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IN-VITRO EFFECTS OF PARACETAMOL ON RHEOLOGICAL PROPERTIES OF ERYTHROCYTES AND ITS ANTIOXIDANT PROPERTIES

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Keywords:

Paracetamol, Erythrocyte Deformability, Antioxidant, Lipid Peroxidation, Erythrocyte Aggregation, Erythrocyte Osmotic Fragility.

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ABSTRACT: Human erythrocytes were pre-incubated in vitro with paracetamol and then erythrocyte's rheological properties of deformability, osmotic fragility and aggregation were evaluated after being compromised by various means. Paracetamol (1, 2, 4mg/ml) significantly prevented the loss of deformability through 5µm diameter pores of erythrocytes dehydrated with hypertonic buffer or with calcium ionophore A23187 at 4 mg/ml. When the potassium ionophore valinomycin was used to induce cell dehydration, paracetamol (4mg/ml) also prevented partially the loss of deformability. Paracetamol (4mg/ml) also decreased significantly erythrocyte osmotic fragility induced by hypotonic saline, and prevented significantly the aggregation of erythrocytes induced by dextran 70. The antioxidant properties of paracetamol was studied by chemical assays and found to be as follows: total antioxidant capacity (88.9 µg AAE/mg), reducing power (A^0 at 700 nm= 1.0 compared to 3.0 for Vit. C), DPPH scavenging activity (IC_{50}= 62.5 µg/ml compared to 5.8 for Vit. C), $OH^{\cdot}$ scavenging activity (IC_{50}= 40.2 µg/ml compared to <10 for catecholamin), iron chelating (IC_{50}= 144.6 µg/ml compared to 17.3 for EDTA) and Paracetamol at 1, 2, 4, 5 mg/ml also prevented significantly lipid peroxidation in a concentration dependent manner of human erythrocytes incubated with H_2O_2. The observed rheological and anti-lipid-peroxidant effects of paracetamol could be explained by its antioxidant activity, thus protecting membrane integrity and deformability. The results suggest that paracetamol could have a rheologically useful role on erythrocytes, preventing dehydration, aggregation and lipid-peroxidation, that may contribute positively to its analgesic and antipyretic effects.

INTRODUCTION: Paracetamol (acetaminophen) is a widely used analgesic and antipyretic drug, which in overdose can lead to severe liver and renal damage [1, 2, 3, 4]. Paracetamol is metabolized primarily by conjugation with glucuronic acid and sulfate in the liver.

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Less than 5% of the given dose is metabolized by hepatic cytochrome P-450 to produce a highly reactive toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is detoxified in the hepatocytes by the glutathione system [1, 2, 3, 4]. Once glutathione reserves are depleted, such as in overdose, multiple perturbations ensue that ultimately lead to cell damage.

In addition to cellular damage inflicted by NAPQI metabolite, paracetamol itself may directly contribute to toxicity. Paracetamol (even in the absence of cytochrome P_{450} conversion to NAPQI

in most studied cells) has been found to interfere with growth factor signal transduction, mitochondrial respiration, ribonucleotide reductase, transcription factor (NF-kB) activation by serum growth factors, and also to have a variety of other effects such as binding and inhibiting DNA repair and synthesis and cell proliferation, down regulation of *c-myc* and *bcl-2* mRNA, increasing chromosomal breaks, binding to cellular glutathione and ferrous iron and altering some hormone-regulated processes [1, 3].

Despite of extensive investigations, there is uncertainity about the mode of action and toxicity of paracetamol, as there is considerable debate about the hepatotoxicity of therapeutic doses and the mechanism by which paracetamol achieves its effects on fever and pain [1, 2, 4]. Although, there is considerable evidence that the analgesic effect of paracetamol is central and is due to activation of descending serotonergic pathways, its primary site of action may still be peripheral.

Since it is accepted that paracetamol have central and peripheral effects, it is worth studying its effects on rheological properties of erythrocytes. Erythrocyte rheological properties influence the interaction of platelets with vascular structures as well as macro- and micro- circulation, that may eventually contribute to the analgesic and antipyretic effects.

