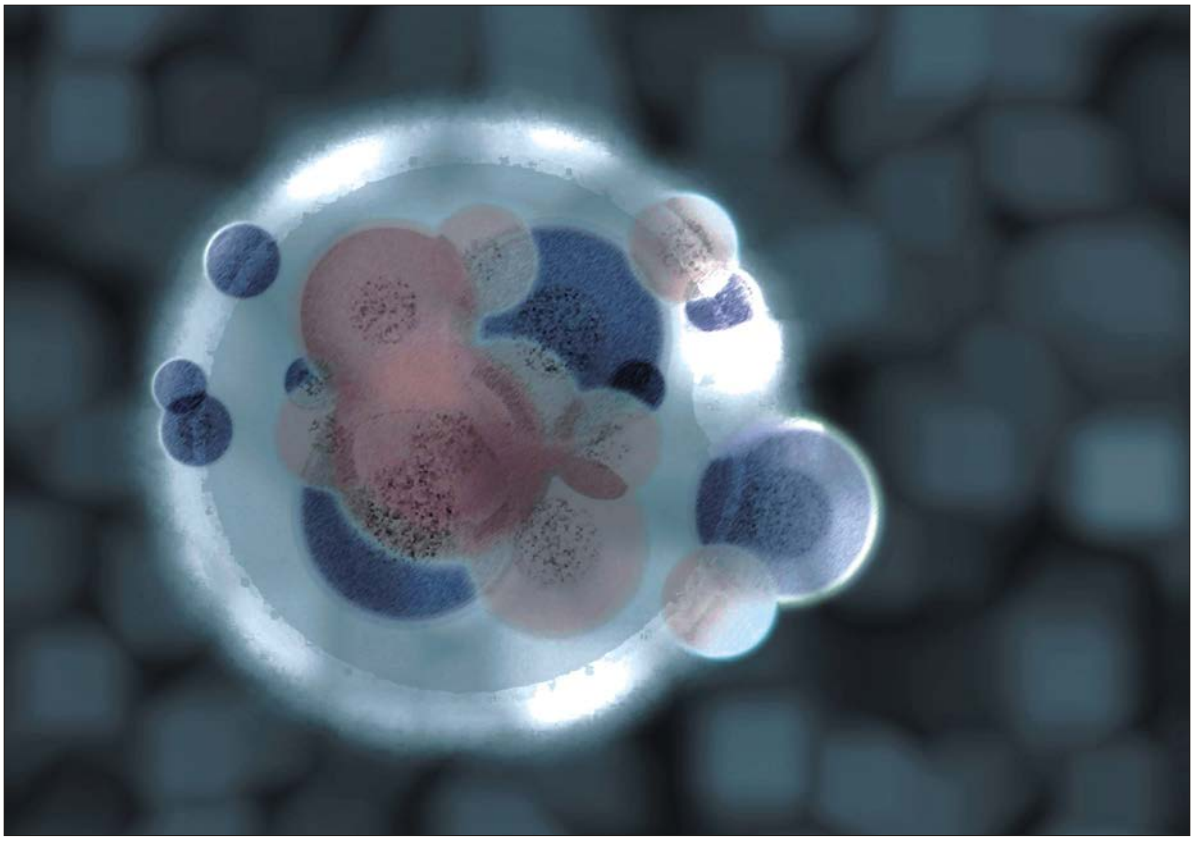

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Determination of the Toxicity Effect of Sorbitan Esters Surfactants Group on Biological Membrane

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Abstract: The aim of this study is to investigate the effect of one group of surfactants including sorbitan esters (span 20, 40, 60 and 80) on biologic membrane by using human red blood cells as a model. In this study, the hemolytic effect of four surfactants from span category were evaluated. Surfactants solution were prepared in Mclvane's buffer in specific concentration. Red blood cells were mixed with surfactants solution incubated in three different time and three temperature. The UV absorbances of the samples were determined by UV spectrophotometer. Each test was done in 9 times. Emulsification index, foam producing activity, also determined for each surfactant solution. The results showed that these agents had hemolytic effects. Span 20 caused more hemolysis and membrane demolishing effect than the others. Their hemolysis effects were enhanced by increasing temperature and time of incubation. According to hemolysis results, foam activity test and E_{24}, the interaction between span 80 and biological membrane is the least and it means has least toxic effect on cells. Anyway because these agents are used as excipients in pharmaceutical preparations, their membrane toxic effect should be considered.

