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Morphological and Functional Alterations in Human Red Blood Cells Treated with Titanium Citrate

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ABSTRACT

The morphological and functional effects of titanium (*Ti*) *citrate on human erythrocytes were studied by scanning electron microscope* (*SEM*), *sulphate uptake via band* 3 *protein and by determining the reduced and oxidised glutathione* (*GSH and GSSG*, *respectively*) *concentrations. The rate constant for sulphate uptake was significantly lower after Ti citrate treatment. Ti citrate* (0.001 *and* 0.0025 *mM*) *significantly decreased erythrocyte GSH and increased GSSG concentrations. At* 0.005 *mM Ti citrate*, *the intracellular GSH could not be tested due to significant cellular damage. SEM of erythrocytes treated with* 0.001 *mM and* 0.0025 *mM Ti citrate showed structural membrane defects but almost normal cellular diameters. At even higher Ti citrate concentrations* (0.005 *mM*), *erythrocytes showed obvious morphological alteration and shape changes compromising the cells physiological functions. In conclusion*, *although the Ti concentrations used in our experiments are physiologically high*, *the cumulative effect of prolonged exposure to much lower doses of Ti*, *as might occur during total hip replacement*, *should be considered for further experimental testing.*

Keywords*: *Anion Transport*, *Band* 3 *Protein*, *Erythrocytes*, *Erythrocyte Membrane*, *GSH*, *Titanium Citrate

1. Introduction

Titanium (Ti) is largely present in nature in the form of oxides and it is contained in different minerals. It is the ninth element in order of diffusion and the fourth in order of abundance among the metals, preceded only by aluminum, iron and magnesium. Modern extraction technologies have allowed the use of Ti both in the aereonautic-spatial industry as well as some medical fields, particularly orthopedics, cardio-vascular surgery and dental surgery. Among the many qualities that make Ti useful for implantation, are its long-term hardness and biocompatibility. The success of using pure Ti in medicine is due to its unique physical and chemical characteristics, not readily found in other metals, such as its X-ray transparency that helps in diagnosing the state of prosthesis conservation.

Ti compounds are currently the subject of much biological research [1,2]. A recent study has investigated Ti compounds as anticancer agents because they are easily disseminated throughout the body [3]. One the few studies showed that osteoblast-like cells respond to titanium particles through increased expression of the proinflammatory cytokine interleukin-6 in a process requiring phagocytosis and intracellular signaling pathways. Other than this specific research, very limited data are available on the toxic effects of soluble Ti (IV) complexes in erythrocytes and in particular the effect on band 3 protein. Band 3 protein is the most abundant membrane protein in human erythrocytes, being present at approximately 1 million copies per cell [4], and it facilitates the electroneutral exchange of Cl^- and HCO_3^- across the membrane.

Three anion exchanger isoforms are known, AE1, AE2 and AE3, which differ in their tissue expression. AE1 is found in erythrocytes and in the kidney, AE2 is found in a wide variety of tissue and AE3 is found in the brain, the retina and the heart. All the anion exchanger family consist of two domains, an N-terminal cytoplasmic domain that contains binding sites for glycolytic enzymes and haemoglobin and a C-terminal

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membrane domain [5,6]. The membrane domain is highly conserved, it spans the lipid bilayer 12 - 14 times and mediates anion transport. The cytoplasmic domain is anchored to the cytoskeleton and plays a structural role by connecting the cytoskeleton to the membrane [7,8].

Band 3 protein is one of the main phosphorylated membrane proteins. It is phosphorylated mainly at three sites in the cytoplasmic domain, the most important being tyrosine-8, followed by tyrosine-21 and tyrosine-46 [9]. Band 3 protein phosphorylation is accompanied by rigidification of the membrane skeleton, suggesting the visco-elastic properties of human erythrocytes may be regulated by band 3 tyrosine phosphorylation [10].

The aim of our study was, therefore, to expose human erythrocytes to different concentrations of Ti citrate (IV) in order to study: 1) its effects on the mechanisms of anionic transport mediated by band 3 protein, 2) its oxidative effects by the determination of -SH groups and intracellular concentrations of GSH and GSSG and 3) its action on the erythrocyte membrane by scanning electron microscopy (SEM).

