

## In vitro efficacy of frozen erythrocytes: implementation of new strategic blood stores to alleviate resource shortage (issue revisited)

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**Background/aim:** Currently, the provision of blood products largely depends on walking blood banks and limited amounts of stored blood with short shelf lives. We aimed to compare the efficacy of erythrocyte concentrate (ECs) by pre- and postfreezing in vitro tests.

**Materials and methods:** In our study, 10 ECs were glycerolized, frozen, thawed, and then deglycerolized using the Naval Blood Research Laboratory method. In addition to using the standard tests, ATP and 2,3-DPG levels and the viability of erythrocytes were also determined.

**Results:** The prefreezing mean viability rates of erythrocytes changed from  $89.7 \pm 13.7\%$  to  $98.6 \pm 1.8\%$  after thawing and deglycerolization. Prefreezing and day 0 ATP levels ( $1.64 \pm 0.15 \mu\text{mol/g Hb}$  and  $1.81 \pm 0.14 \mu\text{mol/g Hb}$ , respectively) were similar. The 2,3-DPG levels decreased from  $18.09 \pm 4.78 \mu\text{mol/g Hb}$  measured before the procedure to  $10.41 \pm 4.58 \mu\text{mol/g Hb}$  on day 0. The mean hemolysis rates and supernatant Hb levels changed from  $0.21 \pm 0.11\%$  to  $0.36 \pm 0.12\%$  and  $1 \pm 0.5 \text{ g/L}$  to  $1.5 \pm 0.5 \text{ g/L}$ , respectively.

**Conclusion:** The test results showed the efficacy of the frozen-thawed ECs to be used in humans for a broad spectrum of clinical indications. As a part of a contingency plan, national frozen blood reserves need to be established.

**Key words:** Cryopreservation, erythrocyte concentrates, blood transfusion, flow cytometric analysis

### 1. Introduction

Freezing erythrocytes was first attempted by Félix-Archimède Pouchet in 1866, and fractionation after thawing was reported (1). In 1950, Smith postulated that glycerol could prevent erythrocyte damage due to freezing and was the first to freeze erythrocytes without glycerol-related loss of cell function. Mollison, using the system discovered by Smith, performed the first successful transfusion of frozen erythrocyte concentrate (EC) in 1951 (2–4). The US Food and Drug Administration (FDA) approval for frozen ECs with glycerol to be stored for later use at  $-80^\circ\text{C}$  for 10 years was announced in September 1987. In 2010, the EC shelf life was extended to 30 years in European countries (5,6). Following the deglycerolization of frozen ECs after years of storage, their shelf life can be

extended to 7 or 14 days by adding saline, adenine, glucose, and mannitol (SAG-M) or AS-3 solution, respectively (7–10).

The storage of frozen ECs has become an essential part of national blood crisis policies in several countries, as frozen ECs can be thawed and transfused rapidly in emergency situations without the need for laboratory tests. ECs are also cryopreserved for patients with rare blood types and are autologous blood for patients with red blood cell alloantibodies; clinical conditions that require cytomegalovirus (–) blood can also be considered in this context (11).

Although fresh ECs can be stored for up to 42 days by introducing additive solutions, they can be preserved for an extended time period by freezing in an efficient and

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reliable manner (11). Thus, cryopreservation of expired ECs, instead of disposal, and their use after thawing can not only prevent the inappropriate waste of resources but will also allow the use of ECs in times of need.

This study presents the *in vitro* assay data of 10 ECs that were frozen at  $-80\text{ }^{\circ}\text{C}$  with 40% glycerol using the Naval Blood Research Laboratory (NBRL) method and were thawed after 24 h at the Blood Training Center and Blood Bank of Gülhane Military Medical Academy. Additionally, the need to establish the national blood policy of Turkey, including the use of frozen ECs at times of peace and war, was addressed by reviewing the experiences gained by other countries during the historical development of frozen ECs.

## 2. Materials and methods

After receiving ethical committee approval and informed consent from all participants, 450-mL whole blood donations from 10 donors were collected in 3 bags (Kansuk Blood Bags with 63 mL of CPD anticoagulant, CE 0197, Turkey). Whole blood samples were centrifuged at  $22\text{ }^{\circ}\text{C}$  and 4000 rpm for 20 min using a Sorvall RC12BP centrifuge (Thermo Scientific RC12BP, USA). Plasma was extracted by using a bag press (Bioelettronica, Italy). ECs were then transferred into Kansuk Blood Bags containing 100 mL of SAG-M.