Key words: Non ionic surfactants, span, hemolysis, cell membrane, emulsification index

INTRODUCTION

Surfactants have many characteristics comprising groups with hydrophilic and hydrophobic characters with different usages in pharmaceutical formulations, such as co-solvent, humectant, emulsifying, solubilizing agent and enhancer. Regarding their hydrophilic part, they are divided into four groups, anionic, cationic, amphoteric and non-ionic (Dehghan Noudeh *et al*., 2008).

Absorption enhancing ability of surfactants in formulations with low absorption like peptides or proteins is used for drug delivery in non-injectable formulations. A broad spectrum of surfactants is used as enhancers including bile salts, anionic detergents, glycerides and lysophospholipids (lysolecithins); however, the efficacy of non-ionic surfactants with moderate polarity is better. On the other hand, it is reported that non-ionic polar surfactants do not have toxicity, while surfactants with moderate polarity showed toxic effects (Gould *et al*., 2000).

Morphologic and biochemical studies on membrane of absorption sites showed that surfactants enhance membrane transport followed by acute toxicity but these effects were reversed after a long time. As a result, there is a pivotal relationship between permeability enhancement activity and acute toxicity; moreover, permeability enhancing effect of surfactants is not only related to their nature, but also depends on other characteristics like electrical charge, polarity and the membrane (Golembeck *et al*., 1998; Gould, 1996).

Permeability enhancers are agents that decrease or remove extra cellular layer resistance reversibly and allow the drug to pass through and between epithelial cells toward blood and lymph. Recently, enhancing drugs permeability through cellular membrane becomes one of the main topics in pharmaceutical researches (Muranishi, 1990).

One of the suggested mechanisms is inducing partial but reversible gap within cells membranes' and consequently increasing the permeability by surfactants or other enhancers. Various models exist for evaluation of membrane toxicity of surfactants including single cell models using erythrocytes, erythrocyte ghosts, or liposomes. The erythrocyte model has been widely used as it presents a direct indication of toxicity of injectable formulations as well as general indication of membrane

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toxicity. Another advantage of erythrocytes model is that blood is readily available and that cells are easy to isolate from the blood; moreover, its membrane has similarities with other cell membrane (Robertis and Robertis, 1995).

Evaluating the toxicity of permeability enhancers using biological membranes play an important role. Consequently, in present study we decided to determine the effects of sorbitan mono lorate (span 20), sorbitan mono palmitate (span 40), sorbitan mono stearate (span 60) and sorbitan mono oleate (span 80) on cellular membrane.

MATERIALS AND METHODS

Materials: This study was conducted 3 to 4 months ago. All materials were of reagent grade unless otherwise mentioned. Span 20, 40, 60 and 80 were prepared from Fluka, (Netherlands). Sodium chloride, di-sodium hydrogen phosphate, citric acid (monohydrate), di-sodium phosphate and liquid paraffin were purchased from Merck (Germany). Drabkin's agent was supplied from Chimi-Daru (Iran).

Buffer and reagents preparation: McIlvaine's buffer was prepared as follows: solution 1, containing 21 g of citric acid (100 mM) and 8.775 g of sodium chloride (150 mM) made up to 1000 mL with deionized water, was mixed with solution 2, containing 28.4 g of di-sodium hydrogen phosphate (200 mM) and 8.775 g of sodium chloride (150 mM) made up to 1000 mL with deionized water, to produce the required pH of 7.0. Solution's pH was measured by electrical pH-meter (TWT Metrohm, Germany).

Preparation of red blood cells suspension: Human blood was collected from a healthy individual with 46.7% hematocrit and added to four heparinized tubes. After centrifuging at 3000 rpm for 10 min (Hermle 230 ZA, Germany), plasma and buffy coat were removed and the erythrocytes were washed three times in at least five times of their volume with McIlvaine's buffer, pH = 7.0. Afterward, by adding McIlvaine's buffer, an erythrocyte suspension with 12% hematocrit were prepared and kept in 4°C for experiments (Gould *et al*., 2000).

Hemolytic method: A suspension of erythrocyte (200 μL) within a micro-tube was incubated for the required times with an equal volume of the test sample of surfactants mixture, including span 20, 40, 60, or 80, prepared in McIlvaine's buffer, at 25, 37, or 42°C. After incubation, the mixture were spun in a microcentrifuge at 3000 rpm for 35 sec (Spectrafuge 161 M, England) and 200 μL of the resulting supernatants was added to 3 mL of Drabkin's reagent. To assay for the amount of haemoglobin released, the absorbance of samples were assessed in 540 nm wavelength using spectrophotometer (Shimadzu, 3100, Japan). Positive controls consisted of 200 μL of uncentrifuged mixtures of erythrocyte suspensions and 200 μL of buffer, which were added to 3 mL Drabkin's reagent to obtain a value for 100% haemolysis. A negative control, included to assess the level of spontaneous haemolysis, comprised 200 μL buffer mixed with 200 μL erythrocytes and after centrifugation for 35 sec, a 200 μL sample of supernatant was added to 3 mL of Drabkin's reagent. Haemolysis percentage for each sample were calculated by dividing sample's absorbance on positive control absorbance (compete haemolysis) multiplied 100 (Gould *et al*., 2000).