2. Materials and Methods

2.1. Erythrocyte Preparation

Human blood was obtained, after informed consent, from 30 healthy volunteers. The blood was collected in heaprinnized tubes, washed in 150 mM NaCl, 25 mM HEPES, pH 7.40 and centrifuged three times for 5 min at 3000 rpm. At each step the buffy coat was carefully removed and the cells were suspended at 3% hematocrit and incubated for 1 h at 25˚C in the following medium: 140 mM choline chloride, 10 mM NaCl, 25 mM HEPES, 10 mM glucose, pH 7.4, ± increasing concentrations of Ti citrate (0, 0.0005, 0.001, 0.0025 and 0.005 mM). After incubation, the suspensions were centrifuged and the erythrocytes were divided into three aliquots. These were used to study sulphate kinetics, to indirectly verify the oxidative effects exercised by Ti citrate on the -SH groups of the membrane proteins, for measuring the intracellular concentrations of GSH and GSSG and to observe the red blood cell shape by SEM.

Ti citrate (IV) was obtained by mixing Ti III chloride with sodium citrate in the ratio 1:1.2, at pH 3. The Ti citrate solution was used after verifying the absence of precipitates and after solutions become completely clear, to avoid interference with our experimental protocol [11-13].

2.2. Sulphate Transport Measurement

For kinetic studies, the erythrocytes were resuspended in 3% hematocrit and incubated at 25˚C in the following medium: 115 mM Na_2SO_4, 5 mM $Na_3(C_6H_5O_7)2H_2O$, 5 mM KCl, 25 mM HEPES, 5 mM glucose ± increasing concentrations of Ti citrate (0, 0.001, 0.0025 and 0.005 mM), pH 7.40. At specified intervals, 5 ml samples of the suspension were removed and added to a test tube containing 5 μM 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate (DIDS) stopping medium and incubated on ice. DIDS binds specifically and irreversibly to the band 3 protein and inhibits sulphate transport in human red blood cells. In the same experiments, DIDS was added to the red blood cell suspension to give a final concentration of 5 μM.

After withdrawal of the last sample, the erythrocytes were washed three times in a sulphate-free medium at 0˚C to remove extracellular sulphate. Cells were then lysed with distilled water and trichloroacetic acid. The membranes were removed by centrifugation and sulphate ions in the supernatant were precipitated by adding glycerol and distilled water (1:1), 4 mM NaCl and HCl (37%) (12:1), and 124 mM $BaCl_2$ dihydrate, to obtain a homogeneous barium sulphate precipitate. The intracellular sulphate concentration was measured by atomic absorption spectrophotometry at 425 nm. Using a standard curve, obtained by precipitating known sulphate concentrations, we converted the absorption to mM intracellular sulphate and calculated the rate constant in min-1 by a non-linear, least square, curve-fitting procedure applying the following equation:

$$C_{(t)} = C_\infty(1 - e^{-rt}) + C_0$$

where C_0, C_t and C_∞ represent the intracellular sulphate concentrations measured at times 0, t and ∞ [14,15], e indicates nepero number (2.7182818) and r is a constant.

2.3. GSH Measurement

The GSH concentration was measured in erythrocytes before and after treatment with 0.001 and 0.0025 mM Ti citrate using an immunodiagnostic assay [16], intended for the quantitative determination of glutathione in EDTA-blood. At 0.005 mM Ti citrate, the intracellular GSH was not possible to test because of damage to the erythrocyte membrane.

In this assay, the sample was treated with a dilute solution of Ti citrate and divided into two aliquots: 1) The reduced fraction was measured by adding 50 μl of the diluted sample, 100 μl reaction buffer and 100 μl derivatization solution. After incubation for 20 min at 60˚C, during which time GSH was converted to a fluorescent product, 100 μl of the precipitation solution were added to remove higher molecular weight substances. The samples were precipitated for 10 min at 2˚C - 8˚C and centrifuged for 10 min at 6000 rpm, then 200 μl of supernatant

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were added to 200 µl of the reaction buffer in autosampler vials. 2) The total glutathione was measured by adding 50 µl of the diluted sample, 20 µl of the reduction solution, 100 µl of the internal standard and 100 µl of the derivatization solution. The sample was then handled like the reduced fraction. After that, 20 µl of the supernatant were injected into the HPLC system. The separation by HPLC followed an isocratic method at 30˚C using a reversed-phase column in two runs.

