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The Lyophilization of Dispersed Systems: Influence of Freezing Process, Freezing time, Freezing Temperature and RBCs Concentration on RBCs Hemolysis

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ABSTRACT In this work, we studied the influence of different parameters controlling cooling stage on biological dispersed system injury. The human red blood cell (RBCs) was chosen as work model. The study examined the influence of two freezing processes on RBCs hemolysis, one process producing big crystals, the other producing small crystals. Using both processes, we examined the effect of freezing temperature, freezing time, and RBCs concentration on injuries to RBCs. Freezing damage was assessed by the hematocrite measure before freezing and after thawing. The process producing a small number of big ice crystals (Pa) seems–in relation to the one producing a large number of small ice crystals (Pb)–to be less traumatic for the RBC, although the two are not statistically different. Freezing temperature and freezing time influence the preservation of RBCs. At 0 and –20°C there were high preservation and total hemolysis, respectively. At –5°C and –10°C, the RBC hemolysis depends on freezing temperature and freezing time. The RBCs hemolysis rates increases when freezing time increases and when freezing temperature decreases. The rates of RBCs preserved decreases with RBCs concentration some with either the freezing process used (Pa or Pb). More, an accentuation of the difference between the two used freezing processes on RBCs hemolysis was retrieved. The analysis of the conductivity evolution within the RBCs suspension frozen showed that the destruction of the RBCs is had essentially to the solution effects. When the crystallization eutectic takes place, the RBCs are already completely destroyed.

KEYWORDS Freezing process, Red blood cells, Crystallization, Lyophilization

INTRODUCTION

The biological (e.g., blood and cellular dispersions) and pharmaceutical (e.g., emulsions, classical suspension, and particulate drug carrier suspensions) dispersed systems often present stability problems as incompatibilities, drug release during storage and change of the particle structure. The freeze-drying process is often the solution recommended when the classical methods of stabilization or the formulation techniques

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prove fruitless. However, the protection of dispersed systems from the damaging effects of freezing remains indispensable. In the case of the biological dispersed systems, two mechanisms have been cited to explain injury of dispersed cells: intracellular ice formation at rapid cooling rates and solution effects at slow cooling rates (Han & Bischof, 2004). The methods used to warn cells injury are essentially: (i) addition of cryoprotective chemicals agents and (ii) determination of optimal cooling rates (Acker & McGann, 2003). Cryoprotective chemicals agents are essentially added to protect cells from eutectic crystallization (defined as simultaneous solidification of unfrozen fraction into solids) (Izutsu et al., 1995; Han & Bischof, 2004).

The freezing process can be characterized by the size of ice crystals obtained after freezing. The ice crystal sizes obtained check an important role in the porous structure of the obtained drayed product and in the easiness of its redispersion. Two essential phenomena control ice crystal formation: nucleation and crystal growth (Hallett, 1966). The predominance of one or the other of these two phenomena depends on cooling rates and temperature of storage. A temperature of –5°C favors the growth of the crystals and provokes nucleation, giving rise to a small number of big ice crystals (SNBIC). Temperatures of –30°C to –100°C favor nucleation, and the slow cooling rate produces a big number of small ice crystals (BNSIC). In earlier work, we showed the different ice crystallizations produced by these and similar congealing methods applied to an emulsion (Bensouda et al., 1988).

In the case of human red blood cells (RBCs), numerous studies have examined the influence of freezing on cells hemolysis, but to our knowledge no research has explained the influence of the crystal sizes obtained after freezing on RBCs integrity. Indeed, the essential parameter studied during RBC freezing has been cooling rates (Iijima, 1998; Rindler et al., 1999a; Rindler et al., 1999b; Zhao et al., 2004; Han et al., 2005) where spontaneous crystallization depends on different factors.

The objective of this work was to study the influence of the type of ice crystal obtained (BNSIC or SNBIC) on the dispersed systems injury. The RBCs were used as a work model. Also, the influence of freezing temperature, freezing time, and RBCs concentration on RBCs hemolysis were studied using the two freezing processes. The damages provoked by these factors was evaluated by the determination of the hematocrite before freezing and after thawing.