Determination of emulsification index: For estimation of the emulsification index (E_{24}), 5 mL of liquid paraffin was added to 5 mL of different concentrations of surfactants in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The E_{24} was calculated by measuring the emulsion layer formed (Carrillo *et al*., 1996).

Foam formation activity: Different concentrations of surfactants were dissolved to 5 mL disodium phosphate buffer and shaked with vibrator for 5 sec. The samples put aside at 25°C for 1 min. Foam activity (F_h) was measured as foam height in graduated cylinder (Porter, 1994).

RESULTS AND DISCUSSION

The results of haemolysis induced by surfactants were showed in Table 1-9 that Table 1-3, 4-6 and 7-9 are related to haemolysis at 25, 37 and 42°C, respectively. Each concentration show the mean of haemolysis percentage repeated in nine experiments. In order to compare the hemolytic effects of all surfactants, the concentration of each surfactant needed to induce 50% haemolysis was determined (data not shown). Results of emulsification index and foaming formation are presented in Fig. 1 and 2, respectively.

Despite the fact that all about surfactants hemolytic activity is not fully known, but it's proposed that it may consist of according processes: Absorption of surfactant molecules on cellular surface, penetration of surfactant molecules into cellular membrane, induction of alterations within cellular membrane, increasing permeability of cellular membrane, gradual increase of osmotic phenomenon and followed by destruction of cellular membrane and hemolysis (Dehghan Noudeh *et al*., 2008).

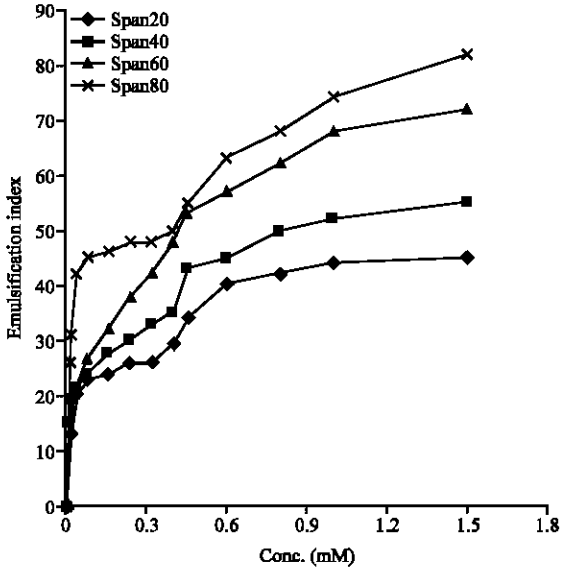

Fig. 1: Emulsification index at different concentrations of spans 20, 40, 60 and 80 (n = 9)

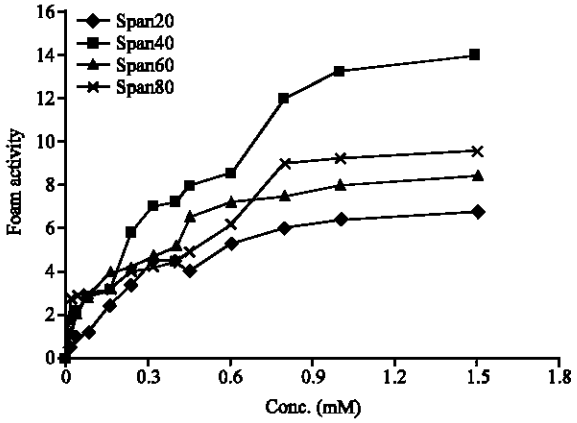

Fig. 2: Foam formation activity at different concentrations of spans 20, 40, 60 and 80 (n = 9)

According to above explanation, two different effects from surfactants in hemolytic studies can be observed, the first one is increasing cellular membrane permeability and the latter is cellular lysis. Surfactants which induce haemolysis can alter the membrane permeability for haemoglobin. This alteration occurs in a specific spectrum of the surfactant concentration and in lower concentrations hemolytic effects can not be seen; in these concentrations cellular membrane is permeable for low molecular weight molecules. Destruction due to surfactants is the result of cellular membrane breakage by alteration of structural molecules of the membrane; subsequently, the membrane permeability for macro molecules similar to smaller molecules increase. In this chain reaction mechanism, surface active agents adhere to erythrocyte surface and enter inside, change the molecular structure of the membrane which results in colloid-osmotic swelling of the erythrocyte and ultimately cellular rupture. Micelle production from surfactant molecules and membrane phospholipids lead to increase in membrane permeability and colloid-osmotic lysis of erythrocyte. Above mechanism highly depends on surfactant concentration and temperature and by increase in these factors the level of haemolysis increases. These mentioned effects support the idea of surfactant usage as absorption enhancer (Bonarska *et al*., 2005).

Haemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer emulsion and cellular membrane destruction. As this haemolysis relates to concentration and potency of surfactants, the model can be used for evaluation of surfactants potency. Biological membrane consists of a lipid bilayer which surrounds whole cell surface and proteins. Lipid bilayer structure is stabilized by non-covalent bonds among acyl groups and ionic bonds between polar heads and aqua. In non-ionic surfactants the interaction with biological membrane needs hydrophobic interaction between alkyl chains of

Table 1: Hemolysis induced by spans 20, 40, 60 and 80 after 15 min at 25°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	3.2	0.22	1.5	0.11	1.1	0.12	2.8	0.21
0.015	4.2	0.21	1.8	0.12	1.1	0.12	3.0	0.26
0.025	4.3	0.26	2.0	0.21	2.0	0.15	3.1	0.12
0.05	5.8	0.42	3.0	0.14	2.5	0.17	3.3	0.42
0.1	6.1	0.52	4.0	0.12	3.6	0.11	3.6	0.21
0.2	7.0	0.18	4.2	0.21	4.0	0.13	3.8	0.13
0.3	7.5	0.72	4.6	0.24	4.6	0.42	3.9	0.12
0.4	8.2	0.85	5.7	0.12	5.0	0.15	4.3	0.22
0.5	8.5	0.12	7.2	0.22	5.6	0.12	4.5	0.26
0.6	10.0	0.42	8.6	0.16	7.0	0.22	4.6	0.41
0.8	12.0	0.17	9.0	0.15	7.5	0.24	5.3	0.23
1.0	12.3	0.36	9.5	0.13	8.2	0.12	5.8	0.22
1.2	12.8	0.34	8.5	0.85	10.3	0.21	7.0	0.43
1.5	13.0	0.28	11.5	0.12	11.0	0.86	7.6	0.42

n = 9

Table 2: Hemolysis induced by spans 20, 40, 60 and 80 after 30 min at 25°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	3.4	0.41	1.9	0.12	1.3	0.12	3.0	0.14
0.015	4.5	0.12	2.0	0.22	1.5	0.12	3.2	0.10
0.025	4.8	0.22	2.2	0.22	1.7	0.14	3.3	0.12
0.05	5.9	0.43	2.2	0.14	2.0	0.21	3.9	0.22
0.1	7.0	0.17	3.5	0.12	2.9	0.15	4.0	0.45
0.2	7.2	0.21	4.0	0.26	3.2	0.22	4.3	0.12
0.3	8.0	0.22	4.2	0.31	4.6	0.41	4.6	0.14
0.4	8.1	0.95	4.8	0.12	5.0	0.12	4.9	0.43
0.5	8.5	0.86	7.5	0.17	7.0	0.17	5.6	0.15
0.6	8.7	0.26	8.6	0.26	9.0	0.21	5.8	0.15
0.8	10.1	0.34	8.7	0.17	8.2	0.85	6.3	0.42
1.0	11.2	0.22	11.0	0.17	9.2	0.34	6.4	0.12
1.2	12.21	0.12	12.2	0.15	9.3	0.31	6.6	0.22
1.5	12.8	0.43	12.5	0.12	9.5	0.12	6.8	0.26

n = 9

Table 3: Hemolysis induced by spans 20, 40, 60 and 80 after 45 min at 25°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	5.0	0.12	1.0	0.12	1.4	0.12	2.2	0.12
0.015	5.1	0.11	1.5	2.00	1.4	0.12	2.0	0.42
0.025	6.2	0.22	2.8	0.26	2.4	0.11	2.1	0.11
0.05	6.8	0.26	3.0	0.12	2.5	0.15	2.4	0.22
0.1	7.1	0.13	3.8	0.15	3.4	0.21	3.0	0.22
0.2	7.6	0.42	4.1	0.12	4.0	0.41	3.5	0.21
0.3	7.8	0.21	4.3	0.26	5.2	0.21	4.2	0.11
0.4	9.0	0.22	5.0	0.12	6.5	0.22	5.0	0.12
0.5	9.2	0.32	5.2	0.22	7.8	0.15	6.4	0.25
0.6	10.8	0.37	6.8	0.12	8.5	0.14	7.5	0.14
0.8	12.2	0.17	9.2	0.33	9.0	0.17	8.2	0.25
1.0	14.0	0.51	12.2	0.21	10.0	0.24	9.0	0.26
1.2	14.8	0.12	12.5	0.41	11.2	0.51	10.5	0.11
1.5	15.0	0.21	12.8	0.15	11.8	0.12	11.0	0.17

n = 9

surfactant and lipoprotein parts of membrane (Swenson and Curatolo, 1992).

