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Effects of glycerol on the thermal dependence of the stability of human erythrocytes

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Abstract Incubation of human blood in saline solution of 0–36% (v/v) ethanol for 30 min produces lysis or stabilization of erythrocytes depending on the ethanol concentration. Under less elevated concentrations of ethanol, erythrocytes are present in expanded shapes (R state) that present lower stability and suffer lysis with increase in the ethanol concentration. Under more elevated concentrations of ethanol, erythrocytes are present in contracted shapes (T state) that have higher stability and suffer lysis at even more elevated ethanol concentrations. This work evaluated the effects of glycerol (0 to 2.0 M) and temperature (7 to 47°C) on the stability of the R erythrocytes, characterized by the ethanol concentration at the mid-transition point (D_{50R}) of the hemolysis curve (D_{50R}). D_{50R} declined sigmoidally with increase in the glycerol concentration or temperature, due to transition of the R to the T state erythrocytes. In 1.5 M glycerol, the erythrocytes stability decreased below 32 but increased above 37°C. The combination of temperature, glycerol and ethanol actions generates a critical value of osmotic pressure below which the R state predominates and above which the T state predominates. At 7°C 1.5 M glycerol decreased the erythrocytes stability against ethanol but increased the erythrocytes stability against hypotonic shock. Those conditions favor the R state, which has a lower stability against ethanol; however, in the absence of ethanol, glycerol determines less water entrance in the erythrocytes, making more difficult its lysis by hypotonicity.

Introduction

The capacity of a biological membrane in keeping its structure against the action of chaotropic conditions is defined as stability. A low stability will determine structural changes that will compromise the membrane functions.

The stability of a membrane can be increased by the capacity of the proper cell in increase the concentration of phospholipids with saturated fatty acids or of cholesterol in the lipid bilayer. The production and concentration of small organic solutes, called as osmolytes, is another cellular strategy to preserve the stability of the organization complexes (Borowitza and Brown 1974; Bowlus and Somero 1979; Pollard and Wyn-Jones 1979; Santoro et al. 1992; Yancey et al. 1982; Yancey 1985).

However, an excessive increase in stability will decrease the membrane fluidity and compromise its function. This means that the membranes need to equilibrate stability and fluidity to preserve the cell functions.

The osmostabilization is a strategy also used in biotechnological procedures as the cryopreservation of erythrocytes and other cells for long periods. The more frequently used osmostabilizing agents are glycerol, sorbitol, trehalose and dextran (Boutron and Arnaud 1984; Lang et al. 1998; Pellerin-Mendes et al. 1997; Santoro et al. 1992; Scott et al. 2005; Wagner et al. 2002).

The nature of the stabilizing action of the osmolyte on the biological membrane is still controversial.

This works aimed to evaluate the stability of erythrocytes against the lysis by ethanol and hypotonic shock, in different temperatures and glycerol concentrations.

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Materials and methods

Blood sample collection

The blood samples (4 ml) were collected by intravenous punctions, after nocturnal fasting (8–14 h), in evacuated tubes (Vacuntainer®) containing 50 μl of 1 g dl^{-1} EDTA as an anticoagulant, from 12 female volunteers (24±3 years), selected among healthy, non-smokers, no alcohol, drugs or medicine users. The study was previously approved by the institutional Ethics Committee.

Reagents and equipments

The reagents used (NaCl, glycerol and ethanol) were purchased from Synth (Diadema, SP, Brazil) with a purity degree of 99.5%, which was dully corrected in the solutions preparation. The volume measurements were done in refractory glass burettes and in automatic pipettes (Labsystems, model Finnpipette Digital). The mass measurements were done in a digital balance (AND, model 870). The incubations were done in a thermostatized water bath (Marconi, model MA 184, Piracicaba, SP, Brazil). The absorbance readings were conduced with a visible spectrophotometer (Micronal, model B-442, São Paulo, SP, Brazil).

Effect of 1.5 M glycerol on the stability of erythrocytes against ethanol in saline solutions at 7, 12, 17, 22, 27, 32, 37, 42 and 47°C

Two sets of assay tubes (Eppendorff®) containing 1 ml of 0 to 36% (v/v) ethanol in 0.9% (w/v) NaCl (saline solution), one in the absence and the other in the presence of 1.5 M glycerol, were hermetically closed and pre-incubated during 10 min at 7, 12, 17, 22, 27, 32, 37, 42 and 47°C. After addition of aliquots of 25 μl of blood to the tubes, they were closed, homogenized, incubated by 30 min at each temperature and then centrifuged at 2,000×g by 10 min. The erythrocytes lysis was followed by measurements of the absorbance at 540 nm (A$_{540}$) of the supernatants in function of the ethanol concentration at each temperature.