To our knowledge, there is no published work reporting on the status of hemorheological parameters under paracetamol toxicity, nor on the effects of paracetamol on erythrocyte rheology. The present study therefore aimed to investigate whether the rheological properties of erythrocyte such as deformability, osmotic fragility and aggregation might be affected by paracetamol. In this study, the effect of paracetamol on erythrocyte deformability was evaluated before and after the deformability was compromised by hypertonic buffer (known to dehydrate erythrocytes resulting in high intracelluar K^+), calcium ionophore A23187 (known to load erythrocytes with Ca^{2+} with consequent loss of intracellular K^+ and water) [5], and potassium ionophore valinomycin (known to deplete erythrocytes from K^+ and water) [5]. We also studied the effects of paracetamol on aggregation of erythrocytes induced by incubation with dextran 70, the osmotic fragility of erythrocytes stressed with hypotonic saline, and the plasma recalcification time (clotting time). We also studied the antioxidant and anti-lipid-peroxidant activities of paracetamol. The present study was concerned with *in vitro* experiments, to avoid the possible effects on erythrocyte rheology of many factors that might play *in vivo*.

MATERIALS AND METHODS:

Deformability studies:

Deformability studies with hypertonic buffer, calcium ionophore A23187 and potassium ionophore valinomycin were performed as described elsewhere for aspirin [5]. Results were expressed as an index of filtration (1F) of the flow time for the erythrocyte suspension relative to buffer and corrected for haematocrit [6, 7]. An increase in IF indicates loss of filterability (deformability). Filtration results (IF) for each concentration of paracetamol were compared with those for the control erythrocytes treated similarly but without the added paracetamol. Stock solutions of A23187 (Calbiochem brand, Behring Diagnostics, La Jolla, USA) in absolute ethanol (1.9 mmol / l) and of valinomycin (Sigma Chemical Co., ltd., poole, UK) in absolute ethanol (4.5 mmol / l) were stored at - 40 ^{0}C and diluted in the appropriate buffer immediately before use. Paracetamol (Jordan Sweden Medical and Sterilization Co., Na'ur, Jordan) in powder form was kept at room temperature and dissolved in the appropriate buffer before use.

Osmotic fragility measurement:

Erythrocyte osmotic fragility was measured as described elsewhere [8], using heparinized blood, after 30 min incubation of erythrocytes at ambient temperature in solutions of 0.1 - 0.9 % w/v NaCl with and without paracetamol (Final concentration of 4 mg/ml). Results of percent haemolysis for each concentration of paracetamol were compared with those for control erythrocytes treated similarly with NaCl solutions but without the added paracetamol.

Erythrocyte sedimentation rate (ESR) measurement: Heparinized venous blood was diluted with dextran 70 to a haematocrit value of 35 % (The final concentration of dextran 70 was 3 %).

The blood was then pre-incubated at ambient temperature for 30 min with paracetamol (Final concentration of 4 mg/ml) and the ESR (mm/h) was measured using westergren method. ESR results for paracetamol were compared with those for control blood treated similarly with dextran 70 but without the added paracetamol.

Plasma clotting time:
Oxalated venous blood obtained from healthy adults was centrifuged at 50g for 5 min within 30 min from venepuncture to get the plasma. Plasma clotting time in the absence and presence of paracetamol (4 mg/ml) was determined by recalcification with calcium chloride (0.025 M) at 37 ^{0}C as described elsewhere [9]. Plasma clotting time results for paracetamol were compared with those for control plasma treated similarly but without the added paracetamol.

Antioxidant testing assays:
Determination of total antioxidant capacity, reducing power, DPPH scavenging activity, $OH^{\cdot}$ scavenging activity and metal chelating ability (Ferrous ion) were performed as described elsewhere [10].

