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Evaluation of the Pre-Hemolytic Concentrations of β-, Methyl-β- and Dimethyl-β-Cyclodextrin on Dog and Goat Erythrocytes

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Abstract: β-Cyclodextrin (β-CD) is a cage-like molecule consisting of seven glucose units and have appropriate size to form inclusion complexes with many hormones, vitamins and drugs. Due to having very poor aqueous solubility, β-cyclodextrin is modified chemically for various applications. Experiments were conducted to examine effects of pre-hemolytic concentration of β-cyclodextrin and its modified derivatives, methyl-β-cyclodextrin (Mβ-CD) and dimethyl-β-cyclodextrin (DMβ-CD), on the osmotic fragility of dog and goat erythrocytes. Blood samples collected from healthy 10 dogs and 10 goats were analyzed within 5 h of collection. Erythrocyte suspensions were mixed with various concentrations of β-CD, Mβ-CD and DMβ-CD. The mixtures were then incubated for 30 min at 37°C and osmotic fragility of erythrocytes was measured. Incubation of all 3 Cyclo Dextrins (CDs) with erythrocyte suspensions of both species, dog and goat, induced a dose dependent increase in the erythrocyte osmotic fragility ($p<0.01$). The hemolytic effect was decreased in order DMβ-CD>Mβ-CD>β-CD. Hemolytic doses of these CDs were lower for goat erythrocytes than those of the dog erythrocytes. When β-CD and its methylated derivatives are even used in pre-hemolytic concentrations, they might also reduce life span of erythrocytes. Due to importance of scientific knowledge on the cellular activity of cyclodextrins and growing number of its potential applications on diagnosis food preparations, our results may help to deal with the hemolytic activity of CDs for *in vivo* studies.

Key words: β-Cyclodextrin, dog, goat, methyl-β-Cyclodextrin, osmotic fragility

INTRODUCTION

Cyclodextrins are cyclic oligosaccarides composed of 6, 7, or 8 glucopyranose units called as α-, β- and γ-CDs, respectively. Each cyclodextrin molecule has a hydrophobic cavity capable of forming a complex with a guest hydrophobic compound, which incorporate into the cavity of cyclodextrin by displacing water (Arikan and Rodway, 2000; Del Vale, 2004). When a hydrophobic guest molecule is encapsulated into the hydrophobic cavity of a cyclodextrin molecule, apparent solubility of the guest molecule in aqueous solution is increased (Loftsson and Duchene, 2007). Therefore, CDs are used in cosmetic (Buschmann and Schollmeyer, 2002), food (Yoshi, 2004) and drug (Brun *et al*., 2006, Park and Jang, 2007) industries to increase aqueous solubility and stability and eliminate unpleasant taste and smell of a range of products.

In particular, β-CD is most commonly used as a complexing agent due to its virtue of cavity size, well-suited for many quest compounds and low cost (Frömming and Szejtli, 1994; Chen *et al*., 2007; Karathanos *et al*., 2007). It can easily form inclusion complexes with many kinds of compounds. However, β-CD has very poor aqueous solubility in comparison to the α- and γ-CDs (Fromming and Szejtli, 1994). Thus, parent β-CD must be modified chemically for various applications. Among the chemically modified β-CD, methylated β-cyclodextrins are commonly used as complexing agents (Loftsson *et al*., 2005). Thus, β-CD, Mβ-CD and DM β-CDs were chosen for the comparative study in present project.