MATERIALS AND METHODS

Freezing

The freezing device is composed of a container (6 liters capacity), a cryogenic probe ETK 50 (–50°C) (Lauda, RFA), a heating thermostat (–40/+100°C) (Techne ED8, RFA), a stirrer (Heidolph, RFA), transparent plastic containers with flat bottoms (3.5-cm diameter), and glycerined ethanol as a bath liquid. All these elements are arranged as shown in Fig. 1.

Conductivity

Digital conductivimeter (Amel134, Italy) and plotter (Laumann DLH 250, RFA).

Biological Dispersed System

Venous human blood from healthy donors was collected in 4.5 mL tubes containing EDTA (BD Vacutainer* K3E 15% 0.054mL, UK). This blood was preserved at 4°C and used within 24–28 h.

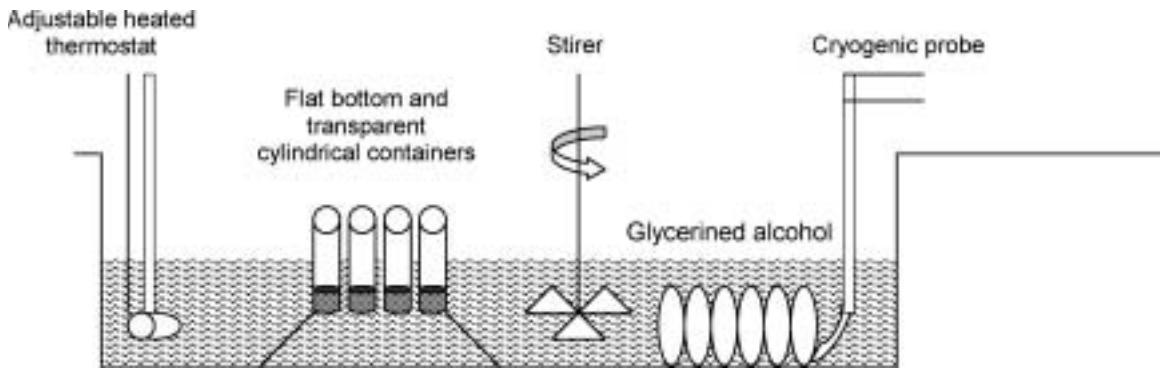

FIGURE 1 Freezing Device.

In all experiments, blood was washed with an isotonic Nacl solution (Laprophan Maroc) and centrifuged at 2000 t/mn for 5 min., followed by removal of the plasma. Washing was repeated three times.

Congealing Processes

Two freezing methods were used to obtain respectively a small number of big ice crystals (SNBIC) (see process a [Pa] in Fig. 2A) and a big number of small ice crystals (BNSIC) (see process b [Pb] in Fig. 2B). Both methods were applied to a 2 mL product volume (200µl of concentrated RBCs and 1.8 mL of isotonic mannitol solution). The isotonic mannitol solution was used in this work to maintain consistency with our previous work. In fact, this product was used in our previous work to identify the types of crystals obtained after freeze-drying according to the chosen freezing process.

Freezing Process Pa

The bath temperature was stabilized at –5°C; the addition of a small ice crystal provoked nucleation, producing a small number of big ice crystals (SNBIC) and raising the product temperature to approximately 0°C. The influence of freezing time and freezing temperature were studied by slowly decreasing the temperature after crystallization of the dispersion.