Exposure of erythrocytes to H_2O_2 with and without paracetamol:
Washed erythrocyte suspensions were prepared by centrifugation of heparinized whole blood from adult volunteers, to remove the buffy coat layer and then washing the packed cells three times with cold phosphate buffered saline (PBS). Washed erythrocyte suspensions were pre-incubated with 2 mM sodium azide for 60 min at 37°C in a shaking water bath to inhibit catalase. Next, equal volumes of cell suspension and 20 mM H_2O_2 were mixed and incubated for a further 60 min at 37°C. Controls contained PBS instead of H_2O_2. Following the incubation period, the suspensions were mixed and used for MDA. Paracetamol at 1, 2, 4 and 5 mg/ml was added to erythrocyte suspensions at 30 min of the pre-incubation period with sodium azide.

Erythrocyte MDA determination: MDA was determined as a measure of lipid peroxidation as described elsewhere [11]. All MDA concentrations were expressed as nmol/gHb.

Statistical methods:
Data are presented as means ± SD. Statistical significance was determined using SPSS statistics 17. The results were compared by paired *t*-test. Differences were considered significant when $p < 0.05$.

RESULTS:
Paracetamol at 4 mg/ml had no effect on deformability of normal erythrocytes from 12 adults incubated with the drug in isotonic buffer with osmolality of 290 mOsmol/kg water for 60 min (**Fig. 1**). When the deformability of normal erythrocytes from 12 adults was compromised by placing the cells in hypertonic buffer with osmolality of 450 mOsmol/kg water, the mean IF (index of filteration) rose from 12.5 ± 2.1 in isotonic buffer to 24.5 ± 1.4 in hypertonic buffer **(Fig.1)**. This loss of deformability was presumably due to the loss of water from the cells by osmosis. Pre-incubation of these compromised erythrocytes with paracetamol at 1,2 and 4 mg/ml for 60 min caused a significant ($p < 0.05$) improvement in deformability at 1, 2 and 4 mg/ml of paracetamol and the mean IF fell to 22.4 ± 2.1, 21.5 ± 2.2 and 19.8 ± 2.1 respectively **(Fig.1)**.

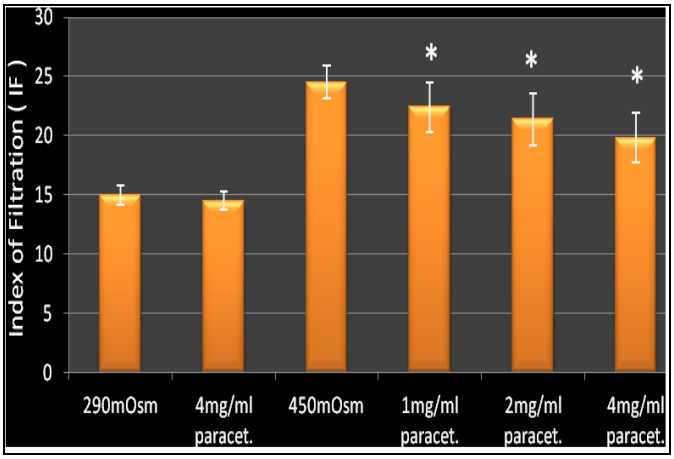

FIG. 1: INDEX OF FILTRATION (IF) OF NORMAL ERYTHROCYTES WHEN INCUBATED AT 37 ^{0}C IN ISOTONIC BUFFER (290 mOsm), IN HYPERTONIC BUFFER (450 mOsm) AT 1, 2, 4 mg/m1 PARACETAMOL. MEAN & SD FOR 12 EXPERIMENTS REPRESENTING 12 INDIVIDUALS. * P < 0.05 – COMPARED TO HYPERTONIC (450mOs) CONTROL ERYTHROCYTES

When the deformability of normal erythrocytes from 10 adults was compromised by placing the cells in isotonic buffer containing the calcuim ionophore (A23187), the mean IF rose from 12.3 ± 3.5 to 214.2 ± 54.4. This loss of deformability was

presumably due to the calcium loading into the erythrocytes with a consequential loss of K^+ and water from the cells according to gardoss phenomenon [6]. Pre-incubation of these compromised erythrocytes with paracetamol at 4 mg/ml for 60 min caused a significant improvement in defomability and thus the mean IF fell to 132.1 ± 39.8 (i.e. 38.3 % improvement) **(Fig. 2)**.

