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Research Article

A Comparison of the Effect of Methyl-β-Cyclodextrin on the Osmotic Fragility of Ovine, Bovine and Human Erythrocytes

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Abstract: Cyclodextrins are used extensively in food, cosmetic, drug and chemical industries to increase aqueous solubility and stability, and reduce or eliminate the unpleasant taste and smell of many products. Experiments were conducted to examine the effect of a pre-haemolytic dose of methyl β-cyclodextrin (MBCD) on the osmotic fragility of erythrocytes collected from sheep, cattle and humans.

Heparinised blood collected from these three species were analysed within 3 h of collection. Erythrocyte suspensions were mixed with MBCD to give concentrations of 2 mM, 4 mM or 6 mM cyclodextrin. The mixtures were incubated for 30 min at 37 ˚C and centrifuged. The osmotic fragility of re-suspended erythrocytes was measured.

The incubation of MBCD with the erythrocyte suspensions induced a dose-dependent increase in the osmotic fragility of erythrocytes obtained from all three species. The beginning of haemolysis in control groups of sheep, cattle and humans occurred at 0.85%, 0.70% and 0.55% NaCl, respectively. However, haemolysis in the MBCD-treated groups began earlier than this in all the three species studied. The osmotic fragility of erythrocytes was significantly higher at NaCl concentrations of 0.70% ($P > 0.01$), 0.75% ($P > 0.001$) and 0.80% ($P > 0.001$) in sheep; 0.55% ($P > 0.01$), 0.60% ($P > 0.001$) and 0.65% ($P > 0.01$) in cattle; and 0.40% ($P > 0.01$), 0.45% ($P > 0.001$) and 0.50% ($P > 0.01$) in humans than in the controls.

In conclusion, these results indicate that a pre-haemolytic dose of MBCD may also induce membrane disruption which elicits removal of membrane components from erythrocytes.

Key Words: Cyclodextrin, erythrocyte, osmotic fragility, ovine, bovine, human

İnek, Koyun ve İnsan Eritrositi Ozmotik Fajilitesi Üzerine Metil-β-Siklodextrinin Karşılaştırmalı Etkisi

Özet: Siklodekstrinler gıda, kozmetik, ilaç ve kimya sanayiinde kullanım alanı bulan bazı etken maddelerin çözünürlüğünü ve stabilitesini artırmak, etken maddelerde bulunan bazı istenmeyen tat ve kokuları maskelemek için yaygın olarak kullanılmaktadır. Bu çalışmada pre-hemolitik dozda kullanılan metil-β-siklodekstrinin (MBCD) koyun, inek ve insan eritrositi ozmotik frajilitesi üzerine etkisi araştırıldı.

Bu üç türden elde edilen heparinli kan, alımı takiben üç saat içinde analiz edildi. Eritrositlerden 2, 4 veya 6 mM MBCD içerecek şekilde süspansiyonlar hazırlandı. Karışım 37 ˚C ısıda 30 dk inkübe edildikten sonra santrifüj edildi. Tekrar sulandırılan eritrositlerin ozmotik frajiliteleri ölçüldü

Her üç türde de eritrosit süspansiyonu ile inkübe edilen MBCD, eritrositlerin ozmotik frajilitesinde doza paralel olarak artan bir ozmotik frajiliteye yol açtı. Hemoliz başlangıcı koyun, inek ve insan kontrol gruplarında sırası ile %0,85, %0,70 ve %0,55 NaCl olarak bulundu. Bunun aksine MBCD uygulanan grupların hepsinde de hemoliz, bundan çok daha erken başladı. Eritrositlerin ozmotik frajilitesi NaCl solüsyonunun %0.70 ($P>0.01$), %0.75 ($P>0.001$) ve %0.80 ($P>0.001$) konsantrasyonlarında koyunda, %0.55 ($P>0.01$), %0.60 ($P>0.001$) ve %0.65 ($P>0.01$) konsantrasyonlarında inekte ve %0.40 ($P>0.01$), %0.45 ($P>0.001$) ve %0.50 ($P>0.01$) konsantrasyonlarında ise insanda kontrol gruplarınkinden daha fazla bulundu.
Sonuç olarak; pre-hemolitik dozda kullanılan siklodextrine bağlı olarak eritrosit frajilitesinde meydana gelen artış, bu makromolekülün düşük dozlarının dahi membran komponentlerini uzaklaştırarak, eritrosit membran dayanıklılığını azalttığına işaret etmektedir.