There have been numerous studies published dealing with the hemolytic effect of various CDs in humans (Irie *et al*., 1982; Ohtani *et al*., 1989; Macarak *et al*., 1991; Leroy-Lechat *et al*., 1994; Panini *et al*., 1996, Bost *et al*., 1997; Hirayama *et al*., 1999) and animals (Shiotani *et al*., 1995; Arikan, 2003; Arıkan *et al*., 2004; Motoyama *et al*., 2006). Hemolytic effects of CDs have already been attributed to the removal of the erythrocyte membrane components, particularly cholesterol (Fauvelle *et al*.,

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1997). Previous studies have also demonstrated that hemolytic effects of β-CD are of the same order as their capability to solubilize cholesterol. Since, cholesterol is a rigidifier of lipid bilayers (Yeagle, 1985), extraction of cholesterol will influence stability of the membrane and eventually result in membrane damage. This corroborated by the fact that pre-hemolytic concentration of β-CD caused shape changes in erythrocytes (Irie *et al*., 1982; Ohtani *et al*., 1989; Shiotani *et al*., 1995). It was reported that the order of hemolytic effects of β-CD and its methylated derivates were as followed; DM β-CD> trimethyl-β-CD>β-CD (Uekama and Irie, 1987) and DMβ-CD>β-CD (Macarak *et al*., 1991). However, there has been no published study indicating the comparative effects of pre-hemolytic concentrations of β-, Mβ- and DMβ-CDs on human or animal erythrocytes.

It is well known that there have been quite variation between animal species in terms of erythrocyte size in diameter (Meinkoth and Clinkenbeard, 2000; Kramer, 2000). It is also a fact that marked species variations exist in erythrocyte susceptibility to hemolysis in hypotonic saline. The susceptibility relates in part to the size of red cells (Fairley *et al*., 1988), since increasing fragility correlates with decreasing cell volume (Schalm *et al*., 1975). Healthy red cells have a mean diameters of 3.2 and 7 μm in goats and dogs, respectively (Meinkoth and Clinkenbeard, 2000; Kramer, 2000). Therefore, blood samples collected from goats and dogs are used as material due to distend variation between erythrocyte sizes of two species.

Although, adverse effects of relatively high concentrations of CDs on erythrocyte have been studied, effects of pre-hemolytic cyclodextrin concentrations have not been fully understood. Hemolytic effects of Mβ- and DM β-CDs have been poorly studied. There has also been limited numbers of study on animals. Thus, hemolytic effects of CDs on domestic animals, especially on goats and dogs, have not been understood. As knowledge of the hemolytic activity of CDs is important in view of the growing number of their potential applications in drug and food industry, present study aimed to compare effects of pre-hemolytic concentrations of β-CD and its methylated derivatives on dog and goat erythrocytes.

MATERIALS AND METHODS

All three cyclodextrins (β-CD, Mβ-CD and DM β-CD) were obtained from Sigma (Sigma Aldrich Co.). Blood samples collected from 10 dogs and 10 goats were used in the experiments. One sample was collected per day and analyzed within 5 h of collection. Anticoagulated (EDTA) blood samples were centrifuged at 1000 × g for 10 min. Erythrocytes were then washed twice with Phosphate Buffer Saline (PBS). After final washing, packed cells were re-suspended in PBS to give a hematocrit of 10%. Finally, erythrocyte suspensions were mixed with β-CD, M β-CD and DM-β-CD, dissolved in PBS to give various concentrations. Optimum concentration for each type of CDs to induce osmotic fragility was also determined by pre-experiments. The mixtures were then incubated in a shaking water bath for 30 min at 37°C, after which osmotic fragility measurement were performed.

Erythrocyte osmotic fragility was determined in PBS containing decreasing concentrations of NaCl as described by Simmons (1980). Hemolytic activity of erythrocytes suspended in PBS was assessed as described by Shiotani *et al*. (1995). Optic density was read at 540 nm on a spectrophotometer. Percentage of hemolysis in each concentration of NaCl was calculated assuming 100% hemolysis in distilled water. The results were expressed as % hemolysis.

Data, obtained from all experiments, were evaluated using one-way ANOVA procedure of Statistical Package for the Social Sciences (SPSS). Means were compared by Duncan's multiple comparisons range test ($p<0.05$). All results were reported as means ± SEM.