When the deformability of normal erythrocytes from 10 adults was compromised by placing the cells in isotonic buffer containing the potassium ionophore (valinomycin), the mean IF rose from 12.6 ± 3.5 to 274.7 ± 170). This loss of deformability was presumably due to the loss of cell K^+ and thus water from the cells caused by valinomycin with a consequential increase in erythrocyte MCHC. Pre-incubation of these compromised erythrocytes with paracetamol at 4 mg/ml for 60 min caused an improvement in deformability and the mean IF fell to 209.2 ± 125 (i.e. 23.8 % improvement), but this improvement was not statistically significant **(Fig. 2)**.

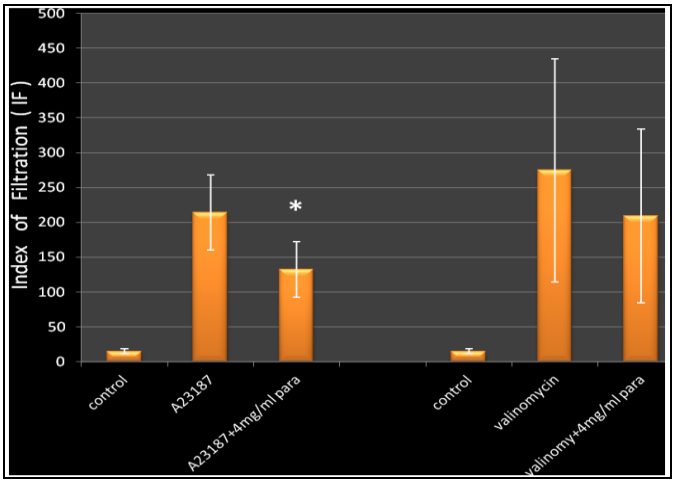

FIG. 2: INDEX OF FILTRATION (IF) OF NORMAL ERYTHROCYTES WHEN INCUBATED AT 37 ^{0}C IN BUFFER ALONE (CONTROL), IN BUFFER CONTAINING A23187 OR VALINOMYCIN AND IN BUFFER CONTAINING A23187 OR VALINOMYCIN WITH 4mg/ml PARACETAMOL. MEAN & SD FOR 10 EXPERIMENTS REPRESENTING 10 INDIVIDUALS. * P < 0.05 – COMPARED TO A23187 OR VALINOMYCIN ALONE.

When the osmotic fragility of normal erythrocytes from 10 adults was measured at various NaCl concentrations in the absence and presence of paracetamol at 4mg/ml, paracetamol caused a significant decrease in fragility **(Fig. 3)**.

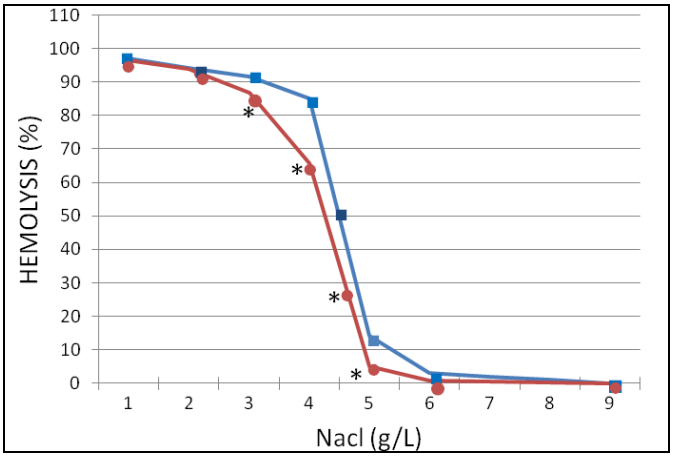

FIG. 3: OSMOTIC FRAGILITY CURVES (MEAN OF 10 EXPRIMENTS) FOR HEPARINISED ERYTHROCYTES INCUBATED FOR 30 min WITHOUT (■) AND WITH 4 mg/ml (●) PARACETAMOL. * P < 0.05 – FOR PARACETAMOL COMPARED TO CONTROL ERYTHROCYTES (■).