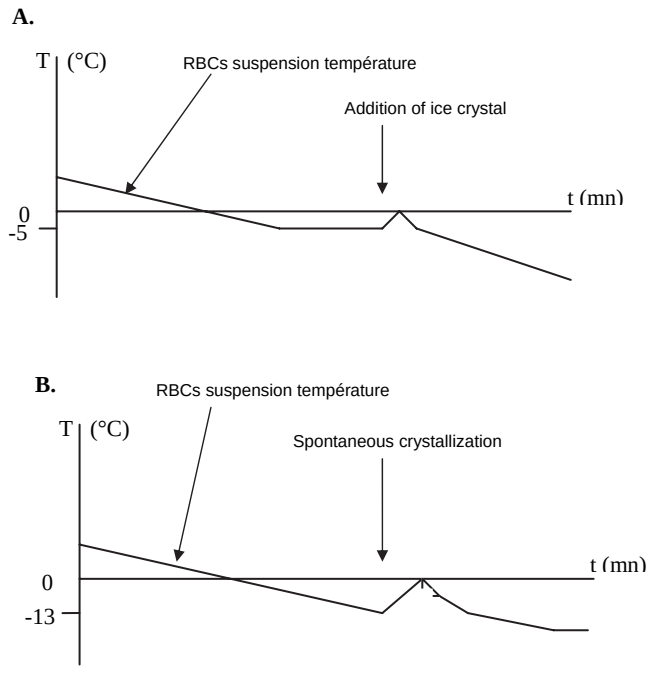

FIGURE 2 **(A) Freezing Process (Pa) Producing a Small Number of Large Ice Crystals, (B) Freezing Process (Pb) Producing a Big Number of Small Ice Crystals.**

Freezing Process Pb

After a slow decrease of the bath temperature (–0.78°C/mn), a spontaneous nucleation appears at about –13°C, producing a big number of small ice crystals (BNSIC) and raising the product temperature to approximately 0°C. A temperature gap is avoided by raising the bath temperature to approx. 0°C. The influence of freezing time and freezing temperature were studied by slowly decreasing the temperature after crystallization of the dispersion.

Assessment Method

The crystallization damage was assessed by the hematocrite measure before freezing and after thawing.

RESULTS AND DISCUSSION

Influence of Freezing Process, Freezing Temperature and Freezing Time

Fig. 3 shows the rates of RBC preservation after freezing with the two processes at different freezing temperatures and freezing times. With Pa, the rates of RBCs destroyed at 0°C varied from 5 to 10 % as freezing time increased. The lethal effect of the freezing process over time was evident at low temperatures. Thus, the speed of cell injury increased as the freezing temperature decreased and when freezing time increases some either the temperature of storage used (0°C, –5°C, –10°C and –20°C). At 0 and –20°C, there was respectively a high preservation and a total hemolysis. At –5 and –10°C, the hemolysis depended on time and freezing temperature. Earlier research has shown similar results about liposomes (Franzen et al., 1986). At –5°C, after 2 h of storage, 45% and 50% of RBC were destroyed with Pa and Pb respectively. After 30 min. at –10°C, 60% and 75% of RBCs were destroyed with Pa and Pb respectively; after 2 h they were completely destroyed. At –20°C, the RBCs were destroyed immediately with Pb and after 15 min. with Pa. Although the Pb seems more lethal than the Pa, they were not statistically different. This difference decreased globally when the freezing temperature decreased and freezing time increased. The difference observed between the two process in percentages of RBCs destroyed, especially at 0°C, can be explained by the