Effect of the glycerol concentration on the stability of erythrocytes against ethanol in saline solution at 7°C

Several sets of assay tubes (Eppendorff®) containing solutions with 0 to 36% (v/v) ethanol (all sets) and 0, 0.5, 1.0, 1.5 or 2.0 M glycerol in 0.9% (w/v) NaCl (each set) were prepared and pre-incubated at 7°C. After addition of 25 μl of blood, the tubes were treated as described in the preceding section.

Determination of the stability of human erythrocytes against hypotonic stress

Two sets of assay tubes (Eppendorff®) containing 1 ml of 0 to 1.0% (w/v) NaCl solutions or 1 ml of 0 to 1.0% NaCl with 1.5 M glycerol were pre-incubated at 7°C during 10 min. After addition of 25 μl aliquots of blood the tubes were treated as described before.

Determination of the hemolysis transition curves

The dependence of A_{540} with the ethanol or NaCl (X) concentration was fitted to the sigmoidal regression line given the Boltzmann equation,

$$A_{540} = \frac{A_1 - A_2}{1 + e^{(X-X_{50})/dX}} + A_2, \tag{1}$$

where A_1 and A_2 represent the absorbance values at the first and second plateaus of the sigmoid, X is the ethanol or NaCl concentration, X_{50} represents the ethanol (D_{50R}) or NaCl (H_{50}) concentration that causes 50% of hemolysis and dX is the amplitude of the sigmoidal transition between A_1 and A_2. The percentage of hemolysis in each assay tube was calculated by the equation:

$$\text{Hemolysis}(\%) = \frac{A_{540\text{nm}}}{A_{\text{max}}} \times 100\%, \tag{2}$$

where A_{max} is A_2 when X is ethanol and A_1 when X is NaCl.

Statistical analyses

The statistical analyses were done using the applicative Origin 7.5 Professional (Microcal, Massachusetts, USA). Each regression analysis was considered significant when p was smaller than 0.05. The comparison of the D_{50R} and H_{50} values was done with use of the Tukey test and ANOVA, respectively, with $p < 0.05$ indicating statistically significant differences.

Results

All the experimental results here described are consequent to incubation of human erythrocytes by a fixed time (30 min) in solutions with different saline concentrations (hypotonic stress) or in saline solutions with different ethanol concentrations, under different temperatures (7, 12, 17, 22, 27, 32, 37, 42 and 47°C) or glycerol concentrations (0, 0.5, 1.0, 1.5 and 2.0 M).

The D_{50R} among the temperatures were always significantly different in both solvents. The dependencies of the D_{50R} values with the temperature were significantly fitted ($p < 0.001$) to decreasing sigmoidal lines (Fig. 1).

The presence of 1.5 M glycerol determined evident changes in the D_{50R} values in relation to the values in the absence of the osmolyte (Fig. 1). Below 32°C, the values of D_{50R} were smaller in the presence of saline solution with 1.5 M glycerol than in saline solution without glycerol. But above 37°C, the D_{50R} values were larger in the presence of saline solution of the osmolyte than in regular saline solution (Fig. 1).

The erythrocytes lysis by ethanol in saline solution was also evaluated in the presence of different glycerol concentrations, but only at 7°C. The values of D_{50R} presented a significant sigmoidal decline ($p<0.05$) with the glycerol concentration (Fig. 2).

The lysis of erythrocytes by hypotonic shock was also evaluated in the absence and in the presence of 1.5 M glycerol at 7°C (Fig. 3), with the erythrocytes stability expressed by the abscissa value (H_{50}) of the half-transition point of the sigmoid (Fig. 3). The presence of 1.5 M glycerol caused a statistically significant decrease in H_{50} at 7°C.