When the normal erythrocytes from 13 adults were incubated with 3% dextran 70 to induce erythrocyte aggregation, the mean erythrocyte sedimentation rate (ESR) rose from a mean of 6.0 ± 4.2 mm/hr before dextran to a mean of 95.8 ± 12.0 mm/hr with dextran. Pre-incubation of these erythrocytes with paracetamol at 4 mg/ml for 60 min caused a significant improvement in ESR, and the mean ESR fell to 20.7 ± 16.0 mm/hr (i.e. 78.4 % improvement).

Addition of 4 mg/ml paracetamol to a normal plasma from 10 adults had no effect on the plasma recalcification time (clotting time), the mean of which in the presence of paracetamol was 156.3 ± 25.3 seconds, compared with a mean of 149.2 ± 23.3 seconds in the absence of paracetamol.

When the antioxidant activity of paracetamol was studied by several chemical assays as shown in **Table 1**, total antioxidant capacity was 88.9 mg AAE/g compared to 184.9 for ginger as positive control, ferric reducing power measured as absorbance at 700 nm was 1.0 compared to 3.0 for Vit. C as positive control, DPPH scavenging activity measured as IC_{50} was 62.5µg/ml compared to 5.8 for Vit. C as positive control, $OH^{\cdot}$ scavenging activity measured as IC_{50} was 40.2 µg/ml compared to <10 for catecholamin as positive control and iron chelating capacity measured as IC_{50} was 144.6 µg/ml compared to 17.3 for EDTA as positive control.

TABLE 1: RESULTS OF ANTIOXIDANT ASSAYS OF PARACETAMOL. AAE DENOTES TO ASCORBIC ACID EQUIVALENT.

	Total Antioxidant Capacity (mg AAE/g)	Reducing power (A^0 at 700 nm)	Scavenging activity of DPPH (IC_{50}=µg/ml)	Scavenging activity of OH· (IC_{50}=µg/ml)	Iron Chelating (IC_{50}= µg/ml)
Paracetamol	88.9 ± 1.1	1.0 ± 0.004	62.5 ± 5.0	40.2 ± 1.8	144.6 ± 7.5
Ginger (positive control)	184.9 ± 20.0	1.4 ± 0.006	64.6 ± 1.0	< 10.0	212.3 ± 2.2
Vit. C (positive control)	-	3.0 ± 0.002	5.8 ± 0.6	-	-
Catecholamin (positive control)	-	-	-	< 10.0	-
EDTA (positive control)	-	-	-	-	17.3 ± 1.0

Incubation of erythrocytes with H_2O_2 for 60 min at 37°C caused a significant increase in intracellular MDA (*i.e.* an increase in lipid peroxidation) from 11.7 ± 5.9 nmol/g Hb without H_2O_2 to 80.6 ± 22.2 nmol/g Hb with H_2O_2 **(Fig. 4)**. When erythrocytes were pre-incubated with 1, 2, 4, and 5 mg/ml paracetamol, all concentrations caused a significant inhibition of MDA production that was concentration dependent **(Fig. 4)**.

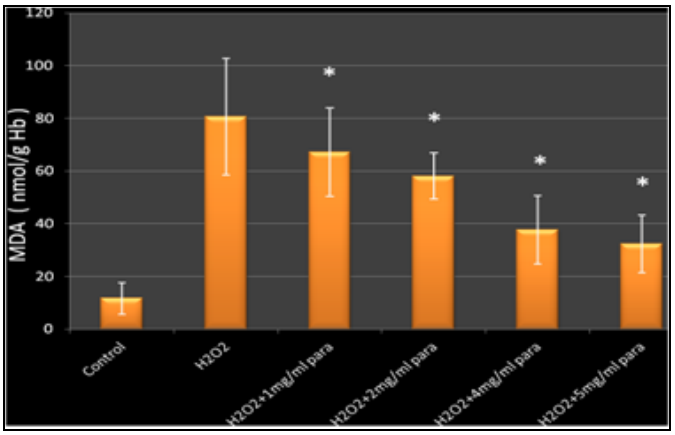

FIG. 4: MDA OF NORMAL ERYTHROCYTES WHEN INCUBATED AT 37 ^{0}C IN BUFFER ALONE (CONTROL), IN BUFFER CONTAINING H_2O_2, AND IN BUFFER CONTAINING H_2O_2 WITH 1, 2, 4, 5 mg/ml PARACETAMOL. MEAN & SD FOR 4 EXPERIMENTS REPRESENTING 4 INDIVIDUALS. * P < 0.05 – COMPARED TO H_2O_2 ALONE.