White blood cell (WBC), hemoglobin (Hb), hematocrit (Htc), pH, supernatant Hb, adenosine triphosphate (ATP), and 2,3-diphosphoglycerate (2,3-DPG) levels and the osmotic fragility and viability of ECs were analyzed before the procedure, after deglycerolization (day 0), and on day 14 of the storage of the thawed components at  $4\text{--}6\text{ }^{\circ}\text{C}$ . SAG-M was separated before glycerolization using an extractor following a 4-min centrifugation at  $20\text{ }^{\circ}\text{C}$  and 1623 RCF ( $\times g$ ). The ECs were weighed and Htc measurements were recorded. The NBRL method was used for glycerolization and deglycerolization (12).

### 2.1. Glycerolization

The 1-day-old ECs were glycerolized in a closed system with 57.1% glycerol using the Haemonetics ACP 215 device (glycerol 57.1% 500 mL, S.A.L.F. Laboratorio Farmacologica, Italy; Haemonetics RBC Glycerolization Set Ref 225, USA), and the glycerolized ECs at the targeted concentration of 40% were obtained. To remove the excessive glycerol, a 10-min centrifugation was performed at  $20\text{ }^{\circ}\text{C}$  and  $1283 \times g$ . The excessive glycerol was transferred into an empty bag using a Terumo sterile tubing welder (TSCD II, Terumo Europa N.V., Belgium). Glycerolized ECs were placed in a cardboard box and kept in deep-freeze (Arctiko AS ULUF750, Denmark) at  $-80\text{ }^{\circ}\text{C}$  for 24 h.

### 2.2. Deglycerolization

Frozen glycerolized ECs were thawed at  $37\text{ }^{\circ}\text{C}$  over a period of 10–12 min (Helmer DH 8, USA). ECs were processed

using Haemonetics ACP 215 with 12% NaCl, 0.2% glucose + 0.9% NaCl, and SAG-M solutions, respectively (RBC De-Glycerol Ref 235, Haemonetics Corporation USA; solution NaCl 12% from Bioluz Laboratoire Pharmaceutique; solution glucose 0.2% + NaCl 0.9% from Bioluz Laboratoire Pharmaceutique; SAG-M from Haemonetics Corporation USA).

### 2.3. *In vitro* measurements

All biochemical analyses were performed at the Medical Biochemistry Laboratory of Gülhane Military Medical Academy. The blood cold-chain principles were strictly followed. pH analyses were performed within 2 min after collecting the samples using an ABL 835 FLEX blood gas analyzer (Radiometer Medical ApS., Denmark). The samples' whole blood counts at the time of receipt were performed using the ABX Pentra XL80 instrument (HORIBA ABX SAS, France). The preparation of resuspended erythrocytes and hemolysate was performed in the method described by Erlandsen et al. (13). All the samples, including supernatant and hemolysate, were kept at  $-80\text{ }^{\circ}\text{C}$  for ATP, 2,3-DPG, and supernatant hemoglobin concentration measurements.

For the quantitative determination of hemolysate ATP, the ENLITEN ATP Assay System Bioluminescence Detection Kit (Promega Corporation, USA) was used. All the processes were performed by following the instructions of the kit. For the quantitative determination of hemolysate 2,3-DPG, the Cusabio Human 2,3-DPG ELISA Kit (CSB-E09861h, CUSABIO, China) was used. All the processes were performed by following the instructions of the kit.

The measurements of free hemoglobin concentrations were performed according to the cyanomethemoglobin method (14,15). Normal-level Hema-Trol Hb control material (NERL HEMA-Trol Whole Blood HGB Controls, Product Number HYC84667, Thermo Fisher Scientific Inc., USA) was used as the standard. Following suitable dilutions, the samples and standards were added to the microplate with Drabkin's solution in the wells. All the standards and samples were analyzed in duplicate. The Synergy HT Multi-Mode Microplate Reader and KC4 Software (BioTek Instruments Inc., USA) were used for luminescence and optic density (absorbance) readings. The formulation of Valeri et al. was used to calculate hemolysis ratio before and after the steps (16).