In this study the hemolytic effects of surfactants increased as temperature increased (Table 11). Note that liquid characteristic and fluidity of bilayer lipid is one of its special features. Therefore, some parts of the membrane can easily move throughout the surface and this characteristic is due to membrane phospholipids which covert to jelly in temperatures lower than physiologic temperature. This conversion of phospholipids helps in more stabilized and regular membrane and increases its resistance. As a result, the amount of haemolysis in is 42°C (Table 7-9) more than 25°C (Table 1-3); the reason is that with increase in temperature the membrane fluidity and accordingly its permeability increase (Bonarska *et al*., 2005).

Also, in solutions with higher concentration of surfactants haemolysis amount were more (Table 1-9). This result can be easily described by Fick's law that according to this law, the diffusion flux from a membrane is proportional to concentration difference of both sides (Muranishi, 1990). In other words, the concentration of intra-membrane surfactant is related to its extra-membrane concentration and by increasing the latter concentration the first one increases until reaching to a specific concentration which leads to membrane destruction and hemolytic effects (Bonarska *et al*., 2005).

The first step in surfactant-membrane interaction is membrane saturation with surfactant's monomers; following the process cellular lysis is possible. The onset is followed by destruction and deconstruction of surfactant-protein-lipid complexes and surfactant-lipid mixture micelles. Adding more surfactant enriches the surfactant-protein-lipid complexes and more mixture micelle production. At the extremity and in critical micellar concentration (cmc) the amount of protein-surfactant complexes, mixture micelles and surfactant's micelles become balanced with free surfactant (Swenson and

Table 4: Hemolysis induced by spans 20, 40, 60 and 80 after 15 min at 37°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	3.8	0.12	2.5	0.12	2.4	0.12	2.8	0.26
0.015	4.1	0.11	3.0	0.22	2.8	0.26	2.9	0.21
0.025	4.5	0.11	3.4	0.11	3.0	0.11	3.2	0.25
0.05	4.7	0.26	3.8	0.26	3.0	0.11	3.4	0.41
0.1	5.4	0.41	5.0	0.25	4.6	0.42	3.9	0.12
0.2	7.1	0.42	6.7	0.31	6.1	0.22	4.0	0.21
0.3	7.6	0.32	7.5	0.41	7.0	0.17	4.3	0.22
0.4	8.1	0.95	7.8	0.24	7.1	0.12	4.8	0.21
0.5	9.1	0.12	9.0	0.21	8.0	0.85	5.5	0.11
0.6	10.2	0.31	9.5	0.11	8.2	0.31	5.7	0.17
0.8	10.8	0.26	10.0	0.11	8.5	0.11	6.0	0.14
1.0	13.1	0.11	12.2	0.21	8.7	0.42	6.1	0.22
1.2	13.9	0.45	13.2	0.41	9.0	0.86	6.5	0.21
1.5	14.5	0.12	13.6	0.11	9.8	0.12	7.2	0.24

n = 9

Table 5: Hemolysis induced by spans 20, 40, 60 and 80 after 30 min at 37°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	6.4	0.11	3.0	0.15	2.5	0.12	3.5	0.12
0.015	7.8	0.21	3.2	0.14	2.8	0.26	3.8	0.11
0.025	10.0	0.42	3.5	0.15	3.1	0.15	3.9	0.17
0.05	10.2	0.31	3.8	0.11	3.2	0.25	4.1	0.12
0.1	10.5	0.15	4.9	0.15	3.6	0.41	4.6	0.42
0.2	11.9	0.14	5.8	0.11	4.2	0.25	4.8	0.21
0.3	12.8	0.26	7.8	0.16	5.3	0.41	5.4	0.44
0.4	13.4	0.22	8.9	0.15	6.7	0.12	6.0	0.22
0.5	15.0	0.21	9.0	0.14	7.3	0.11	6.0	0.21
0.6	15.6	0.11	10.0	0.11	7.8	0.11	6.2	0.25
0.8	15.8	0.26	12.0	0.15	8.6	0.25	6.8	0.26
1.0	17.0	0.21	12.2	0.14	9.0	0.25	7.0	0.17
1.2	17.1	0.22	13.5	0.17	9.8	0.35	7.2	0.21
1.5	17.2	0.31	14.0	0.35	10.5	0.12	8.0	0.71

