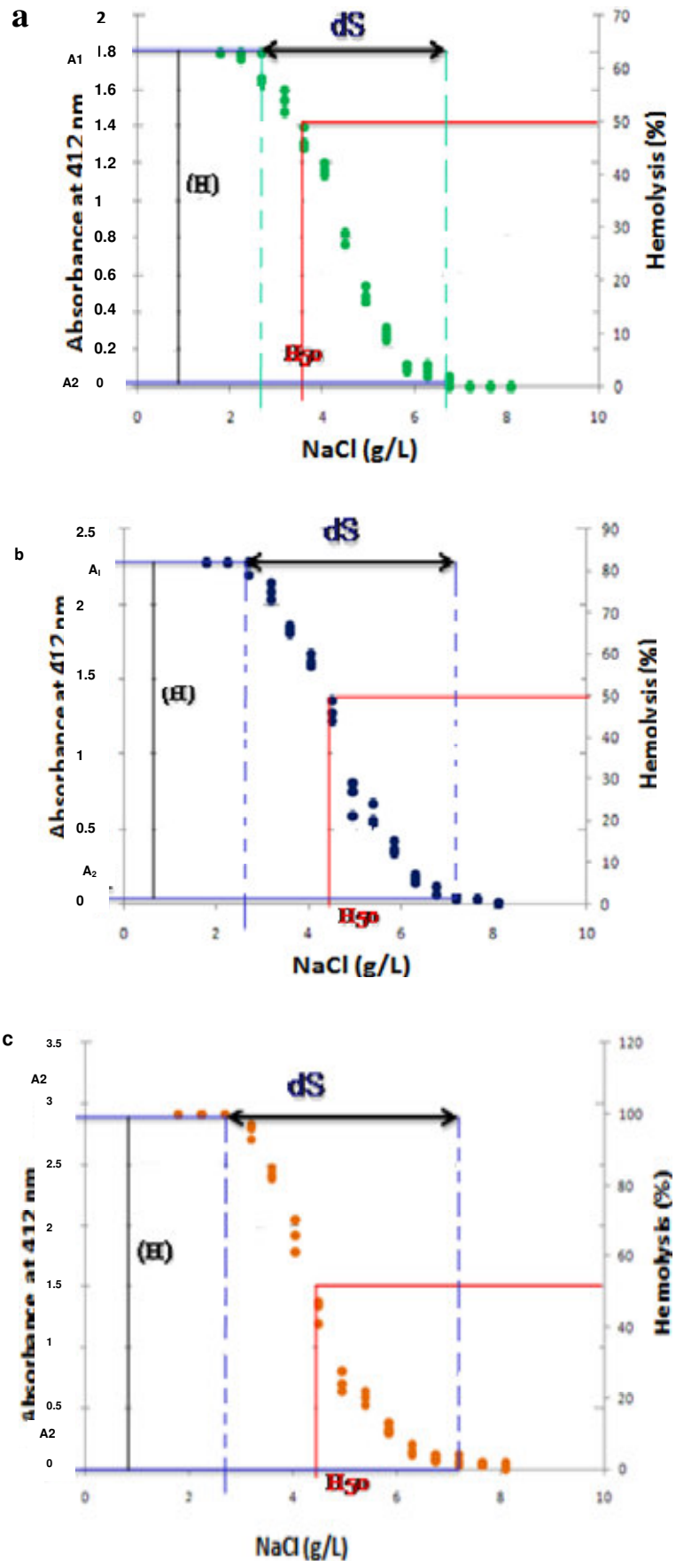
In contrast, a decrease in H is possible and may represent an anti-hemolytic effect through an aggregation or partial precipitation of haemoglobin. H50 and dS undoubtedly describe the effect of the extract on osmotic stability. Erythrocyte stabilization causes a decrease in H50 and/or an increase in dS, and vice versa. Instead of a weakening of the membrane normally obtained in the hypotonic medium, it is a resistance cell which was observed in the first two hours of exposure to the concentrations of *p*-PD ranging from 5 to 2.5 mg/ml (Fig 2).

A statistically significant decrease in *H50* was observed in the presence of the *p*-PD at 5 or 2.5 mg/ml (Table 1), indicating that these two concentrations have an effect of stabilizing erythrocyte membranes. However, a higher concentration of *p*-PD (3 mg/ml) produced significant increases in H_{50} (Table 1), indicating that they tended to destabilize the erythrocyte membrane leading to the release of haemoglobin that could be the cause of the majority of toxic effects.