Erythrocyte samples were transferred to 3-mL tubes containing heparin and were mixed for the evaluation of osmotic fragility. The quantitative tube method was used. The NaCl concentration of the tube in which hemolysis started was recorded. Presence of hemolysis at 0.45%–0.55%,  $>0.55\%$ , and  $\leq 0.30\%$  NaCl concentrations was defined as normal, increased, and decreased osmotic fragility, respectively.

## 2.4. Erythrocyte identification and viability assessment with flow cytometry

Erythrocyte samples were transferred to tubes containing K<sub>3</sub> EDTA. The 2 µL-samples were transferred to a polystyrene tube, 3 mL of phosphate buffer solution (PBS) was added, and samples were resuspended and then washed twice at 1500 rpm for 10 min (following centrifugation, discarding the supernatant, and resuspending the pellets underneath). The pellet at the bottom was resuspended with 250–300 µL of PBS (Becton Dickinson, USA) and prepared for the study. To label the erythrocytes, 2 µL of CD235a FITC (BD Pharmingen, USA) was transferred to another polystyrene tube to be studied, and 100 µL of the erythrocyte sample was added. The cells were vortexed gently for 3 s and were incubated in the dark at room temperature for 45 min. Vortex was performed every 10 min. Cells with disturbed integrity whose membranes became permeable and therefore not viable were penetrated. A volume of 5 µL of cytoplasm-staining nucleic acid dye 7-aminoactinomycin D (7-AAD) was added during the last 10 min of incubation (BD Pharmingen). Following incubation, 500–600 µL of PBS was added to prepare for analysis in flow cytometry. The samples were analyzed using the FACSDiva software of FACSCanto II model flow cytometry (BD Biosciences, USA). Viable erythrocytes were identified as cells stained either positive with CD235a FITC or negative with 7-AAD (Figure 1). A total of 20,000 cells were counted for analysis, and the ratio of viable erythrocytes was expressed as percentage.

## 3. Results

The mean Hb and Htc levels were  $198 \pm 21$  g/L and  $57.8 \pm 6.4\%$ , respectively, in the before-procedure period. The levels decreased to  $144 \pm 24$  g/L and  $43.9 \pm 6.6\%$ , respectively, on day 0. The supernatant Hb levels that denote the hemolysis ratio were similar in both the before-

procedure and the day 0 measurements ( $1 \pm 0.5$  g/L and  $1.5 \pm 0.5$  g/L, respectively); the day-14 supernatant Hb levels were increased ( $3.5 \pm 1.2$  g/L). Accordingly, hemolysis ratios calculated using supernatant Hb and Hb measurements were increased on day 14 (Table 1).

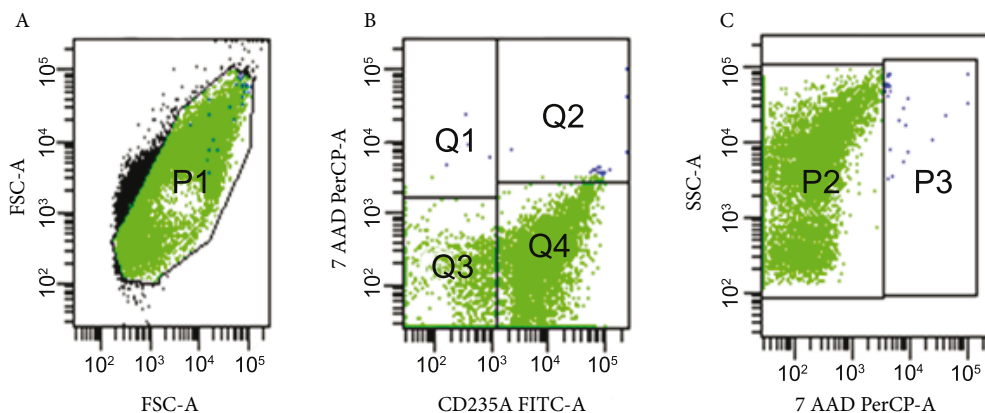
Osmotic fragility test results in all 3 periods of the study were similar to each other. The before-procedure mean osmotic fragility was  $0.57 \pm 0.03$ , compared to  $0.63 \pm 0.02$  and  $0.67 \pm 0.04$  on days 0 and 14, respectively (Table 1).