The chromatograms were scanned by a fluorescence detector and concentrations were calculated by integration of the peak height by the external standard method for the reduced fraction and the internal standard method for the total glutathione fraction. The amount of oxidised glutathione was calculated by subtraction of:

$$\text{glutathione}_{\text{total}} - \text{glutathione}_{\text{reduced}}$$

2.4. Preparation of Erythrocyte Membrane, Isolation of Band 3 Protein and Determination of Sulphydryl Groups

Erythrocytes (with/without Ti treatment) were washed with an isotonic solution (150 mM NaCl, 25 mM HEPES, pH 7.40) and hemolysed by adding 20 volumes of cold hypotonic buffer (5 mM HEPES, pH 7.40). Membranes were obtained by centrifugation at 20,000 g for 30 min at 4˚C. The process was repeated with the same hypotonic buffer until the red blood cell membranes were almost free of hemoglobin [17]. One volume of red blood cell membrane was then incubated with nine volumes of 0.1 N NaOH for 30 min at 0˚C in the presence of 0.2 mM DTT (dithiothreitol) and 20 µg/ml PMSF (phenylmethylsulfonyl fluoride). After incubation, samples were centrifuged at 20,000 g for 30 min at 4˚C.

The pellet containing band 3 protein was washed three times with 5 mM HEPES, pH 7.40, and used for determination of sulphydryl groups. Membrane with/without Ti treatment and containing band 3 protein was incubated with 0.3 ml of 20% SDS (Sodium Dodecyl Sulphate) and 2.8 ml of 100 mM sodium phosphate, pH 8, for 25 min at 37˚C. The pellet suspension was further incubated with 0.1 ml of 10 mM DTNB (2-nitrobenzoic acid) in 100 mM sodium phoshate, pH 8, for 20 min at 37˚C. The sulphydryl group concentration was measured by atomic absorption spectrophotometry at 412 nm [18]. A standard curve was employed to calculate the concentration of thiol groups.

2.5. Scanning Electron Microscopy and Human Erythrocytes

The sample preparation technique for electron microscopy involved: fixation, dehydration, assembly, covering with gold and observation. To analyse the effect of Ti citrate on the morphology of human red blood cells, the samples were incubated in the presence or absence of increasing concentrations of Ti at 25˚C for 1h. Samples were then washed with physiological solution, 166 mM NaCl, and fixed overnight at 5˚C by adding one drop of each sample to plastic tubes containing 1 ml of 4% glutaraldehyde in Sorensen's phosphate buffer 0.1 M to pH 7.4. Samples were washed three times in the same buffer for 30 min and then exposed to increasing concentrations (30%, 50%, 70%, 90% and 95%) of ethanol for about 30 min each.

The process of dehydration was carried out with liquid carbon dioxide until the critical point was reached. Then samples were assembled on a particular type of glass using conductive silver paste and covered with a thin layer of gold (200 - 300 A) in appropriate sputtering. The samples were then ready for observation by SEM.

3. Statistical Analysis

In order to test the existence of homogenity between untreated (control) and Ti treated cells, we applied non-parametric permutation tests. This procedure is more conservative than homologue parametric tests for small samples [19].

4. Results

The experiments were carried out in order to highlight not only possible modifications of sulphate transport mediated by band 3 protein, but also variations in the levels of GSH, the shape of human red blood cell membranes and oxidation of -SH groups. We used low concentrations (0.0005 and 0.001 mM) and high concentrations (0.0025 and 0.005 mM) of Ti citrate. Sulphate influx in the control red blood cells and in cells treated with increasing concentrations of Ti citrate are reported in **Figure 1**.

The rate constant of the Ti citrate (0.001 mM) treated sample was $0.026 \pm 0.001 \times \text{min}^{-1}$ (**Table 1**), compared to $0.039 \pm 0.001 \times \text{min}^{-1}$ in the control sample, a reduction of 33% relative to the control. In **Figure 1**, the curve (■) represents the cells treated with an intermediate concentration (0.0025 mM) of Ti citrate. The last curve shows an increase in the time necessary to reach the saturation equilibrium of sulphate.

In **Figure 1**, the graph (▲) represents the sulphate influx in human red blood cells treated with 0.005 mM of Ti citrate. It shows a remarkable difference in the kinetic profile. In fact it can be seen that sulphate saturation equilibrium was reached suddenly, because band 3 protein lost its function of anion transporter due to damage by the titanium.