n = 9

Table 6: Hemolysis induced by spans 20, 40, 60 and 80 after 45 min at 37°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	4.8	0.21	3.5	0.25	3.1	0.12	3.0	0.41
0.015	5.4	0.42	3.7	0.11	3.2	0.22	3.1	0.12
0.025	6.0	0.26	4.0	0.15	3.5	0.15	3.5	0.21
0.05	7.0	0.17	4.5	0.14	3.8	0.15	4.2	0.25
0.1	7.1	0.11	5.6	0.14	4.8	0.26	4.8	0.21
0.2	7.5	0.22	7.0	0.16	5.0	0.12	5.1	0.41
0.3	8.0	0.34	7.4	0.14	6.5	0.11	5.4	0.45
0.4	10.1	0.11	8.5	0.17	6.8	0.26	5.8	0.25
0.5	12.3	0.26	9.5	0.21	7.0	0.17	6.0	0.21
0.6	14.5	0.21	10.0	0.86	8.2	0.85	6.8	0.26
0.8	15.8	0.22	14.5	0.11	9.2	0.31	7.0	0.25
1.0	16.5	0.21	15.0	0.51	10.2	0.32	7.4	0.12
1.2	17.8	0.11	16.5	0.17	13.0	0.34	7.8	0.81
1.5	18.1	0.11	16.8	0.11	13.5	0.25	9.0	0.25

n = 9

Table 7: Hemolysis induced by spans 20, 40, 60 and 80 after 15 min at 42°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	5.0	0.14	2.5	0.11	2.8	0.26	2.9	0.12
0.015	5.9	0.11	2.5	0.15	3.1	0.12	3.0	0.10
0.025	6.8	0.12	2.7	0.11	3.7	0.11	3.5	0.42
0.05	7.2	0.15	3.0	0.15	4.4	0.15	3.8	0.12
0.1	7.9	0.31	3.2	0.25	5.4	0.42	4.0	0.25
0.2	8.0	0.15	4.3	0.26	5.7	0.12	4.5	0.21
0.3	8.8	0.12	5.4	0.41	6.1	0.21	5.3	0.12
0.4	10.5	0.08	6.0	0.21	6.4	0.12	5.5	0.21
0.5	10.8	0.11	7.2	0.21	6.5	0.22	6.0	0.12
0.6	11.2	0.21	8.7	0.26	6.8	0.26	6.5	0.22
0.8	12.8	0.11	9.8	0.12	7.2	0.25	7.1	0.11
1.0	15.4	0.31	12.1	0.11	9.0	0.21	7.5	0.52
1.2	16.2	0.42	13.0	0.34	9.2	0.32	8.0	0.20
1.5	16.4	0.21	13.5	0.31	9.5	0.16	8.5	0.15

n = 9

Table 8: Hemolysis induced by spans 20, 40, 60 and 80 after 30 min at 42°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	5.6	0.11	3.5	0.26	3.4	0.25	3.2	0.21
0.015	5.8	0.12	4.0	0.11	3.9	0.41	3.8	0.12
0.025	6.9	0.13	5.4	0.11	4.7	0.15	4.2	0.25
0.05	7.1	0.11	6.0	0.41	5.0	0.22	4.8	0.21
0.1	7.2	0.21	6.2	0.12	5.4	0.15	5.0	0.25
0.2	7.8	0.15	7.0	0.21	5.9	0.42	5.2	0.20
0.3	9.5	0.25	8.0	0.22	6.0	0.12	5.6	0.42
0.4	12.3	0.11	8.9	0.17	6.8	0.21	6.1	0.21
0.5	13.2	0.61	9.5	0.11	6.8	0.26	6.2	0.22
0.6	14.5	0.11	10.4	0.12	7.3	0.26	6.6	0.26
0.8	15.0	0.12	12.5	0.11	7.9	0.12	7.0	0.17
1.0	15.8	0.25	13.5	0.42	9.0	0.11	7.2	0.21
1.2	17.4	0.11	14.2	0.11	9.6	0.11	7.5	0.23
1.5	18.0	0.26	14.8	0.21	9.8	0.12	7.8	0.15