RESULTS

Figure 1-3 represents dose dependent effects of β-CD, M β-CD and DMβ-CD on the osmotic fragility of dog erythrocytes. Osmotic fragility of goat erythrocytes incubated with the same CDs is also shown in Fig. 4-6. Incubation of all three cyclodextrins with erythrocyte suspensions of both species induced a dose dependent increase in the erythrocyte osmotic fragility. The hemolytic effects of β-CD and its metil derivatives on dog and goat erythrocytes in PBS are also presented in Fig. 7 and 8, respectively.

Effects of β-CD, Mβ-CD and DMβ-CD on the osmotic fragility of dog erythrocytes: Incubation of 5, 6, 7 and 8 mM β-CD with dog red cell suspensions was induced a dose dependent increase ($p<0.01$) in the osmotic fragility at NaCl concentrations of 0.55 and 0.60% (Fig. 1). When Mβ-CD was used at 2, 4, 6 and 8 mM concentrations, the fragility of erythrocytes was significantly increased ($p<0.01$) by increasing cyclodextrin doses at NaCl concentrations of 0.55 % (Fig. 2). However, when DMβ-CD was used as low as 2, 2.5 and 3 mM concentrations, significant hemolysis ($p<0.001$) was observed at NaCl concentrations of 0.50 and 0.55% (Fig. 3). Initial and complete hemolysis in control groups occurred at 0.50 and 0.35% of NaCl, respectively.

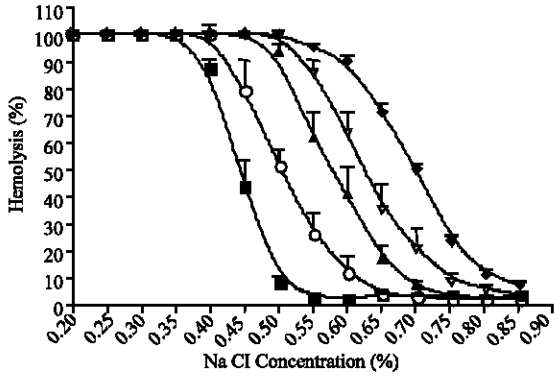

Fig. 1: Effect of β-cyclodextrin on the osmotic fragility of dog erythrocytes. Control (■), 5 mM (○), 6 mM (▲), 7 mM (▽) and 8 mM (◆) β-cyclodextrin. Data were expressed as % hemolysis. Results are the means (±SEM) of 10 independent experiments

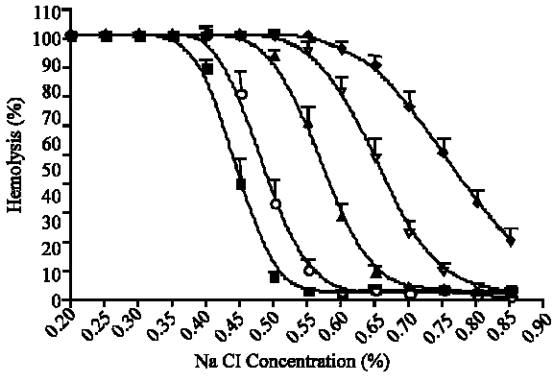

Fig. 2: Effect of methyl-β-cyclodextrin on the osmotic fragility of dog erythrocytes. Control (■), 2 mM (○), 4 mM (▲), 6 mM (▽) and 8 mM (◆) methyl-β-cyclodextrin. Data were expressed as % hemolysis. Results are the means (± SEM) of 10 independent experiments

Effects of β-CD, Mβ-CD and DMβ-CD on the osmotic fragility of goat erythrocytes: Incubation of 1, 2, 3 and 4 mM β-CD with goat erythrocyte suspensions induced dose dependent increase ($p<0.05$) in the osmotic fragility at NaCl concentrations of 0.70 and 0.75% (Fig 4). When erythrocyte suspensions were incubated with 1, 2 and 3 mM Mβ-CD significant increase ($p<0.01$) in erythrocyte osmotic fragility was determined at NaCl concentrations of 0.75, 0.80 and 0.85% (Fig 5). Finally, incubation of Dmβ-CD (1, 2 and 2.5 mM) was also induced a significant increase ($p<0.01$) in the red cell osmotic fragility at NaCl concentrations of 0.80% (Fig 6). Initial and complete hemolysis in control groups occurred at 0.80 and 0.50% of NaCl, respectively.