Discussion

The osmostabilization of erythrocytes occurs with volume contraction and morphological alterations that can be reverted after dilution of the solute excess (Bakaltcheva et al. 1996; De Loecker et al. 1993; Lang et al. 1998; Pellerin-Mendes et al. 1997). This indicates that erythrocytes can exist in two morphological states, an expanded or relaxed (R) state, present at the natural conditions of the blood, and a condensed or tight (T) state, present under high concen-
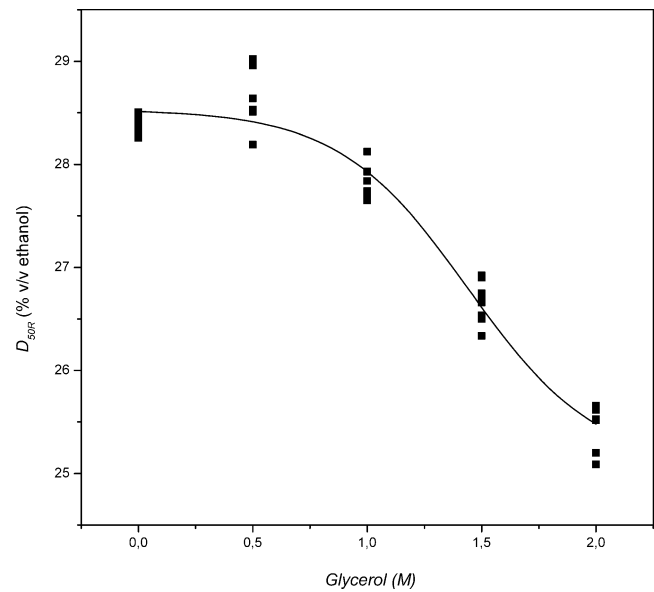
Fig. 2 Dependence of the half-transition point (D_{50R}) with the glycerol concentration ($n=42$, $p<0.001$) in saline physiological solution at 7°C

trations of osmolytes. Each morphological state would be formed by several different shapes.