It has been shown that the electrochemical oxidation of simple aliphatic amines is quite complex and may lead to several different products (Barbier et al., 1990; Deinhammer et al., 1994).

According to Mann, Barnes and Smith (1970), the mechanism of formation of polymeric film (ploy p-PD) protective of haemolysis involves four major steps. The monomer p-PD is oxidized with the loss of an electron and the formation of a cation radical. This step is followed by the cleavage of the C-N bond with formation of a primary carbonation, which attacks another molecule of p-PD. After the expulsion of the proton from the protonated amine, an additional loss of an electron and C-N bond cleavage take place (Mann et al., 1970). In the present study, we believe that the protective effect of the low concentrations of p-PD less than 2,5 mg/ml on the erythrocyte membranes depends on the mechanism of formation of Polyp-PD (Lakard et al., 2003).

The haemolytic action of the concentrations greater than 2.5 mg/ml, may be related to the cytotoxic effects. Since the molecule of p-PD is very reactive, it may form


a
dS
A1
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H50
Absorbance at 412 nm
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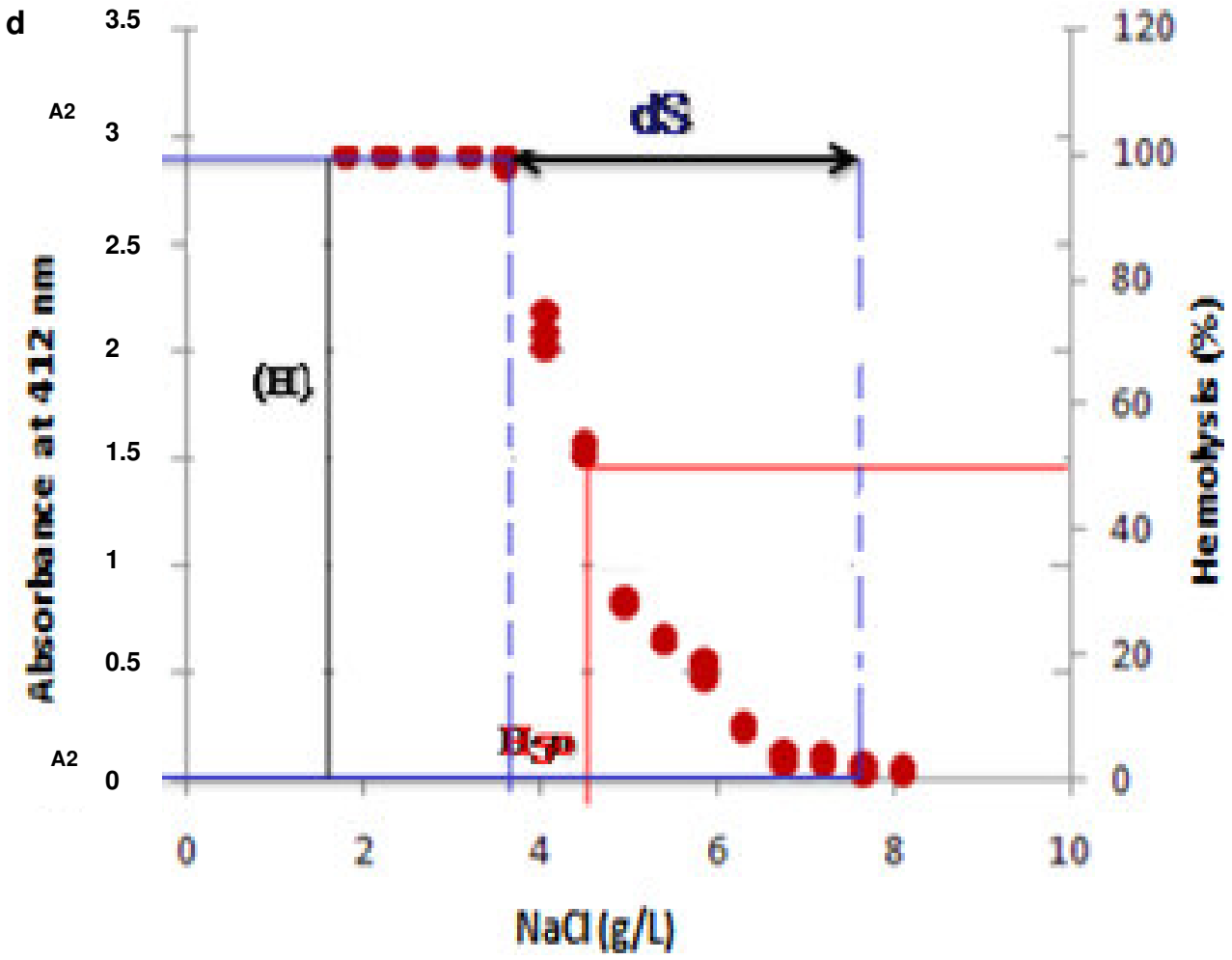
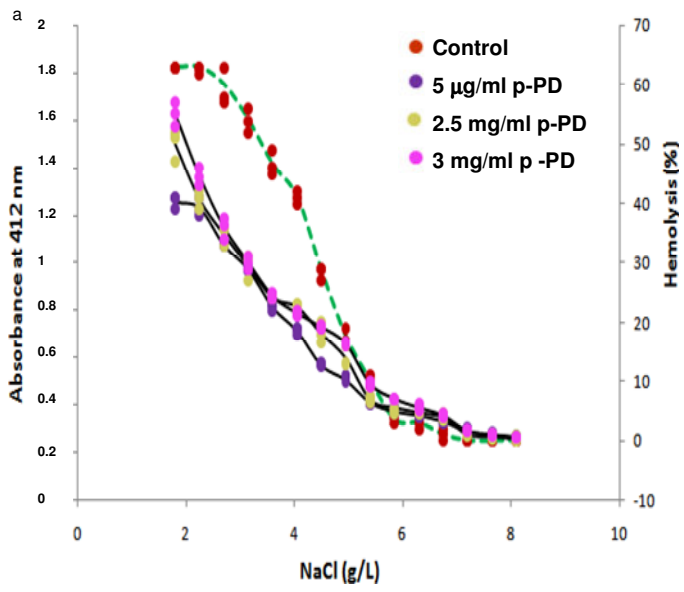
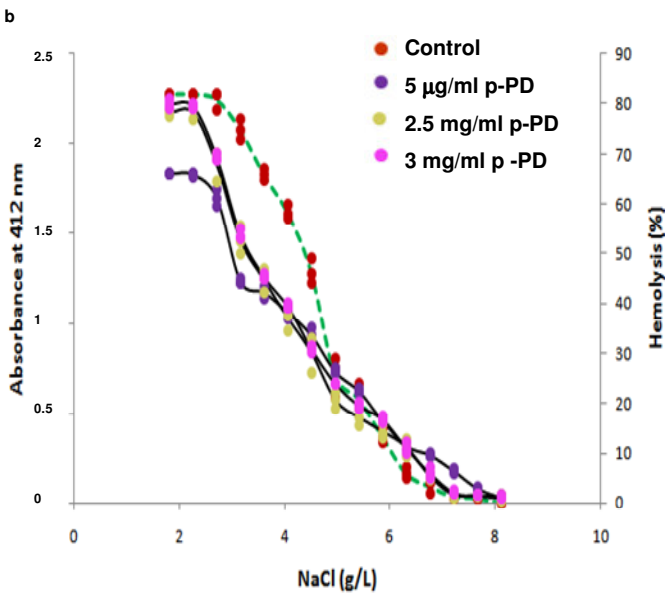


Figure 1. A typical osmotic fragility curve for human erythrocytes for 30 min (a) 1 h (b), 1 h 30 min (c) and 2 h (d). Data were fitted to a sigmoidal curve, from which we obtained the mean maximum (*A1*) and mean minimum (*A2*) values of absorbance, the concentration of NaCl promoting 50% of hemolysis (H_{50}), the intensity (H) and the amplitude (dS) of hemolysis transition.