n = 9

Table 9: Hemolysis induced by spans 20, 40, 60 and 80 after 45 min at 42°C

	Surfactant							
	Span 20		Span 40		Span 60		Span 80	
Conc. (mM)	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD	Hemolysis (%)	±SD
0.0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
0.01	6.0	0.11	4.3	0.26	3.9	0.11	3.8	0.12
0.015	7.1	0.14	5.0	0.12	4.4	0.11	4.2	0.21
0.025	8.0	0.18	5.7	0.11	4.8	0.21	4.5	0.23
0.05	8.1	0.22	6.4	0.11	5.0	0.22	5.0	0.22
0.1	10.0	0.15	7.0	0.17	6.0	0.41	5.6	0.42
0.2	11.5	0.14	7.5	0.85	6.2	0.21	5.9	0.21
0.3	12.8	0.11	7.8	0.22	6.8	0.26	6.0	0.21
0.4	13.9	0.22	9.0	0.21	7.1	0.11	6.8	0.51
0.5	15.2	0.11	9.7	0.15	8.3	0.41	7.0	0.17
0.6	16.0	0.24	10.8	0.26	9.5	0.11	8.0	0.18
0.8	16.5	0.14	13.1	0.16	11.2	0.41	8.1	0.22
1.0	17.0	0.11	14.5	0.50	12.3	0.11	8.4	0.22
1.2	17.9	0.26	15.8	0.11	15.0	0.11	9.5	0.21
1.5	18.5	0.22	16.8	0.22	15.6	0.32	10.4	0.81

n = 9

Curatolo, 1992). Present results showed that hemolytic effects of surfactants increase as the latency of incubation and the amount of contact duration with erythrocytes increase (Table 1-9). It is reported that the more is the contact duration of erythrocytes with a solution, including a surface active agent solution, the more is the amount of cellular lysis (Tragner and Csordas, 1987).

Adherence of surface active agents to erythrocyte's membrane which is followed by their entrance leads to alteration of the molecular structure of cell membrane, osmotic-colloid swelling and erythrocyte membrane rupture. Above mechanism depends on surfactant concentration, temperature and duration of contact with erythrocyte; and by increasing these factors membrane permeability and haemolysis, that happen due to micelle production from surfactant and membrane phospholipids bilayer, increase (Araki and Rifkind, 1981).

Another aspect of this study was to evaluate the membrane toxicity of surfactants. As the agents or any other substance which have the ability to destruct the erythrocytes membrane can have similar effects on other cells membranes, evaluating erythrocytes membrane stability is a proper criterion for determination of surfactant toxicity. According to present result, haemolysis was observed by increasing the incubation period and temperature. In 1 mM and temperature of 37°C span 80 caused 7% of erythrocytes destruction, while span 20, 40 and 60 caused 17.0, 12.2 and 9.0% of destruction, respectively. The hemolytic activity of experienced surfactants in this study increase in higher concentrations and in a specific concentration, in critical concentration for micelle formation, reached to its utmost and after this point remained steadily (Table 1-9). Hence, the ability to increase membrane permeability and after it osmotic cellular lysis are due to mixture micelle formation in bilayer membrane. Evaluating the erythrocyte haemolysis showed that span 80 had lower destruction level and less toxicity on cellular membrane. Erythrocyte haemolysis method is used to evaluate surfactant and cellular membrane interactions, enhancing activity and emulsifying ability. Accordingly, span 80 with lower toxicity should be preferred to be used as a surface active agent and needs more studies on its enhancing abilities and formulatory properties. As we showed span 20 (HLB = 8.6) with low hydrophobic and high hydrophilic properties has more capability for membrane destruction, while span 80 with lower hydrophilic properties has less destruction capability. Tranger and Csordas (1987) that evaluated haemolysis effects of some non-ionic surface active agents reported that in a series of surfactants from one family, the ones with higher hydrophobic contents and lower HLB have lower haemolysis (Schott, 1999). Accordingly, it can be concluded that higher content of hydrophobic part may lead to reduction in permeability and hemolytic effects (Table 10).

Table 10: Some properties of spans

Surface active agent	Hydrophobic chain (R)	Weight of hydrophilic part per weight of lipophilic part (mH mL^{-1})	Molecular weight (MW)
Span 20	$C_{12}H_{23}O_2$ (lorate)/199.32	4.94	1226
Span 40	$C_{16}H_{31}O_2$ (palmitate)/255.42	3.64	1282
Span 60	$C_{18}H_{53}O_2$ (stearate)/283.46	3.01	1310
Span 80	$C_{18}H_{33}O_2$ (oleate)/281.46	3.12	1308

Table 11: Effect of temprature on hemolysis percentage of spans

Spans	Temperature (°C)	Incubation time (min)	Hemolysis (%)
20	25	15	13.0
40			11.5
60			11.0
80			7.6
20	37	15	14.5
40			13.6
60			9.8
80			7.2
20	42	15	16.4
40			13.5
60			9.5
80			8.5

In non-ionic surfactants hemolytic effects depends on HLB and the percentage of lipophilic part. Surfactants hydrophobic part has a great impact on their properties and affects the size of their micelles and micelle-membrane interaction. Micelle size can affect the cellular membrane permeability, followed by colloid-osmotic lysis through mixture micelle production in bilayer membrane (Araki and Rifkind, 1981; Ohnishi and Sagitani, 1993).