The existence of these two states was considered in the Fig. 4 model (Aversi-Ferreira 2004; Bernardino Neto 2006; Finotti 2006; Reis 2007). Under smaller concentrations of ethanol in saline solution, the erythrocytes suffer a lysis transition with increase in the ethanol concentration (Fig. 5a), that represents the lysis transition of the R state
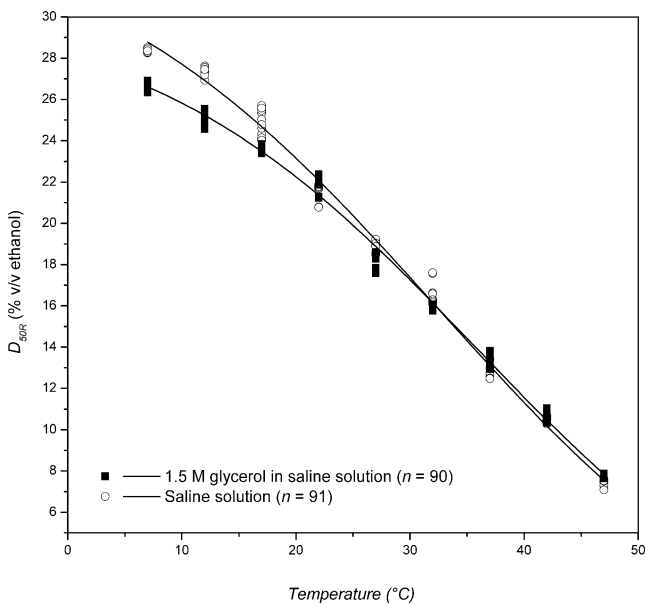
Fig. 1 Effect of 1.5 M glycerol on the thermal dependence lines of the half-transition points (D_{50R}) for the lysis of human erythrocytes by ethanol in saline solution. In both situations the data were fitted to sigmoidal dependence lines ($p<0.001$)
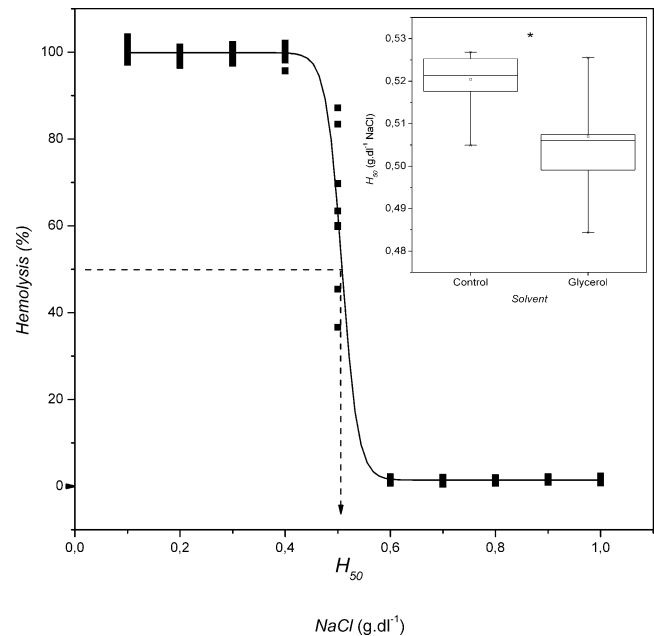
Fig. 3 Determination of the stability of human erythrocytes against hypotonic chock (H_{50}) at 7°C. *Inset*: comparison of the H_{50} value in 1.5 M glycerol ($n=8$) with that in the absence ($n=8$) of the osmolyte (control). *$p<0.05$ indicating statistically significant difference
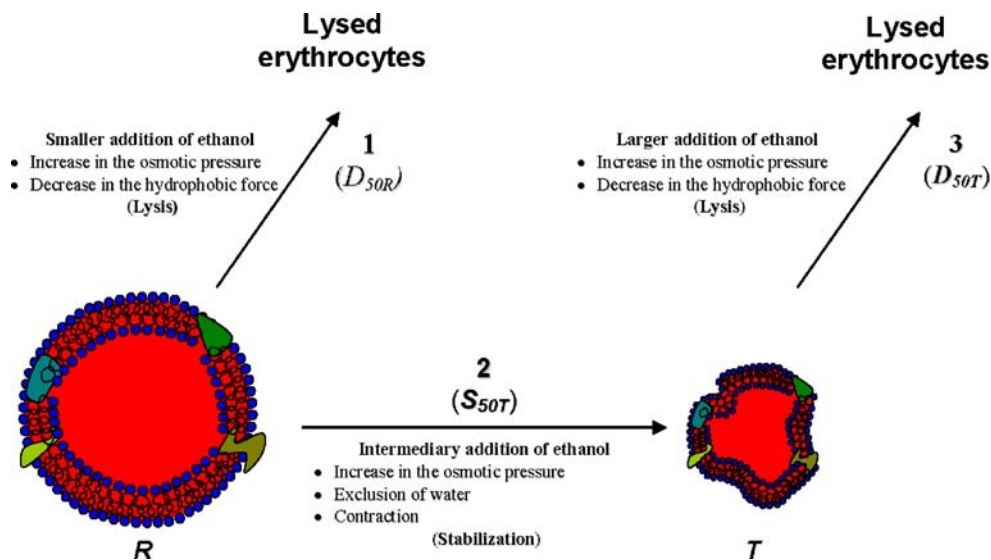
Fig. 4 Actions of ethanol on human erythrocytes in physiologic saline solution. The erythrocytes would exist in expanded morphological states (*R*) and in condensed states (*T*). Each morphological state would be constituted by a conjunct of different shapes. Low ethanol concentrations would cause lysis of the *R* state (*route 1*). Intermediary concentrations of ethanol would cause dislocation of erythrocytes from the *R* state to the *T* state (*route 2*), by increase in the osmotic pressure. High ethanol concentrations would cause lysis of the *T* state (*route 3*). D_{50R}, S_{50T} and D_{50T} represent the half transition points of the ethanol actions

of the erythrocytes (route 1 of Fig. 4). However, under larger ethanol concentrations, also in saline solution, the lysis curve of the erythrocytes by ethanol presents a stabilization chasm (Fig. 5b). This chasm was attributed to the junction of a stabilization curve (descendent portion of the curve at the chasm region) with a lysis transition curve of that stabilized state (ascendant portion of the curve at the chasm curve; Gouvêa-e-Silva 2006). Indeed, the erythrocytes were seen by light microscopy in an expanded state

before the first lysis transition (Fig. 5a), but contracted at the bottom of the stabilization chasm of Fig. 5b (Gouvêa-e-Silva 2006). The descendent and ascending portions of Fig. 5b curve represent respectively the stabilizing transition (route 2 of Fig. 4), with generation of the *T* state, and the lysis transition of the *T* state of the erythrocytes (route 3 of Fig. 4).

The kind of the activated route would be determined by factors as the nature of the solute incorporated in the saline solution, the temperature and the osmotic pressure.