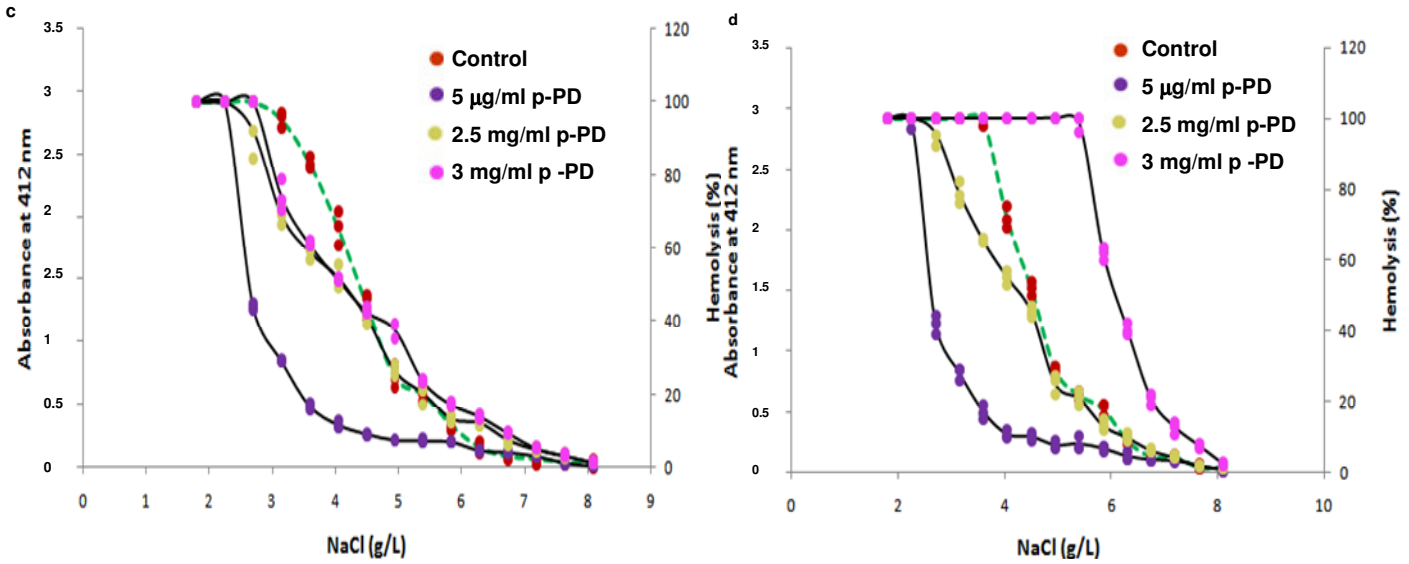


Figure 2. Comparison of lyses transition curves of human erythrocytes for all volunteers in the absence (control) and presence of the three concentrations of p-PD (5, 2.5 and 3 mg/ml) used for 30 min (a), 1 h (b), 1 h 30 min (c) and 2 h (d).

Table 1. Mean ± sd values of H, H_{50} and dS for lyses of human erythrocytes in the absence (control) and the presence of different concentrations of *p*-PD at different times periods of incubation.

Sample	N	H	p(H)	H_{50}	p (H_{50})	dS	p(dS)
				1 h			
Control	3	2.34±0.03	*	4.46±0.03	*	4.50±0.02	*
p-PD 5	3	1.75±0.01	*	3.00±0.01	*	5.40±0.01	*
p-PD 2.5	3	2.16±0.01	*	3.20±0.01	*	4.95±0.01	*
p-PD 3	3	2.27±0.03	*	3.25±0.03	*	4.95±0.01	*
				1 h 30 min			
Control	3	2.79±0.01	*	4.40±0.01	*	4.50±0.02	*
p-PD 5	3	2.38±0.03	*	2.60±0.03	*	5.40±0.01	*
p-PD 2.5	3	2.75±0.02	*	4.20±0.02	*	5.40±0.01	*
p-PD 3	3	2.20±0.001	*	4.20±0.01	*	4.95±0.02	*
				2 h			
Control	3	2.85±0.01	*	4.60±0.03	*	3.15±0.03	*
p-PD 5	3	2.57±0.03	*	2.70±0.03	*	4.50±0.01	*
p-PD 2.5	3	2.74±0.01	*	4.25±0.02	*	4.95±0.01	*
p-PD 3	3	2.26±0.02	*	6.10±0.04	*	2.70±0.02	*

* Statistically significant differences (p<0, 05) between control and each test.

complexes with membrane proteins and phospholipid membranes of erythrocytes and then modifying the membrane permeability.

In summary, we can conclude that the low concentrations of p-PD protected human erythrocytes against hypotonic shock. It is important to acknowledge that this increase in stability may not be a physiologically positive effect, since it may be associated with a decrease in membrane fluidity. In conclusion, it seems that the p-PD has simultaneously protector and haemolytic effects depending on the concentration used and the time of exposure.

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