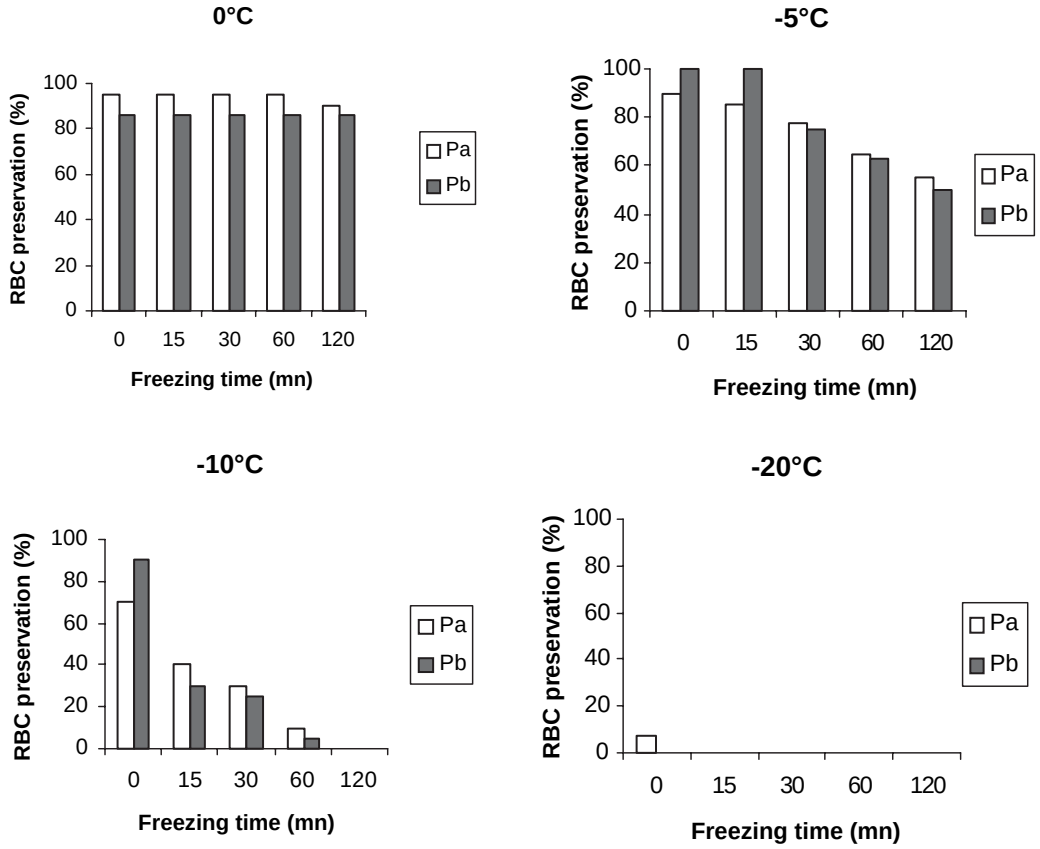

FIGURE 3 **Evolution of the Rate of RBCs Preserved (%) According to Freezing Time and Freezing Temperature. The Rate of Preserved RBCs Was Calculated by Hematocrite Measure Before Freezing and After Thawing.**

amount of the unfrozen fraction more elevated just after freezing of RBCs dispersion (De Loecker et al., 1998; Han & Bischof, 2004). For freezing temperatures below 0°C and when the freezing time is increased, other mechanisms can be evoked. Lovelock (Lovelock, 1953) reported that at slow cooling, the major mechanism in RBC injury is solution effect. This effect corresponds to the increase in the electrolyte concentration of the unfrozen portion, caused by the growth of the ice crystal. Other research (Han & Bischof, 2004) reported that slow cooling rates accentuated this effect. The 1998 work of De Loecker et al. on RBCs and hepatocytes showed that RBCs destruction by crystallization remains likelier. Kristiansen's 1992 work on liposomes reported that eutectic crystallization might be detrimental to a dispersed system during a freeze-drying process. However, his theory has not been confirmed by tests on cells.

In an attempt to explain the mechanism of RBCs injury, follow-up on conductivity evolution within the frozen RBCs suspension was performed with a digital conductimeter (Amel134, Italy). The containers that would hold the electrode to measure conductivity and the subsequent hematocrite evolution were prepared and frozen in identical conditions. Hematocrite measurements are performed on the frozen containers removed each to intervals fixed in advance. The frozen RBCs are practically destroyed after 76 mn of freezing in these conditions. Conductivity values reached zero ≈7 mn after. The RBCs destruction seems to owe essentially to solution effect and not to eutectic crystallization. In fact, when the eutectic crystallization takes place, the RBCs are already completely destroyed. This effect is observed both when freezing time increases and freezing temperature decreases.

Influence of Initial Red Blood Cells Concentration

Five different RBCs concentrations (3.125, 6.25, 12.5, 25 and 50%) were prepared without a cryoprotective agent in the isotonic mannitol solution and frozen by processes Pa and Pb then brought to –5°C during 1 h. This freezing time and freezing temperature were chosen to better illustrate the effect of RBCs concentration on RBCs preservation. The results obtained are shown in Fig. 5.