Fig. 5 Dependence of the erythrocytes lyses with the ethanol concentration in physiologic saline solution at 37°C. The lysis by ethanol of the *R* state erythrocytes is characterized by the ethanol concentration that promotes 50% of lysis (D_{50R}) of the *R* state (**a**). The generation of the stabilized *T* state, promoted by increase in the ethanol concentration, characterized by its half transition point (S_{50T}), followed by the ethanol induced lysis of that *T* state, characterized by its half transition point (D_{50T}), are shown on the *right* (**b**) (Gouvêa-e-Silva 2006)
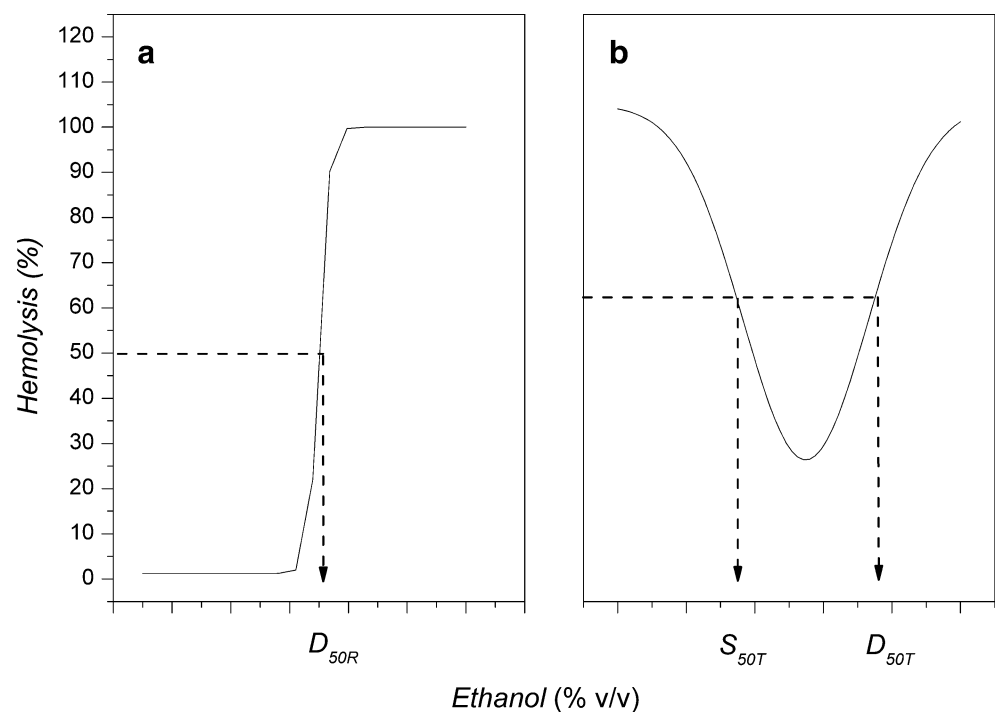
The ethanol promotes lysis of the R state of the erythrocytes in all temperatures. Since the ethanol has a small hydrophobic portion (the ethyl group, CH_3–CH_2–), joined to a hydrophilic group (the hydroxyl group, –OH), it is an amphiphilic agent, capable to associate to apolar groups and to the water molecules at the same time. This structural nature makes the ethanol to diminish the hydrophobic force in aqueous solutions of ethanol, what has a great meaning in the structural equilibriums of biological complexes as proteins, membranes and chromosomes. Such attenuation in the hydrophobic force ocasionates a decrease in the stability of the biological complexes and it is this action that characterizes ethanol as a chaotropic agent (Fonseca et al. 2006; Tanford 1970). On the other hand, the ethanol also increases the osmolarity of the medium, what is associated to a second kind of effect of ethanol, the osmostabilization of the biological complexes, effect that is very important in the morphologic equilibriums of erythrocytes (Fig. 5b) only in more elevated concentrations of ethanol (Gouvêa-e-Silva 2006; Reis 2007). This action of ethanol would be responsible for activation of route 2 (Fig. 4).

Heat is also a chaotropic agent that increases the vibrational energy of the chemical groups of a biological complex, in such a way to diminish the intensity of the forces that stabilize their structures (Betz et al. 2007; Fonseca et al. 2006; Tanford 1970).

The progressive decreases in the D_{50R} values with the temperature increase in both solvents were better adjusted by sigmoids (Fig. 1), what means that the heat first potentiates the chaotropic nature of ethanol (intermediary region of the sigmoid), but after antagonizes this action (second plateau of the sigmoid). Heat is inducing any kind of alteration, probably in the erythrocyte structure, which is responsible by the potentiation followed by the antagonization of the chaotropic action of ethanol.