Procedures that were performed during the deglycerolization process resulted in leucodepletion, as the before-procedure WBC count of  $(5.6 \pm 3.2) \times 10^9/L$  decreased to  $(0.1 \pm 0.04) \times 10^9/L$  in the after-procedure period. After the deglycerolization process, the mean pH levels decreased from  $7.0 \pm 0.03$  to  $6.5 \pm 0.02$  (day 0) and  $6.3 \pm 0.04$  (day 14) (Table 1).

ATP levels, among the criteria like 2,3-DPG that show the oxygen-carrying capacity of erythrocytes, were similar in the before-procedure period and on day 0 ( $1.81 \pm 0.14$  µmol/g Hb and  $1.64 \pm 0.15$  µmol/g Hb, respectively). However, 2,3-DPG levels were decreased in the after-procedure period. Before the procedure, the mean 2,3-DPG level was  $18.09 \pm 4.78$  µmol/g Hb, compared to  $10.41 \pm 4.58$  and  $4.54 \pm 3.23$  µmol/g Hb on days 0 and 14, respectively (Table 2).

## 3.1. Viable erythrocyte ratios assessment by flow cytometric analysis

Before the procedure, the mean total erythrocyte percentage was  $86.7 \pm 8.9\%$ , compared to  $98.5 \pm 1\%$  and  $98.6 \pm 0.6\%$  on days 0 and 14, respectively. Before the procedure, the mean glycophorin A (+) cell percentage (CD235a) was  $71.7 \pm 17.6\%$ , versus  $90.2 \pm 7.8\%$  and  $81.7 \pm 6\%$  on days 0 and 14, respectively. Before the procedure, the mean 7-AAD (–) (viable) cell percentage was  $89.7 \pm 13.7\%$ , versus  $98.6 \pm 1.8\%$  and  $10.6 \pm 2.5\%$  on days 0 and 14, respectively (Table 2).



**Figure 1.** Flow cytometric analysis of the erythrocytes. The location of all cells was determined based on FSC (size) and SSC (granularity). **A)** Total erythrocyte population. **B)** Cells in the lower right quadrant are cells carrying the glycophorin A antibody but not the 7-AAD antibody (viable). **C)** The P2 quadrant shows live erythrocytes, while P3 peripheral shows nonviable erythrocytes with 7-AAD antibodies.

**Table 1.** Hb levels, Htc levels, osmotic fragility, supernatant Hb levels, and hemolysis ratios of ECs before the procedure and on days 0 and 14. \*B-p: Before procedure.

Cases	Hb, g/L			Htc, %			Osmotic fragility			Supernatant Hb conc., g/L			Hemolysis, %		
	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14
1	220	183	197	64.6	54.1	56	0.55	0.65	0.65	1.63	2.05	3.52	0.29	0.39	0.62
2	216	172	181	61.6	52	52.2	0.55	0.65	0.65	1.43	1.63	2.78	0.26	0.33	0.53
3	173	168	165	50	49.5	48.2	0.60	0.60	0.75	1.89	1.98	5.22	0.43	0.41	1.09
4	217	145	158	61.9	45.1	47.1	0.55	0.65	0.65	0.90	1.17	3.99	0.16	0.28	0.87
5	196	132	155	58.3	41.5	46.1	0.55	0.65	0.65	0.55	1.08	2.18	0.11	0.28	0.49
6	206	140	159	60	43	46.6	0.60	0.60	0.65	1.43	2.27	2.42	0.27	0.56	0.53
7	202	143	187	60.2	43.9	55.7	0.55	0.65	0.65	1.26	1.45	4.32	0.24	0.35	0.80
8	151	125	135	43.3	37.4	39	0.55	0.60	0.75	0.59	1.70	5.57	0.15	0.47	1.43
9	203	127	127	59.4	39.3	38.5	0.65	0.65	0.65	0.48	1.52	3.04	0.09	0.41	0.83
10	201	106	108	58.5	32.8	32.7	0.60	0.65	0.65	0.33	0.37	2.25	0.06	0.12	0.72
Mean	198	144	157	57.8	43.9	46.2	0.57	0.63	0.67	1.0	1.5	3.5	0.21	0.36	0.79
SD	21	24	28	6.4	6.6	7.6	0.03	0.02	0.04	0.5	0.5	1.2	0.11	0.12	0.29

**Table 2.** Glycophorin A (+) cell, total erythrocyte (CD235a), and viable erythrocyte [7-AAD (-)] percentages assessed by flow cytometry, ATP, and 2,3-DPG levels of ECs before the procedure and on days 0 and 14. \*B-p: Before procedure.