DISCUSSION: The flow of blood in the micro- and macro-circulation critically depends on the ability of erythrocytes to undergo deformation when subjected to a shear stress. This Deformability is the basic rheological property of the erythrocyte. The high deformability of the normal human erythrocyte is a consequence of its low cytoplasmic viscosity, its high ratio of membrane surface area to cell volume and its viscoelastic cell membrane [12]. Loss of erythrocyte deformability may arise from impairement of any one, or a combination of these properties. Dehydration of the erythrocyte with a consequential loss of deformability has a significant rheological consequences as loss of cellular water is known to cause an exponential increase in cytoplasmic viscosity. Since cellular water will follow osmotically any change in the intracellular concentration of ions, maintenance of the erythrocyte's K^+ concentration is also rheologically important [13]. As overhydration of the erythrocyte can decrease the ratio of membrane surface area to cell volume, hypotonic stress of erythrocytes measured as erythrocyte osmotic fragility is also considered rheologically important [13]. Erythrocyte aggregation measured as erythrocyte sedimentation rate (ESR) is another widely used rheological test that monitors whole blood viscosity [12].

Erythrocyte filterability measured in the present study as an index of filteration (IF) through 5 µm pore diameter polycarbonate membranes, is a popular method for measuring erythrocyte deformability [12]. This method was found to be more sensitive to changes in erythrocyte MCHC than to MCV [14], thus reflecting changes in erythrocyte water and cation content.

The results of the present study showed that paracetamol could not increase the deformability of normal erythrocytes unless this rheological property is compromised by either hyperosmolality, calcium ionophore (A23187) or potassium ionophore (valinomycin). Hyperosmolality induced a moderate degree while the ionophores induced an extreme degree of rheological stress on erythrocytes at 60 min of incubation **(Fig. 1 and 2)**.

These effects are consistent with similar effects of these ionophores observed previously [5, 6, 15].

The improvement by paracetamol of the deformability of erythrocytes stressed by hypersomolality could be explained by the ability of the drug to prevent the loss of water from the cells **(Fig. 1)**. This however could be due to the saturation (concentration) of the cell interior with the osmotically active drug with a concomittent entry of water improving the hydration state of the cell. This is consistent with the observations in the literature that showed binding of paracetamol to cellular glutathione, ferrous iron and probably other cellular proteins that may lead to cytotoxicity in overdose cases [1, 2, 3]. Binding to ferrous iron also shown in the present study **(Table 1)**.

The inhibition by paracetamol of the rheological stress induced by calcium ionophore (A23187) indicates that paracetamol may antagonize the action of this ionophore. This antagonism could be by preventing Ca^{2+} loading in to the erythrocyte and/or inhibiting calcium-induced K^+ loss from the cell [6]. Since there are no reports in the literature suggesting that paracetamol could inhibit Ca^{2+} influx in the human erythrocyte, and since the present study showed that paracetamol doesn't prevent plasma recalcification (clotting) indicating no Ca^{2+} binding to the drug, it seems that this antagonism could be due to the antioxidant activity of paracetamol that has been reported in human erythrocytes [16, 17, 18, 19], and also shown in the present study **(Table 1)**, with consequent anti-lipid-peroxidation activity that has been reported previously [16, 18], and also shown in the present study **(Fig. 4)**.