The physical meaning of that heat action would be the existence of a state conversion in the erythrocytes. The synergistic action between ethanol and the intermediary values of temperature of our interval (intermediary region of the sigmoid of Fig. 1) contributes to the exacerbation of the lysis of what would be the R state of the erythrocytes (route 1 of Fig. 4). But at the superior temperature values of our interval (second plateau of the sigmoid of Fig. 1), the synergistic action of ethanol and heat contributes to decrease the lysis of the R state due to conversion of the erythrocytes from the R to the T state (route 2 of Fig. 4).

Indeed, the activation of the route 2 (Fig. 4) is favored by increase in the osmotic pressure produced by increase in the temperature, since the increase in the temperature was associated to a decrease in the values of S_{50T} (Reis 2007).

If this is true, the incorporation of an osmolyte to the medium would also activate the formation of the T state of the erythrocytes.

In the presence of 1.5 M glycerol, the dependence of D_{50R} with the temperature was also negative, non-linear and better adjusted by a sigmoidal curve (Fig. 1). Under the intermediary values of temperature of our interval, the temperature also potentiated the lysis of what would be the R state of the erythrocytes (intermediary region of the sigmoid). But under the superior temperature values of the interval, the increase in the temperature antagonized the lysis of the erythrocytes, probably by activation of the route 2 (Fig. 4).

But there was a difference between the curves of thermal dependence of the erythrocytes in saline solution of glycerol in relation to the curve only in saline solution (Fig. 1).

The addition of 1.5 M glycerol to the system promoted antagonistic effects on the stability of erythrocytes depending on the temperature (Fig. 1). Below 32°C, glycerol potentiated the chaotropic effect of ethanol, but above 37°C it acted in an antagonic manner, promoting stabilization of the erythrocytes (Fig. 1).

The explanation for these effects would be in the conjunction of the actions of ethanol, glycerol and temperature.

Below 32°C, the conjunction of the actions of the system ethanol, glycerol and temperature promoted exacerbation of the chaotropic action of the ethanol, although this chaotropic action had declined with the increase in the temperature in the presence of glycerol in relation to the saline solution without glycerol (Fig. 1).

Above 37°C, the conjunction of actions of that system promoted stabilization of the erythrocyte.

In whatever situation, ethanol is also an osmolyte and the increase in the temperature increase the osmotic pressure, what contributes to the osmostabilization of the erythrocyte. That is the reason by which under more elevated concentrations of glycerol and more elevated temperatures (route 2 of Fig. 4) there is stabilization of the erythrocytes (Gouvêa-e-Silva 2006; Reis 2007).

With the increase in the temperature, the system reach a critical value of osmotic pressure that would dislocates the equilibrium from the R to the T state of the erythrocytes (route 2 of Fig. 1). The incorporation of another osmolytes as glycerol or sorbitol will make the system to reach this critical value of osmotic pressure at a smaller concentration of ethanol, what would produce a significant diminution in the S_{50T} values, as indeed occurs (Reis 2007).

If this kind of effect illustrated in Fig. 4 is generated by the conjunction of the actions of ethanol, temperature and glycerol on the osmotic pressure, the dependence of D_{50R} with the osmolyte concentration also would exhibit a cooperative nature. Indeed, the dependence of D_{50R} with the glycerol concentration, at a unique temperature and in saline solution, presented a non linear nature that was better

adjusted to sigmoidal curve. The intermediary glycerol concentration of the curve activated the erythrocytes lysis, while the superior concentrations deactivated the lysis (Fig. 2).

This indicates that the elevation in the glycerol concentration is also a factor that promotes the deactivation of route 1 and activation of route 2 (Fig. 4).

This effect must be consequent to the conjunction of the actions of ethanol, temperature and glycerol.

However, each one of these agents can present specific individual actions in the system.

This is the case of ethanol, which is acting, in whatever situation, as a chaotropic and an osmostabilizer.

It is also the case of the temperature, which is in whatever situation acting as a chaotropic, but also as an osmostabilizing agent by promotion of elevation in the osmotic pressure.

