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13. ABSTRACT (Maximum 200 words)

The Bionetics Corporation staffed and maintained a research laboratory to support red blood cell preservation research at the Blood Research Detachment of the Walter Reed Army Institute of Research, 1413 Research Boulevard, Rockville, MD 20850. Contract staff completed data collection on two clinical trials evaluating candidate red cell preservation systems intended for eight week storage. Preliminary data evaluation showed that neither the hypotonic storage medium system tested in the first trial nor the prestorage leukocyte reduction system evaluated in the second trial met the FDA criterion of at least 75% twenty-four hour post transfusion red blood cell survival at the end of eight weeks of storage. We initiated a third clinical trial to evaluate the effects on red cell survival of twenty-four hours at room temperature either early of late in the storage of AS-5 preserved red cells. We also completed an *in vitro* study evaluating the effect of warming both early and late in storage of CPDA-1 preserved red cells. Warming for twenty-four hours at 25°C appears to accelerate the storage lesion equivalent to a week in the refrigerator. The Bionetics Corporation advanced the Blood Research Detachment's mission.

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16 Oct 97

PI - Signature Date

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INTRODUCTION

Nature of the Problem:

Because combat is synonymous with bloodshed and blood replacement saves lives, the US Army Medical Research and Material Command maintains facilities and programs to develop improved blood products.

The Background of the Previous Work:

The US Army has, for decades, conducted research in red blood cell preservation and the production of acellular hemoglobin solutions for use in combat casualty care. From 1974 through 1992, that research took place at the Letterman Army Institute of Research (LAIR) located at the Presidio. The LAIR facility was closed as the result of Base Realignment and Closure actions and the Blood Research Detachment was relocated to leased laboratory space at 1413 Research Blvd., Rockville, Maryland. On 19 September 1994 The Bionetics Corporation (TBC) was awarded a contract to operate and maintain equipment and provide technical support to the Blood Research Detachment. For the first two years of the contract, the contractors supported the Hemoglobin Production Facility (HPF) and integral analytical chemistry laboratory which provided quality control and characterization testing. The HPF produced several hundred liters of a precision hemoglobin based blood substitute material for research. The acellular hemoglobin solutions manufactured by the HPF were based on biochemical modification of stroma-free hemoglobin as described in the literature. [1,2,3 and 4] During the last several months of HPF operation, the contract staff also supported the US Navy lipisome encapsulated hemoglobin research.[5,6] HPF related activities were terminated on 20 September 1996 and the facility closed.

Contract staff has continuously operated and maintained the blood cell preservation research of the Blood Storage Laboratory (BSL), a fully equipped red cell research laboratory staffed by 3 full-time and 1 part-time employee. The Blood Banking Specialist member of this

team is also the Project Manager. A list of contract staff as of the close of the FY is included at Appendix 1.

The currently Food and Drug Administration (FDA) licensed anticoagulant, preservative solutions allows storage of red blood cells at 4^0 C for 42 days after collection. Work by Meryman *et. al.*[7,8] and Greenwalt, Dumaswala and colleagues [9,10] indicates potential for extended storage. Greenwalt and colleagues have recently developed an experimental additive solution which preserves red cells for 56 days with average red cell survival at least 75%.[11]

It is estimated that 1 million units of blood expire per year in the United States. It is further estimated that if expiration were extended to 8 weeks, one-third of the expired units now discarded would be transfused.[12] Extended shelf-life of liquid stored blood would have significant utility to the Armed Services Blood Program as it supports the Department of Defense blood transfusion requirements world-wide. The major advantages of an eight-week extended shelf life red cell product include, reducing resupply requirements by up to 30%, reducing the blood collection requirements to replace outdated products, and increasing the practicality of rotating aged but unexpired red cell products from the theater of operations to CONUS treatment facilities where they are more likely to be used. The net result is reduced costs through enhanced utilization of a scarce, perishable resource.

The Purpose of the Present Work:

The BSL evaluates the effectiveness of candidate red blood cell anticoagulant preservative systems and liquid storage strategies and their potential for further development. Much of the work is done in conjunction with other principal investigators in the BRD, WRAIR. The BRD supports the Military Blood Program by providing data which will evaluate the safety, effectiveness, and practicality of new products or procedures related to the collection, processing and distribution of red blood cells.

During this reporting period, the BSL has performed research under three clinical trial protocols, a phlebotomy type protocol which permits collection of blood from volunteers for *in vitro* research and a single *in vitro* research project. The BACKGROUND, METHODS, RESULTS, and DISCUSSION / CONCLUSION from each research effort will be described separately in order to maximize continuity. The phlebotomy type protocol activities will be

described first, followed by descriptions of the three clinical trials in order, and ending with the single *in vitro* project. Data collection and initial data analysis has been completed on the first two clinical trials; the third clinical trial received full approval during the fourth fiscal quarter and is in progress. The ultimate goal of each clinical trial is to determine if the mean 24 hour post-transfusion survival of the red cells at the end of the storage period exceeds 75% in a minimum of ten volunteers. Furthermore, at least 99% of the cells collected must also remain intact on the final day of storage.