Cases	Total erythrocytes, %			CD235a (+), %			7-AAD (-) erythrocytes (viable erythrocytes), %			ATP, µmol/g Hb			2,3-DPG, µmol/g Hb		
	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14	B-p.*	Day 0	Day 14
1	80.3	97.7	99.5	97.1	92.4	92	99.7	99.9	7.6	1.629	1.601	0.888	26.208	4.195	1.693
2	92.1	99.4	97.5	94.1	92	83.1	91.9	99.6	12.1	1.644	1.428	0.735	18.687	11.599	8.487
3	78.8	98.5	98.5	69.9	91.7	84.8	99.5	99.4	6	1.817	1.532	0.418	14.481	13.141	3.411
4	75.8	95.9	99.5	61.7	69.5	81	98.6	96.9	12.2	1.674	1.570	0.738	17.756	14.410	8.491
5	86.8	98.6	98	83.6	95.9	69	99.6	99.3	11.4	1.82	1.618	0.371	22.242	13.331	8.302
6	97.6	99.7	98.3	75.7	96.4	80.2	98	99.8	10.5	1.915	1.822	0.847	11.764	8.578	4.849
7	87.7	99	98.6	52.2	90	79	99.2	98.7	11.9	2.043	1.583	0.835	23.025	18.505	1.016
8	74.8	98.5	98.9	68.5	87.2	86	67.2	99.2	9.3	1.748	1.569	0.637	19.723	9.147	6.708
9	97.1	99	98.6	74	93.5	84	68.8	99.6	10.3	1.977	1.916	0.718	13.031	5.194	1.086
10	96.4	99.3	98.8	40.2	94.1	78	74.7	94.1	14.9	1.807	1.786	0.716	14.002	6.049	1.355
Mean	86.7	98.5	98.6	71.7	90.2	81.7	89.7	98.6	10.6	1.81	1.64	0.69	18.09	10.41	4.54
SD	8.9	1	0.6	17.6	7.8	6	13.7	1.8	2.5	0.14	0.15	0.17	4.78	4.58	3.23

**4. Discussion**

There are currently 2 methods available for long-term storage of erythrocytes: the low-glycerol method for freezing blood products in liquid nitrogen at -196 °C, and

the high-glycerol method for storage at -80 °C (6). The most commonly used EC-freezing technique is the high-glycerol technique, which was defined by Valeri et al.; it involves 40% (w/v) glycerolization of the erythrocytes

followed by storage at  $-80\text{ }^{\circ}\text{C}$ . When ready for use, the frozen ECs are thawed and deglycerolized, which we also did in our study (12).

Although this technique has been available for more than 4 decades, it has never reached the desired popularity due to its labor-intensiveness, cost, infrastructure necessary for the procedure, maintenance, and limited shelf life of thawed erythrocytes. However, this scenario has been changed by the use of the NBRL method, which has resulted in significantly less processing time and increased shelf life, from 24 h to 14 days after thawing (17).

ATP and 2,3-DPG are important determinants of erythrocyte  $\text{O}_2$  transport quality. Chaplin reported that the frozen-thawed EC ATP levels were close in content to the fresh donor EC levels (18). In our study, the ATP levels were similar in both the before-procedure period and on day 0. Our 2,3-DPG results on days 0 and 14 were lower when compared to the before-procedure measurement ( $10.41 \pm 4.58$ ,  $4.54 \pm 3.23$ , and  $18.09 \pm 4.78\text{ }\mu\text{mol/g}$ , respectively). Additionally, our day 0 results were in accordance with the report of Valeri et al. which compared 4 different methods of freezing ECs (12).

Supernatant Hb, hemolysis, pH, and osmotic fragility levels in ECs were also examined in our study. Our hemolysis percentages and supernatant Hb levels on day 0 were close to the before-procedure levels. The osmotic fragility ratios were increased on day 0. The increased fragility ratios were due to factors (multiple washings/centrifugation, use of cryoprotectant agent, exposure to low temperature, hypertonic solutions during deglycerolization, etc.) that decrease the membrane stability. However, increased osmotic fragility ratios had no effect on erythrocyte functions except for the decreased shelf life after deglycerolization.