But this would not be the case of glycerol, which in saline solution does not promote lysis of the erythrocytes inside the concentration range used in this work (Finotti 2006). However, 1.5 M glycerol in saline solution potentiates the chaotropic action of ethanol in the lower temperatures of the thermal interval considered (Fig. 1). This effect was also observed when glycerol (Finotti 2006) or sorbitol (Bernardino Neto 2006) were used at a 1 M concentration.

What would be the origin of the synergistic action of the osmolyte in the promotion of hemolysis by ethanol?

The answer to this question must be in the sigmoidal nature of the dependence curve of D_{50R} with the glycerol concentration (Fig. 2). The lower concentrations of glycerol are stabilizing the R state while the higher glycerol concentrations are stabilizing the T state of the erythrocytes.

The stabilization of the R state at the lower concentrations of glycerol will favor the chaotropic action of ethanol on this state, once it is a state with a lower stability than the T state of the erythrocytes.

This stabilization of the R state of the erythrocytes by glycerol favors the route 1 (Fig. 4), which represents the lysis of the R state by the chaotropic action of ethanol.

On the other side, it also justifies the erythrocytes stabilization by glycerol against hypotonic stress at 7°C (Fig. 3). While ethanol promotes lysis by its capacity to interact directly with he apolar groups of the membrane (Bakaltcheva et al. 1996; Chi and Wu 1991; Zavodnik et al. 1994), the hypotonicity promotes lysis by its capacity to favor the entrance of water and a volume increase in the erythrocyte (Jain 1973, 1986; Perk et al. 1964). The osmotic pressure generated by the presence only of 1.5 M glycerol, in the absence of ethanol and at a lower temperature (7°C) must prevent the water entrance and the rupture of the erythrocyte.

The osmostabilization of cells have been studied since Polge et al. (1949) discovered that the addition of glycerol to a sample of sperms increases the number of viable sperms after cryopreservation.

Our work does not deal with the ideal conditions to the cryopreservation of cells. Although the stability of the R state of the erythrocytes against ethanol decreases with the incorporation of glycerol to the solution with the decrease in the temperature until 7°C, at this temperature glycerol protects the erythrocytes against hypotonic shock. This protection is related to the capacity of glycerol to reduce the amount of intracellular water, minimizing the damages caused by freezing, but it is also related to its capacity to reduce the freezing temperature of the solvent. A more specific discussion about the benefic and toxic actions of glycerol in the cryopreservation involves questions of kinetic nature (De Loecker et al. 1993; Mazur et al. 1974; Mazur and Miller 1976; Mazur and Armitage 1984; Morris et al. 2006), which were not evaluated in this work.

The merit of our work is in the evaluation of the effects of glycerol at the more elevated temperature, what makes sense in physiological contexts as the treatment cerebral edema (Berger et al. 2005; Sakamaki et al. 2003) and the necessity to preserve the life in the presence of the global heating and the proper necessity to increase the longevity in face of the decrease in the number of births (Deocaris et al. 2006).

Conclusions

The values of D_{50R} decrease with the temperature increase in the absence and in the presence of 1.5 M glycerol according sigmoidal transition lines. This agrees with the idea that the temperature induces a two-state stability transition in the erythrocytes, from a lower stability (R) to a higher stability (T) state.

The values of D_{50R} also decreased with the increase in the glycerol concentration according a sigmoidal curve. This also agrees with the idea that glycerol also induces a two-state stability transitions in the erythrocytes, from a lower stability (R), present under the lower glycerol concentrations, to a higher stability (T) state, present under the higher glycerol concentrations.

The presence of 1.5 M glycerol diminishes progressively the stability of the erythrocytes with the increase in the temperature between 32 and 7°C, but increases the erythrocytes stability above 37°C. This behavior can be due to the existence of a critical osmotic pressure generated by the conjunct action of ethanol, glycerol and temperature. Below this osmotic pressure there would be predominance of the R state, but above that osmotic pressure there would be predominance of the T state of the erythrocytes.

Although the presence of 1.5 M glycerol diminishes the stability of the erythrocytes against the action of ethanol, it increases the stability against hypotonicity at 7°C, since the H_{50} values were significantly lower in the presence of

1.5 M glycerol than in the absence of this osmolyte. At this temperature, the conjunction of the actions of ethanol, temperature and glycerol would determine the predominance of R state of the erythrocytes, which has a lower stability and suffers more easily the chaotropic action of ethanol. However, in the absence of ethanol, the presence of glycerol will determine a lower entrance of water in the erythrocytes, what would make more difficult its lysis by hypotonicity.

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