This is in consistence with observations, shown by others, that calcium loading into normal erythrocytes by A23187 induces lipid-peroxidation and protein-degradation with consequent cross-linking of membrane proteins that are similar to the effects induced by exposure of erythrocytes to oxidative stress [20], that leads to loss of deformability. Therefore, it seems likely that the loss of deformability by A23187 in the present study was due to the oxidative stress, and the deformability improvement by paracetamol was due to its antioxidant activity with consequent anti-lipid-peroxidation and anti-protein-oxidation, this is also in consistence with others who found that an effective antioxidant supplementation was able to improve the hemorheology (i.e. erythrocyte deformability, blood viscosity, whole blood filterability, erythrocyte membrane fluidity, erythrocyte malonyldialdehyde) in alcoholics that was suggested due to either prevention of the ethanol-related lipid-peroxidation and xanthine oxidase system activation and/or due to maintaining membrane integrity [21].

This mechanism however, could also be responsible for the improvement by paracetamol of the osmotic fragility of erythrocytes shown in **Fig 3**. Paracetamol in this case protects and stabilizes erythrocyte membrane integrity against hemolysis induced by hypotonic stress. This is in consistence with others who showed that paracetamol inhibits oxidant induced hemolysis of human erythrocytes [17].

Improvement by paracetamol of the deformability of erythrocytes stressed by valinomycin, although not reaching to a significant level, could be due to the ability of the drug to prevent the loss of intracellular K^+ and/or water **(Fig. 2)**. As the loss of intracellular K^+ and water in this case is a passive process, the saturation of the cell interior with the osmotically active paracetamol antagonising the action of valinomycin could be responsible for the prevention of cell dehydration caused by valinomycin. However, it is also likely that paracetamol may participate a negative charge to the cell (or membrane) inside, binding K^+ ions and preventing their loss, thus competing with valinomycin for K^+ ions [15]. As paracetamol was found by others [22, 23] to decrease the activity of Na^+/K^+ ATPase in erythrocytes in *in vivo* and *in vitro* studies, this effect could also be in play in the present experiment with valinomycin enhancing dehydration of the cell and thus opposing the improving effect of paracetamol on deformability and preventing it to reach a significant level.

Improvement by paracetamol of the ESR (from 95.8 to 21.7 mm/hr) of erythrocytes aggregated by dextran 70, could be due to the participation of a negative charge by the hydroxyl group of paracetamol at the cell membrane, thus increasing

the zeta potential of erythrocytes, or to the binding of paracetamol to dextran macrmolecules, thus competing with the negative binding sites on erythrocyte membrane for dextran molecules [15]. This significant anti-erythrocyte-aggregation activity of paracetamol, decreasing the viscosity and thus increasing the fluidity of the blood, may explain the reported increased haemorrhagic risk of paracetamol in patients treated with warfarin [24 - 27].

The observations in the present study on paracetamol are also in line with similar observations in the literature regarding some flavonoids (similar in structure to paracetamol in regard to phenolic cycle bearing hydroxyl group(s) having an antioxidant and anti-inflammatory activities that showed inhibition of the rheological stress induced by A23187 and valinomycin and also improvement of erythrocyte osmotic fragility and aggregation [15].

The absence of the effects of paracetamol on plasma recalcification time (clotting time) in the present study indicates that paracetamol does not bind or interact with calcium or the other proteins involved in the plasma clotting system.

The results of the present study were obtained using normal erythrocytes that were dehydrated *in vitro* using hypertonic buffer or ionophores and then filtered through 5 μm diameter pores, the degree of the rheological stress induced in these experiments was non-physiological, although the types of stress were relevant to clinical disorders [12, 13, 28]. Therefore care must be taken when extrapolating the present findings to clinical studies. Also, the effect of paracetamol was observed at relatively high concentrations and probably in the toxic range compared with the accepted therapeutic plasma concentrations of 10-20 μg/ml and the accepted toxic minimum concentration of 120 μg/ml [29].

CONCLUSION: The results of the present study suggest that paracetamol could have a hemorheologically useful role in blood, preserving erythrocyte deformability, and preventing erythrocyte dehydration, aggregation and lipid-peroxidation, that may contribute positively to its analgesic and antipyretic effects. Such action could also be useful in rehydrating erythrocytes in pathological conditions such as sickle cell disease [30].

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