Htc and Hb levels of frozen ECs should be  $\geq 38\%$  and  $36\text{ g/dL}$ , respectively, in order to meet the quality control criteria of the ACP 215 system (<http://www.nbrl.org/SOP/ACP215/ACP215All.html>). In our study, both Htc and Hb levels met these criteria. Additionally, these findings were close to the results of a multicenter study that assessed the efficacy of EC processing with the ACP 215. After deglycerolization, the mean pH level was 6.5 in our study. This result was also in accordance with the pH test results of this multicenter study (9).

In vivo and in vitro qualities of frozen ECs have been well established by many studies (9,19). However, viability of erythrocytes has also been studied by flow cytometry analysis in our study. Interestingly, the mean viability of erythrocytes was 89.7% before the procedure and 98.6% on day 0. This result can also be attributed to the repeated washing (5 times) of the cells during the deglycerolization process. The washing process removes nonviable erythrocytes, which may have contributed to

the results observed in our study. The washing process also removes biologically active substances that may contribute to immunomodulatory effects in the recipients of blood products and leads to significant reduction in transfusion reactions (17).

WBC counts were  $<0.1 \times 10^9/\text{L}$ . However, the flow cytometry analysis showed that these cells counted by the automated blood count instrument were not, in fact, WBCs. The freezing process itself and repeated washings of ECs decrease the WBCs as much as filtered ECs.

The AS-3 solution has been validated for a shelf life of 14 days and was used in all other studies (8,9,11). To our knowledge, only the SAG-M additive solution, which is validated for 7 days, is available in Turkey. The data depicted in Tables 1 and 2 include day 14 SAG-M values. The day 14 results show significant differences compared to day 0 results. If the freezing of ECs is to be implemented in Turkey, the AS-3 solution with ACP 215 is definitely required for the sake of frozen-thawed ECs with a longer shelf life.

Although the FDA approved their use after storage for up to 10 years, the exact storage limit of frozen ECs has not been delineated. Peyrard et al. demonstrated the efficacious use of frozen ECs after a storage period of 10 to 20 years. They also reported that, despite recommendations, no official mandatory time limit regarding the validity period of rare frozen EC units exists in France (6).

The essential role of frozen ECs has been recognized and is currently in use in the United States, Germany, the Netherlands, France, and some other countries (3,6,20). It has become a part of their national contingency plans, which include military conflicts, bioterrorism, epidemics, pandemics, and natural disasters.

Many reports related to the clinical use of frozen ECs have been published in recent years. For example, in France, 1957 ECs have been thawed and transfused since 1994, among which 118 were cryopreserved for  $\geq 10$  years and 8 for  $\geq 20$  years. Regarding these transfused ECs, no clinical or biologic evidence of hemolysis of thawed ECs or transfusion reactions have been reported (6). Currently, Australian troops deployed in Afghanistan are supported by the Dutch military blood reserves in that area. They were supplied 132 frozen-thawed ECs, 75 deep-frozen plasma units, and 22 frozen-thawed platelet units for the treatment of 17 casualties. Afterwards, the Australian Defence Force and the Australian Red Cross blood service started to investigate the applicability of frozen blood products for therapeutic purposes to their national regulatory framework (20).

Although a worst-case scenario had become true in the Gölçük earthquake, its overall effects on medical infrastructure and related effects on the casualties have not been reported yet. Abolghasemi et al. reported that

during the earthquake in Bam, almost all medical facilities were damaged as thousand were killed and injured (21). Thus, strategic plans for all worst-case scenarios have been developed, involving vigilant and mobile systems for frozen ECs for the Turkish National Defense and National Blood Crisis Policy.

Criticism regarding the cost of technical equipment and all additional requirements may limit the use of frozen ECs. This cost, however, seems negligible under austere circumstances where there are no applicable alternatives

to frozen ECs. Lessons learned by the Turkish military indicate that saving lives far outweighs the cost of all efforts.

A huge body of literature data and experience has been already gathered over the years on the preparation, storage, and use of ECs. Given the earthquake-prone and strategic location of Turkey, there is a clear and urgent need to establish both military and civilian frozen EC stocks with the cooperation of the Turkish Armed Forces, the Turkish Red Crescent, and other related organizations.

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