Anahtar Sözcükler: Siklodekstrin, eritrosit, ozmotik frajilite, koyun, inek, insan

Introduction

Cyclodextrins are cyclic oligosaccharides consisting of 6, 7, or 8 glucopyranose units, usually referred to as α-, β- and γ-cyclodextrins, respectively. They are used in food, cosmetic, drug and chemical industries to increase aqueous solubility and stability, and reduce or eliminate the unpleasant taste and smell of a range of products (1-4). Hydrophobic guest molecules such as cholesterol can be incorporated into the cavity of cyclodextrin by displacing water. The resulting complex is water-soluble, although the guest molecule can be released relatively easily (5). Cavity size is the major determinant as to which cyclodextrin is used in complexation. α-Cyclodextrins have small cavities which are not capable of accepting many molecules. γ-Cyclodextrins have much larger cavities that allow many molecules to be incorporated. However, the cavity diameter of β-cyclodextrins is well-suited for use with many compounds. Therefore, β-cyclodextrins are most commonly used as a complexing agent (6).

It is well known that β-cyclodextrin in particular has very poor aqueous solubility. Thus, natural cyclodextrins must be modified chemically for various applications. Among the chemically modified cyclodextrins, methylated cyclodextrins are commonly used as complexing agents (7). Therefore, methyl-β-cyclodextrin (MBCD) was chosen by us to study.

Previous studies have demonstrated that cyclodextrins are very efficient at stimulating the removal of cholesterol, phospholipids and proteins from a variety of cells in culture (6,8,9). It has been suggested that the extraction of proteins by cyclodextrin may really be an extrusion process in which proteins are shed from the membrane into the aqueous phase through an erosion of the lipid regions of the membrane (10). Thus, cyclodextrins are used as effective extracellular cholesterol acceptors to monitor the efflux of cholesterol through the plasma membrane of living cells (11-14)

The adverse effect of cyclodextrin on human erythrocytes has been thoroughly studied. Erythrocyte membranes are composed of proteins associated with a lipid bilayer matrix. Their fractions consist of 43% lipids, 49% proteins and 8% carbohydrates; 25% of total membrane lipids are cholesterol (15). Methylated β-cyclodextrins were found to induce haemolysis at relatively high concentrations (6). The haemolytic activity of cyclodextrin has already been attributed to an extraction of lipids from the erythrocyte membrane (10,16). However, pre-haemolytic concentrations of β-cyclodextrins were demonstrated to cause shape changes in human erythrocytes (10,17). Thus, the present work was designed to test the effect of pre-haemolytic concentrations of cyclodextrin on erythrocyte osmotic fragility.

Although the effects of cyclodextrin on the osmotic fragility of erythrocytes have not yet been investigated, considerable research has been done on osmotic fragility itself (18-20). These studies postulated that marked species variations exist in erythrocyte susceptibility to haemolysis in hypotonic saline. This susceptibility is related in part to red cell size, since increasing fragility correlates with decreasing cell volume (21). Therefore, three species were used to determine the effect of erythrocyte sizes on cyclodextrin-induced haemolysis. Normal red cells are biconcave discs having a mean diameter of approximately 4.5 μm, 5.8 μm and 8 μm in ovine, bovine and human blood, respectively (21,22).

The first objective of this study was to determine the effectiveness of a pre-haemolytic dose of MBCD on the osmotic fragility of ovine, bovine and human erythrocytes. The second objective was to examine the effect of erythrocyte size on MBCD-induced hemolysis. The third objective was to assess the effect of time on MBCD-induced erythrocyte osmotic fragility.

Materials and Methods

Preparation of erythrocyte suspensions

Methyl-β-cyclodextrin was purchased from Sigma. Blood samples from eight sheep, seven cattle and seven humans were mixed with heparin and analysed within 3 h of collection. The blood samples were centrifuged at 1500 x g for 10 min to separate the plasma and erythrocytes. The retrieved erythrocytes were washed three times with the isotonic phosphate buffered saline (PBS) (154 mM NaCl, 10 mM sodium phosphate, pH 7.4). The buffy coat was carefully removed with each wash. After final washing, the packed cells were re-suspended in PBS to give a haematocrit value of 33%. Finally, cell suspensions were mixed with MBCD to give 2 mM, 4 mM and 6 mM cyclodextrin concentrations. The mixtures were incubated for 30 min at 37 ˚C and centrifuged at 1500 x g for 10 min. The osmotic fragility assays of re-suspended erythrocytes were performed in

PBS containing increasing concentrations of NaCl. Bovine erythrocytes were also incubated with 6 mM MBCD for 1, 5, 10, 15, 20 or 30 min to assess the effect of time on the cyclodextrin-induced fragility of the erythrocytes. Hemolysis was measured at the NaCl concentration of 0.6%.