Another potential property of surface active agents is their ability in induction and stabilizing emulsions. Emulsifying index has direct relationship with surface tension and agent ability in micelle production. In this study, increasing the concentration of all surfactants leads to increase in emulsions stability; however, this trend was not the same in all surfactants (Fig. 1). This effect started in low concentration (0.020 mM) and reached to its maximum effect in 1.50 mM. Emulsifying index in 1.50 mM concentration for span 20, 40, 60 and 80 were 46, 54, 69 and 89, respectively. The difference between observed data of surfactants was significant ($p<0.01$ for all experiments). According to hemolytic data and emulsifying index, span 80 had the least toxicity and the best properties for emulsification to be used in formulations. Foaming ability of surfactants is a propriety

which may help proving the existence of surfactants in a solution; furthermore, this ability can be used in order to compare the detergency properties of detergents with high ability of foaming production. Foam production and stability depends on type and concentration of surfactants, more and stable foam is produced by ionic surfactants comparing with non-ionic. In a homolog series of surfactants more foam is produced by increasing the content of hydrophobic parts of surfactant molecule until reaching to a maximum point (Dehghan Noudeh *et al*., 2008). Present results also showed that Span 60 and 80 with higher hydrophobic contents had more ability to produce foam (Fig. 2).

CONCLUSION

According to the results of this study we must use spans at concentrations lower cmc in formulations. According to the results, the use of spans with low hemolytic effect like as span 80 is preferred in pharmaceutical preparations.

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REFERENCES

Araki, K. and J.M. Rifkind, 1981. The rate of osmotic hemolysis: A relationship with membrane bilayer fluidity. Biochim. Biophys. Acta, 645: 81-90.

Bonarska, D., S. Witek and J. Sarapuk, 2005. Hemolysis of erythrocytes and erythrocyte membrane fluidity change by new lysosmtropic compounds. J. Fluoresc., 15: 137-141.

Carrillo, P.G., C. Mardaraz, S.I. Pitta-Alvarez and A.M. Giulietti, 1996. Isolation and selection of biosrfactant producing bacteria. World J. Microbiol. Biotechnol., 12: 82-84.

Dehghan Noudeh, G.H., P. Khazaeli and P. Rahmani, 2008. Study of the effects of polyethylene glycol sorbitan esters surfactants group on biological membranes. Int. J. Pharmocol., 4: 27-33.

Golembeck, E., A. Alonso and N. Correa, 1998. Effect of polyoxyethylen chain length on erythrocyte hemolysis induced by nonionic surfactants. Chem. Biol. Interact., 113: 91-103.

Gould, L.A., 1996. Some factors influencing the effects of surface active agents on membranes. Ph.D Thesis, University of London, 1996 Depart. of Pharmacy London, UK.

Gould, F.A., A.B. Lansly and P. Marthin, 2000. Mitgation of surfactants erythrocyte toxicity by egy phosphatidylcholine. J. Pharm. Pharmacol., 52: 1203-1209.

Muramishi, S., 1990. Absorption enhancers. Crit. Rev. Ther. Drug Carr, 7: 1-34.

Ohnishi, M. and H. Sagitani, 1993. The effect of nonionic surfactant structure on hemolysis. J. Am., 70: 679-684.

Porter, M.R., 1994. Handbook of Surfactants. 2nd Edn., Chapman and Hall, London, ISBN: 0751401706, pp: 126-168.

Robertis, F.A. and E.M.H. Robertis, 1995. Cell and Molecular Biology. 1st Edn., Cell Membrane Sunders, London, pp: 239-245.

Schott, H., 1999. Hydrophilic-Lipophilic Balance Solubility Parameter and Oil-Water Partition Coefficients Universal Parameters of Nonionic Surfactants School of Pharmacy. 1st Edn., Temple University, Philadelphia USA., pp: 11-22.

Swenson, E.S. and W.J. Curatolo, 1992. (C) Means to enhance penetration: (2) Intestinal permeability enhancement for proteins, peptides and other polar drugs: Mechanisms and potential toxicity. Adv. Drug Del. Rev., 8: 39-92.

Tragner, D. and A. Csordas, 1987. Biphasic interaction of Triton detergents with the erythrocytemembrane. Biochem. J., 244: 605-609.