Figure 2 shows a progressive decrease in the percentage of sulphydryl groups in band 3 protein with in-

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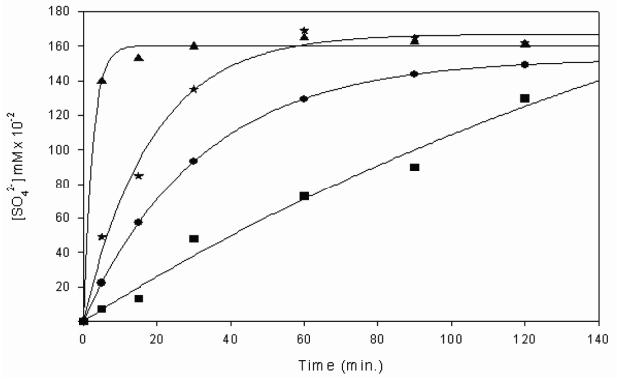

Figure 1. Sulphate influx in human erythrocytes measured in the absence of Ti citrate (★) or in the presence of 0.001 mM (●), 0.0025 mM (■) or 0.005 mM (▲) Ti citrate. On the abscissas, the time of collection of the cellular suspension is reported in minutes. On the ordinates, the corresponding concentrations of the intracellular ion sulphate are given, expressed in mM × 10^{-2}. The experimental conditions were 25˚C and pH 7.40. Data are reported with S.D. (standard deviation).

Table 1. Rate constants and percetual inhibition of sulphate uptake.

Erythrocytes	Rate constants × min^{-1}	% of inhibition
Control	0.039 ± 0.001	
+ 0.001 mM Ti citrate	0.026 ± 0.001	33.33
+ 0.0025 mM Ti citrate	0.016 ± 0.001	58.97
+ 0.005 mM Ti citrate	/	/

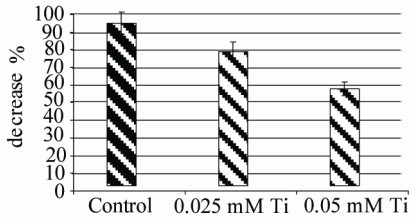

Figure 2. Reduction of sulphydryl groups of band 3 protein in erythrocytes treated and untreated with different Ti concentrations (0, 0.001 and 0.0025 mM). Values are given as means ± S.D. (standard deviation).

creaseing Ti concentration in treated erythrocytes. The decrease of -SH group content, observed in treated erythrocytes, might be the consequence of peroxidation and oxidation processes elicited by free radical activity.

As shown in **Table 2**, the GSH concentration in human red blood cells was reduced after treatment with Ti citrate (0.001 and 0.0025 mM), as compared to controls.

Table 2. Gsh and gssg concentrations in erythrocytes treated with Ti.

	Control	+ 0.001 mM Ti citrate	+ 0.0025 mM Ti citrate
GSH (μmol/l)	735.26 ± 145	697.29 ± 103	602.70 ± 128
GSSG (μmol/l)	270.66 ± 36	287.89 ± 32	292.49 ± 19
GSH/GSSG ratio	2.71 ± 1.2	2.42 ± 1.2	2.06 ± 1.1

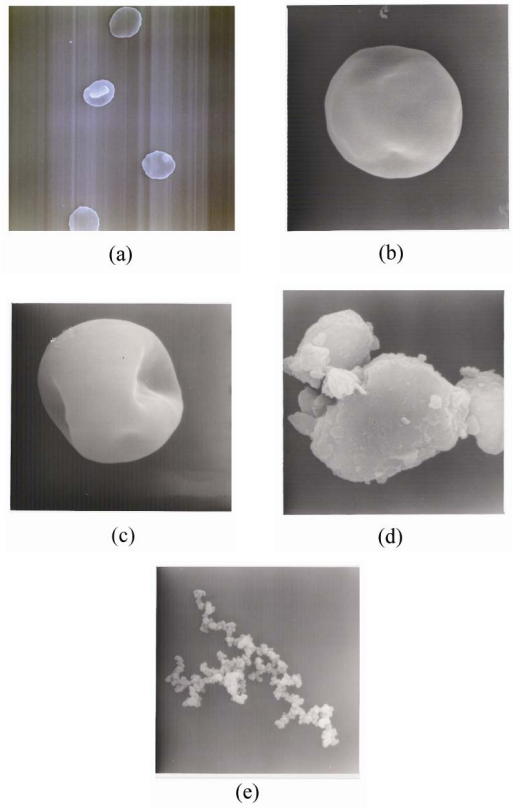

Figure 3. Effects of Ti citrate on the morphology of human erythrocytes. Human erythrocytes observed by SEM: (a) untreated erythrocytes, 1700x, (b) erythrocytes treated with 0.0005 mM of Ti citrate, 10000x, (c) erythrocytes treated with 0.001 mM of Ti citrate, 15000x, (d) erythrocytes treated with 0.0025 mM of Ti citrate, 10000x, (e) erythrocytes treated with 0.005 mM of Ti citrate, 10000x.