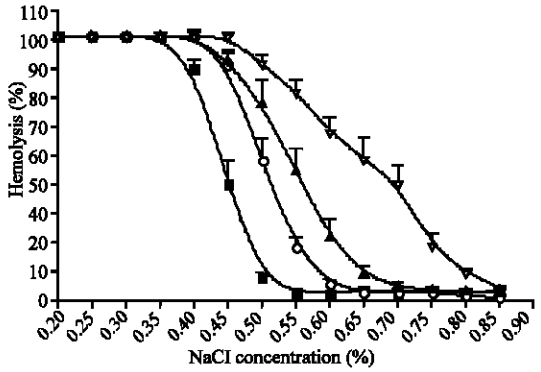

Fig. 3: Effect of dimeethyl-β-cyclodextrin on the osmotic fragility of dog erythrocytes. Control (■), 2 mM (○), 2.5 mM (▲) and 3 mM (▽) dimethyl-β-cyclodextrin. Data were expressed as % hemolysis. Results are the means (±SEM) of 10 independent experiments

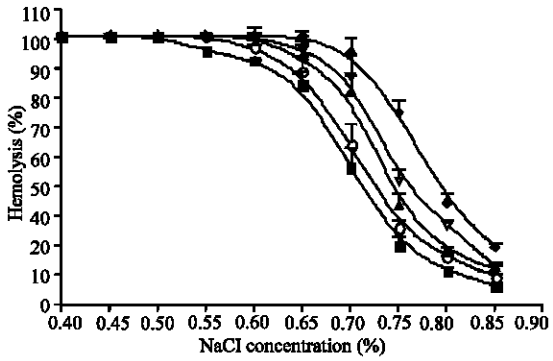

Fig. 4: Effect of β-cyclodextrin on the osmotic fragility of goat erythrocytes. Control (), 1 mM (■), 2 mM (○), 3 mM (▲) and 4 mM (▽) β-cyclodextrin. Data were expressed as % hemolysis. Results are the means (± SEM) of 10 independent experiments

Hemolytic activity of β-CD, Mβ-CD and Dmβ-CD in PBS: In addition to the erythrocyte osmotic fragility, hemolytic activity of β-CD and its methylated derivatives was also determined for both dog and goat erythrocytes.

When dose dependent hemolytic activities of the β-CD, Mβ-CD and DMβ-CD were compared, DM β-CD was more effective than β-CD and Mβ-CD (Fig. 7-8).

In dogs, efficient increase ($p<0.01$) in hemolytic activity was, in particular, observed at 4, 5, 6 and 7 mM CD concentrations (Fig. 7). Initial hemolysis in β-CD, M β-CD and Dmβ-CD treated groups was at 6, 4 and 2 mM, respectively. However, complete hemolysis was determined at 14, 14 and 6 mM for β-CD, Mβ-CD and DM-β-CD, respectively.

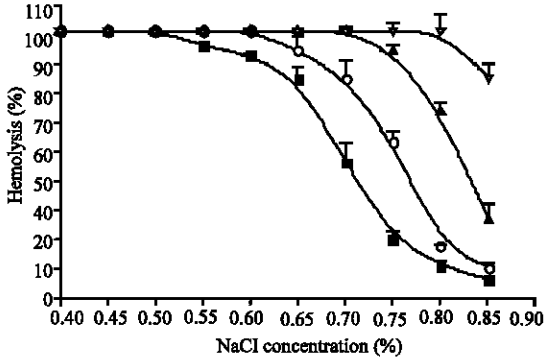

Fig. 5: Effect of methyl-β-cyclodextrin on the osmotic fragility of goat erythrocytes. Control (■), 1 mM (○), 2 mM (▲) and 3 mM (▽) methyl- β-cyclodextrin. Data were expressed as % hemolysis. Results are the means (± SEM) of 10 independent experiments

