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In vitro erythrocytic membrane effects of dibenzyl trisulfide, a secondary metabolite of *Petiveria alliacea*

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ABSTRACT

We investigated the in vitro effect of dibenzyl trisulfide (DTS), a secondary metabolite of *Petiveria alliacea*, on erythrocyte elasticity, relaxation time and membrane morphology. Blood samples from 8 volunteers with hemoglobin AA were exposed to 100, 200, 400, 800 and 1000 ng/ml of DTS respectively and the elasticity and relaxation time measured. There were statistically significant, dose-dependent increases in elasticity and relaxation times. The changes in membrane morphology observed also increased with increased concentration of DTS. This suggests that DTS interaction with membrane protein resulted in increased elasticity, relaxation time and deformation of the erythrocyte membrane.

1. Introduction

Dibenzyl trisulfide (DTS) is one of the polysulfide secondary metabolites isolated from the medicinal plant *Petiveria alliacea* [1]. It has been reported to have antimicrobial, acaricidal, insecticidal, immuno-modulatory, anti-proliferative/cytotoxic activities [2]. The seed methalonic extract caused an increase in the frequency and force of contraction of the rat uterus [3].

DTS has an IC_{50} value of 120 ng/ml on SH-SY5Y neuroblastoma cells and it induced cell–cell contact with crenated morphology of the erythrocytes without lysis at concentrations higher than those effective on cancer cells. This change in morphology is thought to be due to the interaction of DTS with ankyrins, the proteins that help maintain the integrity of the cytoskeleton of the erythrocyte [4]. One of the main determinants of the shape and integrity of the red blood cytoskeleton is membrane proteins [5] and changes in the interactions of the membrane proteins could affect the deformability of erythrocytes.

We therefore decided to investigate the in vitro effect of DTS on erythrocyte elasticity and relaxation time as well as changes in the morphology of erythrocytes with normal hemoglobin AA genotype.

2. Materials and methods

2.1. Subjects

Eight healthy subjects (4 males and 4 females) aged between 25 and 40 years with normal hemoglobin genotype HbAA were used for the study. The study was explained to the participants and their informed consent obtained before recruitment.

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2.2. Test compound

Dibenzyl trisulfide was obtained from Aldrich Chemical Co. (Germany) as a synthetic compound with 99% purity. 1 mg of DTS was dissolved in 100 ml of ethanol to form a stock solution with a concentration of 0.01 mg/ml of DTS. Aliquots of 10, 20, 40, 80 and 100 μl were then pipetted from the stock solution into eppendorf tubes to give concentrations of 100, 200, 400, 800 and 1000 ng/ml respectively.

2.3. Sample collection and preparation

8 ml of venous blood was drawn from the antecubital vein of each subject into vacutainer tubes containing K^+EDTA (1.5 mg/ml) anticoagulant and stored at room temperature (25 °C) until measurements were undertaken. The hematocrit of each sample was measured after which 1 ml of the blood was put into each of the 6 Eppendorf tubes. The first tube served as the control as no DTS was added. The second tube had 100 ng/ml of DTS added, third tube had 200 ng/ml, fourth tube had 400 ng/ml, fifth tube had 800 ng/ml and the sixth tube had 1000 ng/ml of DTS added. The mixture was allowed to incubate and mix properly for 20 min before measurements were made.

2.4. Tests

The hematocrit was measured with a Coulter Counter (COULTER® A^C T diff ™, Coulter Corporation, Miami, Florida). The value of the hematocrit was fed into the BioProfiler before the elasticity and relaxation times were measured.

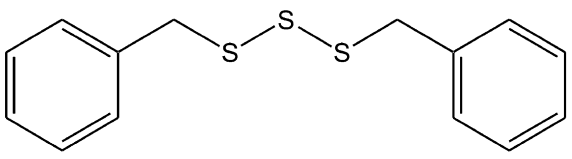
The elasticity and relaxation times were determined with BioProfiler (Vilastic Scientific, Inc.) It gives single measurements of viscosity, elasticity and relaxation time of whole blood at shear rates of 2.51 s^{-1}, 12.6 s^{-1} and 62.8 s^{-1}. Measurements were done at native hematocrits [6] and shear rate of 62.8 s^{-1} at a temperature of 37 °C. All tests were performed within 3 h of venepuncture.

Slides were prepared in triplicate from the blood samples with the different DTS concentrations and stained with Wright's method using a Hematek 2000 slide stainer. They were then examined under 100× objective oil immersion and the pictures taken.

2.5. Statistical analysis

Statistical analysis was performed using Stata Version 10.1.

Data were expressed as mean ± standard error of mean and analysis of variance (ANOVA) used to determine statistical significance which was taken at the 95% confidence interval with $p < 0.05$ considered statistically significant.



Structure of dibenzyl trisulfide

3. Results

The hematocrit value for the subjects ranged between 36% and 49%.

The mean and standard error values for elasticity and relaxation times are shown in Table 1. There were statistically significant dose-dependent increases in elasticity between the control and samples, except at 200 ng/ml, as well as for relaxation time between the control and samples from 200 ng/ml to 1000 ng/ml. Concentrations above 800 ng/ml did not produce any further changes in both elasticity and relaxation time. The data is presented graphically for elasticity in Fig. 1 and relaxation time in Fig. 2.

Fig. 3 shows the normal erythrocytes which served as the control (no DTS added). The erythrocytes show discoid shape and smooth membranes and, for the most part, are evenly separated due to the normal polarity of the cells.

Fig. 4 shows crenated erythrocytes when exposed to 100 ng/ml of DTS.

Fig. 5 shows increased number and severity of crenation as well as erythrocyte fragmentation on exposure to 200 ng/ml of DTS.

4. Discussion

The effect of increasing concentrations of DTS on erythrocyte morphology observed in the present study is in agreement with an earlier study [4] that reported sickling changes in erythrocyte morphology, thought to be due to the interaction of DTS with ankyrins, the membrane proteins that help maintain the integrity of the cytoskeleton of the erythrocyte.

We also observed crenated, fragmented and folded erythrocytes. These changes in erythrocyte membrane morphology were concentration dependent, increasing with the concentration of DTS.

The data presented also showed that there is a significant dose-dependent effect between the concentration of DTS and erythrocyte elasticity and relaxation. As expected, DTS interacted with the membrane cytoskeleton protein possibly ankyrin, resulting in some morphological changes that lead to an increased elasticity of the membrane. Diminished erythrocyte deformability leads to increased elasticity. The change in membrane elasticity was observed from a concentration of 100 ng/ml, a concentration range below the 120 ng/ml IC_{50} value for DTS on neuroblastoma cells. DTS has been reported to have anti-proliferative/cytotoxic activities [2], and work is

Table 1
Elasticity and relaxation time at different concentrations of dibenzyl trisulphide.

Concentration (ng/ml)	Elasticity (mPa s)	Relaxation time (s)
0 (control)	1.01 (0.10)	0.021 (0.002)
100	2.15 (0.84) *	0.028 (0.004)
200	1.91 (0.36)	0.031 (0.005) *
400	2.98 (0.49) **	0.040 (0.005) ***
800	3.97 (0.57) ***	0.049 (0.007) ***
1000	3.98 (0.69) ***	0.048 (0.008) ***

Standard error in brackets.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.