Osmotic fragility measurement

The osmotic fragility assays were performed as described by Parpart et al. (23) in phosphate buffer solutions (10 mM sodium phosphate, pH 7.4) containing various concentrations of NaCl. Optical density was read at 540 nm on a UV-1280 Shimadzu Spectrophotometer (Shimadzu Corporation, Australia). The percentage of haemolysis in each concentration of NaCl was calculated, assuming 100% haemolysis in distilled water. The results were expressed as % haemolysis.

Statistical analysis

Different treatments were assessed by analysis of variance (ANOVA) and Duncan's multiple range test. Significance was defined as $P < 0.05$. All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS). All results are reported as means ± S.E.M.

Results

The effects of MBCD on osmotic fragility, evaluated from the curves of haemolysis as a function of NaCl concentration, are shown in Figure 1. Incubation of cyclodextrin with the erythrocyte suspensions induced a dose-dependent increase in the osmotic fragility of erythrocytes obtained from sheep, cattle and humans. The beginning of haemolysis in control groups of sheep, cattle and humans occurred at 0.85%, 0.70% and 0.55% NaCl, respectively. However, haemolysis in the MBCD-treated groups started earlier than this in all the three species studied. The osmotic fragility of erythrocytes was significantly higher at NaCl concentrations of 0.70% ($P > 0.01$), 0.75% ($P > 0.001$) and 0.80% ($P > 0.001$) in sheep; 0.55% ($P > 0.01$), 0.60% ($P > 0.001$) and 0.65% ($P > 0.01$) in cattle; and 0.40% ($P > 0.01$), 0.45% ($P > 0.001$) and 0.50% ($P > 0.01$) in humans than in the controls.

The effects of time on the MBCD-induced fragility of bovine erythrocytes, evaluated from the haemolysis curve as a function of 0.6% NaCl concentration, are shown in Figure 2. The incubation of 6 mM MBCD with the erythrocyte suspensions was induced to very rapid haemolysis. Over 70% of the haemolysis occurred within 5 min, and maximum haemolysis occurred within 20 min.

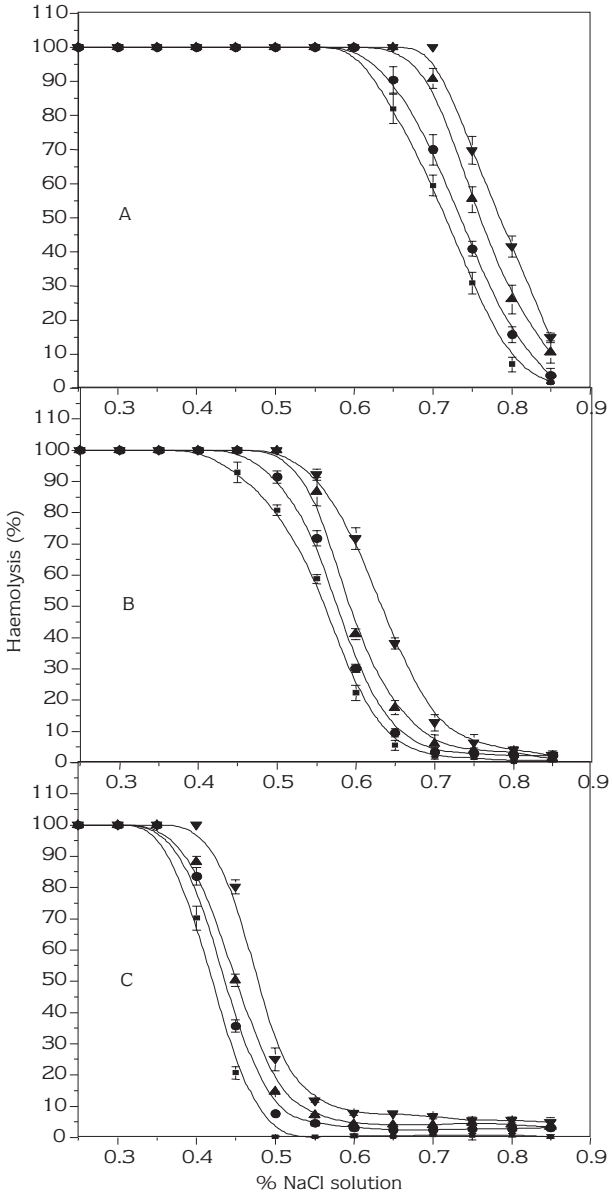

Figure 1. Effect of MBCD on the osmotic fragility of ovine (A), bovine (B) and human (C) erythrocytes. Erythrocytes were incubated without (■) or with 2 mM (●), 4 mM (▲) or 6 mM (▼) MBCD. Results were expressed as a percentage of total haemolysis.