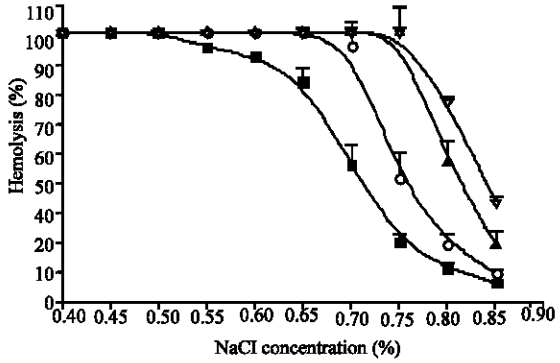

Fig. 6: Effect of dimethyl-β-cyclodextrin on the osmotic fragility of goat erythrocytes. Control (■), 1 mM (○), 2 mM (▲)) and 2.5 mM (▽) dimethyl-β-cyclodextrin. Data were expressed as % hemolysis. Results are the means (± SEM) of 10 independent experiments

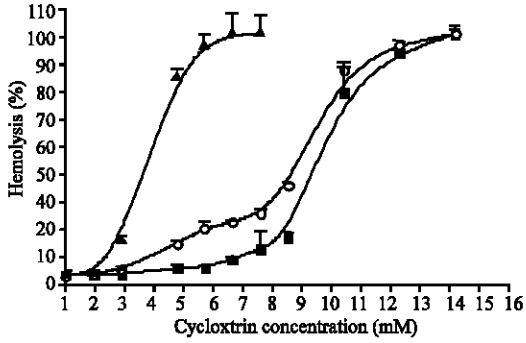

Fig. 7: Hemolytic activity of β-, methyl- and dimethyl-β-cyclodextrin on dog erythrocytes in PBS. β-cyclodextrin (■), methyl-β-cyclodextrin (○) and dimethyl-β-cyclodextrin (▲). Data were expressed as % hemolysis. Results are the means (± SEM) of 10 independent experiments

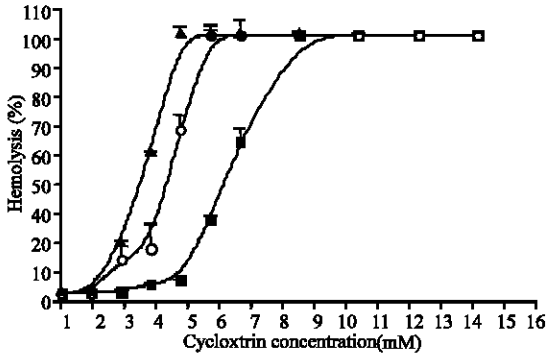

Fig. 8: Hemolytic activity of β-, methyl- and dimethyl-β-cyclodextrin on goat erythrocytes in PBS. β-cyclodextrin (■), methyl-β-cyclodextrin (○) and dimethyl-β-cyclodextrin (▲). Data were expressed as % hemolysis. Results are the means (± SEM) of 10 independent experiments

In goats, initial hemolysis in β-CD, Mβ-CD and DM β-CD treated groups was at 5, 2 and 2 mM concentrations, respectively. However, complete hemolysis was observed at 10, 6 and 4 mM concentrations for β-CD, Mβ-CD and β-CD, respectively. This study demonstrated that hemolytic effects of β-CD, Mβ-CD and DM β-CD decrease in order DM β-CD> M β-CD> β-CD on dog and goat erythrocytes.