Intracellular GSH was not determined at 0.005 mM Ti citrate due to major alterations in the erythrocyte membrane

The oxidative effect highlighted by reduced concentrations of GSH, and indirectly the kinetic study of the sulphate ion exchange, in human red blood cells Ti citrate is corroborated by their morphology as seen by SEM. At Ti concentrations of 0.0005 mM and 0.001 mM SEM

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revealed small membrane alterations but an almost normal cellular diameter (**Figures 3(b)** and **(c)**), compared with the control (**Figure 3(a)**). Increasing concentrations of Ti citrate (0.0025 and 0.005 mM) (**Figures 3(d)** and **(e)**) showed increased morphological damage of the erythrocyte population.

5. Discussions

Band 3 protein mediates anion-exchange and acid-base equilibrium through the red blood cell membrane. It is an integral membrane protein crossing the bilayer lipid membrane many times. Because of its position, the protein is continuously damaged by chemical agents and drugs circulating in the blood flow. Previous studies reported that some metals enhance degradation of band 3 protein after treatment with chemical substances likely to affect the sulphate permeability and modulate anion influx through the erythrocyte membranes [20].

In this set of experiments, sulphate uptake measured in control erythrocytes increased steeply at the intial stage reaching equilibrium in 30 minutes. This process was much slower across the membrane of Ti citrate treated cells. The decrease in the rate constant of influx in treated cells compared to control cells was more prominent after treatment with 0.0025 mM of Ti citrate (**Figure 1**). **Table 1** indicates significant inhibition (33% and 58%) of rate constant sulphate influx across the red cell membrane after Ti citrate treatment. The structural integrity of band 3 protein has a close relationship with the functional aspects of this anion channel protein [15], and these results suggest a conformational change in the anion channel protein during Ti treatment making the transport sites more vulnerable to modification (**Figures 3(a-e)**).

Marked degradation of membranes and band 3 protein resulted from using 0.005 mM of Ti citrate. In this case it was not possible to measure the rate constant in treated cells. This probably correlated with the gradual reduction in the percentage of sulphydryl groups (**Figure 2**) due to oxidative damage in the membrane because of a significant reduction in GSH (735 μmol/l in control cells and 287 μmol/l in Ti treated cells, **Table 2**). The GSH reduction measured in Ti-treated erythrocytes may have altered the properties of hemoglobin and increased its tendency to aggregate, consequently modifying the hemoglobin-band 3 protein interaction [21]. In contrast, the binding of the chemical inhibitor DIDS at the anion binding site of band 3 protein is known to block the uptake of anion across the cell membrane. However, this does not deform the membrane. In fact, the GSH deficiency (**Table 2**) measured in Ti-treated erythrocytes altered the properties of haemoglobin and increased its tendency to aggregate, with consequent modification of the hemoglobin-band 3 protein interaction [21,22].

SEM of erythrocytes treated with increasing concentrations of Ti revealed significant morphological differences compared to untreated erythrocytes. In **Figure 3**, only a few of the erythrocytes exposed to lower concentrations of Ti citrate (0.0005 and 0.001 mM) seem slightly deformed in shape compared to control cells. At higher concentrations of Ti citrate (0.0025 mM), most of the erythrocytes showed morphological alterations. With 0.005 mM of Ti citrate the erythrocyte membranes were damaged and cells appeared smaller and more distorted. This is probably due to insertion of Ti citrate into the lipid bilayer of the erythrocyte membrane.

In conclusion, although the Ti concentrations used in these experiments were unphysiologically high compared to Ti circulating, for example, after total hip replacement, our findings warrant examination of the effect of long-term exposure of erythrocytes to Ti and also check whether the structural alterations induced by titanium citrate to human red blood cells can be extended to other cells affecting their functions.

6. References

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