Results showed that, after freezing and thawing by the two processes Pa and Pb, the rate of RBCs preserved decreases when the RBCs concentration

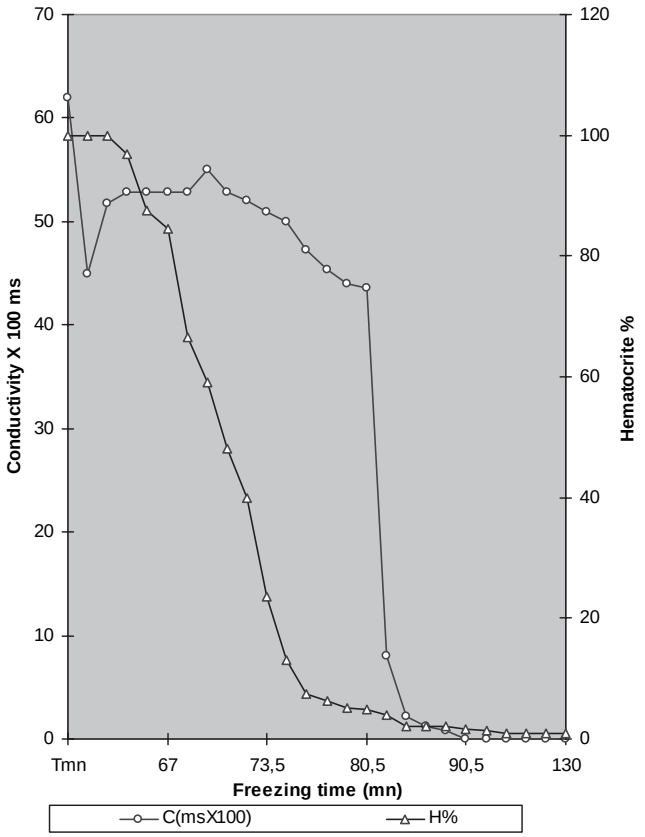

FIGURE 4 **Correlation Between Conductivity Evolution Within the RBCs Suspension and the Rate of RBCs Preservation.**

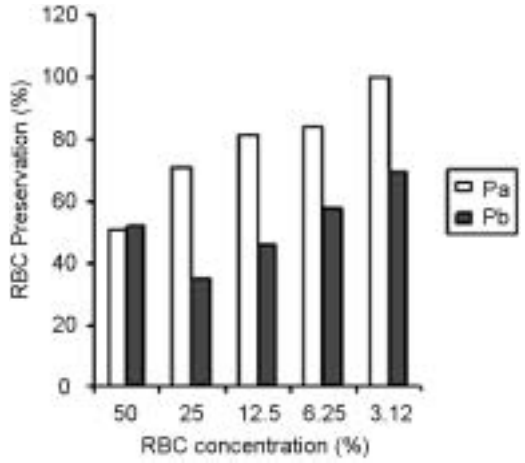

FIGURE 5 **nfluence of RBCs Concentration and Type of Freezing Process on RBCs Preservation. The Suspensions of RBCs Were Frozen by the Two Processes and Maintained at −5°C for 1 h.**

decreases. In 1998, De Loecker et al., using a cryoprotective agent, found similar results on RBCs and hepatocytes. The maximum preservation rates obtained at a low concentration (3.25%) were 100% and 70% with Pa and Pb respectively. At a concentration of 6.25%, these rates were 80% and 60%; at 12.5% rates were 78% and 40%; at 25% they were 70% and 30%, and at 50% the rates were 50% and 50% respectively. The accentuation of differences between the two freezing processes was explained by the size of the unfrozen fraction. The influence of cell concentration on cell injury remains poorly understood, but most important likely is the stress caused by non-physiological cell-to-cell contact during the freeze-thaw cycle (De Loecker et al., 1998).

CONCLUSIONS

In the present work, the cell injuries caused by the two freezing processes were not statistically different. However, Pb seems more traumatic than Pa. The RBCs preservation decreases with the decrease of freezing temperature and with the increase of freezing time, and it increases with cell dilution. The solution effect seems to be the major mechanism responsible of the RBCs injury. The obtained results in this work are able confronted to those obtained with the other pharmaceuticals dispersed systems.

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