DISCUSSION

Previous work in this laboratory (Arikan *et al*., 2004) has demonstrated that exposure of erythrocytes to pre-hemolytic concentration of α, β and γ CDs, induce a dose dependent increase in the osmotic fragility of dog erythrocytes. Another study carried out in our laboratory (Arikan, 2003) indicates that pre-hemolytic doses of M β-CD may also induce damage in ovine, bovine and human erythrocytes. Present study has, therefore, aimed to compare effects of pre-hemolytic concentrations of β-cyclodextrin and its methylated derivatives on dog and goat erythrocytes.

Incubation of dog and goat erythrocytes with pre-hemolytic concentrations of all three cyclodextrins were induced dose dependent increase in the erythrocyte osmotic fragility (Fig. 1-6), which is in agreement with our previous studies carried out using different animals or CDs (Arikan, 2003; Arikan *et al*., 2004). This observation indicates that low concentrations of β-cyclodextrin and its methyl derivatives may also induce erythrocyte membrane damage in dogs and goats.

Erythrocytes have limited life-span due to lack of cell organelles in and any factor affecting erythrocyte

membranes may induce life-span shortening in erythrocytes (Kurata *et al*., 1993). Therefore, when β-CD and its methyl derivatives are even used in pre-hemolytic concentrations, they might also reduce life span of erythrocytes. The effects of chemically modified CDs on the biological membranes must be different from those of the parent CDs (Shiotani *et al*., 1995; Bost *et al*., 1997). Also, in this study, both methylated derivates of β-CD were induced an increase in hemolytic activity in comparison with its parent CD (Fig 7,8) and this phenomenon was particularly marked for the DM β-CD, which have higher water solubility than parent β-CD. The interactions of methylated CDs with biomembranes were studied using human erytrocytes by several researchers. These studies demonstrated that hemolytic effects decrease in the following order; DMβ-CD>TMβ-CD>β-CD (Uekama and Irie, 1987) and DMβ-CD>β-CD (Macarak *et al*., 1991). Present study is in agreement with previous reports and also determines where M β-CD takes part in these methylated derivates. At relatively high concentrations of β-CD and methylated β-CDs were found to induce shape changes of membrane internarization in erytrocytes, which subsequently hemolysis. β-CD and its derivates removed phospholipids, cholesterol and proteins from the cell surface, depending on their inclusion abilities. This order indicates that β-CD and its methylated derivates induced hemolysis are via membrane disruption, which is elicited by the removal of membrane components.

When dog and goat erythrocytes treated with various concentrations of β, M β, DM β-CD, goat erytrocytes were more susceptible than dog erythrocytes. Hemolytic concentration of CD doses were lower in goats than those of dog erythrocytes (Fig. 7 and 8). Diameter of dog erythrocytes are very close to human erythrocytes. Goat erythrocytes have the smallest diameter of mammalian species. Marked species variations exist in erytrocyte susceptibility to hemolysis in hypotonic saline (Schalm, 1975). Due to size are considered to be major factors influencing osmotic fragility (Fairley *et al*., 1988) and increasing fragility correlates with decreasing red cell volume, goat erythrocytes were more suscebtible than dog erythrocytes which exposed to β, M β and DM β-CD.

CONCLUSION

In conclusion, the results from the present study indicate that pre-hemolytic concentrations of β-, M β- and DM β-CDs induce cell membrane disruption that may cause dose dependent removal of membrane components from the both dogs and goats erythrocytes. When β-CD and its methyl derivatives are even used in pre-hemolytic concentrations, they might also reduce life span of erythrocytes. Although, all CD used in the present study were effective, DM β-CD was the most effective one on the erythrocytes. The hemolytic effect was decreased in order DM β-CD>M β-CD>β-CD. Hemolytic doses of these CDs were lower for goat erythrocytes than those of the dog erythrocytes. Due to importance of scientific knowledge on the hemolytic activity of CDs and growing number of its potential applications on diagnosis food preparations, our results may help to deal with the hemolytic activity of CDs for *in vivo* studies.

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