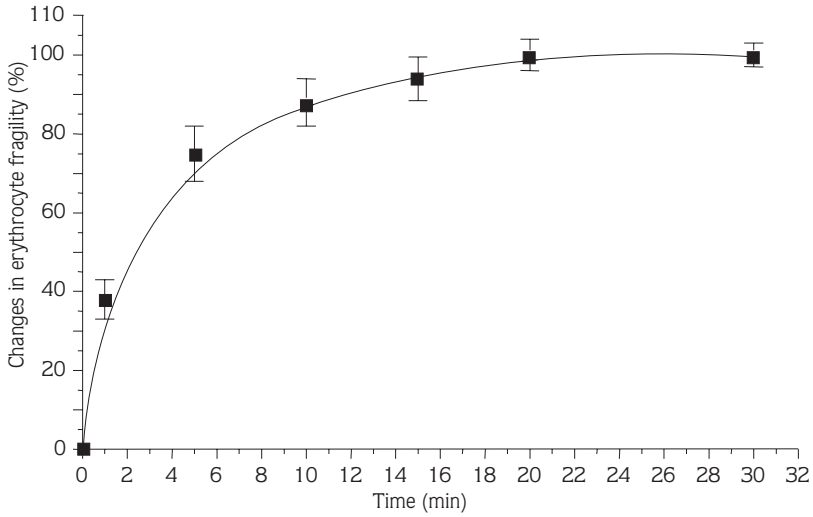

Figure 2. Effect of time on MBCD-induced fragility of bovine erythrocytes. Erythrocytes were incubated with 6 mM MBCD for 1, 5, 10, 15, 20 or 30 min. Haemolysis was measured at the NaCl concentration of 0.6%. Haemolysis prior to treatment and the maximum haemolysis after treatment were assumed to be 0% and 100% respectively. Results are the means ± SEM of four independent experiments.

Discussion

The present study has examined the hypothesis that higher concentrations of cyclodextrin will increase erythrocyte osmotic fragility. It is reported that high concentrations of cyclodextrins induce haemolysis in erythrocytes (16,24,25). Studies with isolated erythrocytes, which have no cell organelles, may provide a simple and reliable measure for cyclodextrin cytotoxicity because the interaction of cyclodextrins with plasma membranes must be the initial step for cell damage. Since marked species variation exists in the mechanical fragility of ovine, bovine and human erythrocytes in hypotonic saline (20,26) and the susceptibility is related in part to red cell size (21), the blood collected from different species was, therefore, used in the present study. Thus, the effect of pre-haemolytic doses of MBCD on the osmotic fragility of ovine, bovine and human erythrocytes was investigated.

The incubation of MBCD with the erythrocyte suspensions induced a dose-dependent increase in the osmotic fragility of erythrocytes obtained from all three species (Figure 1). This observation indicates that low concentrations of cyclodextrins also induced erythrocyte membrane damage. It was previously shown that pre-haemolytic concentrations of β-cyclodextrins caused shape changes in human erythrocytes (5,10,17).

The haemolytic activity of cyclodextrins correlates with their inclusion ability toward membrane lipids rather than their surface activity or intrinsic solubility. This hypothesis is supported by the fact that there is a positive correlation between the haemolytic activity of several cyclodextrins and their capacity to remove cholesterol from different cell membranes (5,6,25,27). It is, therefore, possible that the extraction of membrane compounds from the erythrocyte by cyclodextrin may result in an increase in membrane fluidity, which would induce membrane invagination through a loss of bending resistance and consequently lead to the lysis of erythrocytes.

The haemolysis induced by MBCD on erythrocytes suspended in 0.6% NaCl was studied as a function of incubation time in erythrocyte suspension obtained from bovine samples. As shown in Figure 2, the incubation of red cells with 6 mM MBCD at 37 ºC results in time-dependent haemolysis. More than 70% of haemolysis occurred in the first 5 min of incubation. This observation indicates that cyclodextrin-induced damage to erythrocyte membranes is a very rapid process. This observation is supported by the fact that when labelled human erythrocytes were exposed to a solution of β-cyclodextrins, part of the (^{3}H) cholesterol was extracted from erythrocytes into the supernatant within a few minutes (10). They also reported that the concentrations of cholesterol extracted from erythrocytes into the aqueous phase increased with progressively higher concentrations of β-cyclodextrins.

In conclusion, the results from the present study indicate that a pre-haemolytic dose of MBCD also induced the membrane disruption which elicited removal of membrane components from erythrocytes.

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