The *in vitro* research project has been completed and an abstract from the *in vitro* study was accepted for presentation at the 50th annual meeting of the American Association of Blood Banks 18 - 23 October 1997. A copy of the accepted abstract is at Appendix 2.

A manuscript describing the results of an *in vitro* research project completed the previous fiscal year was prepared and submitted to the journal TRANSFUSION and is currently under review. A copy of the manuscript being reviewed is at Appendix 3. The data from this research formed the basis of the recently approved and ongoing clinical trial.

A. "Phlebotomy Procedures for Use on Human Subjects" WRAIR #514, HURRAD Log #A-6664.

BACKGROUND

Aspects of red blood cell physiology critical to blood storage are species specific; therefore valid *in vitro* studies of the red blood cell storage lesion require freshly collected human blood. The quantities required range from as little as 3.0 mL to as much as a full unit, 450 mL.

METHODS

Volunteers are recruited from within the Detachment, other tenants of the building and the immediately surrounding community and informed fully as to the risks of donation. Potential volunteers were screened for anemia, transfusion transmitted diseases (TTD) and medical conditions which would make blood donation unsafe using the criteria of the American Association of Blood Banks[13] and the Food and Drug Administration 21CFR640.[14] The total amount of blood collected in an 8 week period is limited to 525 mL. A physician certified in

Advance Cardiac Life Support was present at all full unit phlebotomies. Volunteers are compensated for their blood donations IAW 24 USC 30 and AR 40-2.

A streamlined, consolidated personal computer data base was developed in order to maintain documentation of all volunteer related transactions and assure compliance with donation volume and interval limitations. Phlebotomies were performed by trained contract staff and selected active duty personnel.

RESULTS

During fiscal year 1997, the following blood collections were made.

VOLUME	WRAIR	CLIN TRIAL / BRD	TRANSFUSION MED RSCH	TOTAL
1 - 50 mL	185	N/A	41	226
51 - 100 mL	33	N/A	21	56
101 - 200 mL	7	N/A	6	13
Units (450 mL)	7	29	25	61

On one occasion it was discovered retrospectively that a small specimen (<20 mL) was collected from individuals who were borderline anemic. On another occasion, another volunteer was discovered to have donated 548 mL rather than the 525 mL limit within an eight week period.

DISCUSSION / CONCLUSION

Twenty-three percent of the small volume collections and 41% of the full unit collections were performed for the Transfusion Medicine Research Laboratory of the National Naval Medical Center co-located with the BRD. The remainder supported WRAIR sponsored research.

Though it was of some concern to learn retrospectively of the collection of blood from an individual who was border-line anemic, the current procedures of monitoring anemia and other health indicators every six months were retained. The volunteer was advised to refrain from donation for at least a month as a precaution and be screened before any future donations. The

volunteer's hematocrit has returned to acceptable levels and the volunteer has resumed donations. Collection of a small amount of blood from a volunteer with an hematocrit less than 2 percent below the 38% guideline for donation of a whole unit, was, in the opinions of the principal investigator and the medical monitor, very unlikely to injure a volunteer who was otherwise healthy. The semi-annual screening continues to be effective in identifying volunteers who are borderline anemic and thereby offers the opportunity to have the volunteer discontinue donation until the hematocrit is again acceptable. The current procedures preclude collection of a full unit from an anemic volunteer because the volunteer is screened on the day of donation just before blood collection.

The volunteer who donated an excess 5% over the prescribed limit, 548 mL versus 525 mL, suffered no adverse effects. There were no instances where the volume or donation interval limitations were exceeded since the implementation of the consolidated personal computer based database. The blood collection requirements of the BRD were safely provided from a pool of healthy, screened volunteers.

B. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Prolonged Period in the Leukotrap® RC AS-24 System" WRAIR #572, HURRAD Log #A-6986.

BACKGROUND

WRAIR protocols # 572 and # 591, described in the following sections, were performed under terms of a cooperative research and development agreement (CRDA) with the MEDSEP Division of the PALL Filter Corporation. Protocol #572 evaluated an experimental system incorporating a new formulation of chemicals, AS-24, already used in FDA approved solutions for blood storage, and an integral white blood cell removal filter. The object was to determine if this unique preservative formulation coupled with prestorage white cell removal would permit storage for eight weeks.

METHODS

The major differences of AS-24 compared to other already licensed solutions is the presence of 13.0 mM mannitol, elimination of chloride and reduction of tonicity. The reduced

tonicity of the storage medium induces hypotonic swelling of the red cell and is thought to retard the exocytotic loss of membrane and formation of spherocytic red cells thought to be a prelude to hemolysis and elimination from circulation.[8] The white cell removal filter is incorporated to reduce leukocyte mediated red blood cell injury.[15] The FDA approved AS-3 solution, unfiltered, was the control. Fifteen volunteers were recruited for enrollment in the protocol which involved the sequential collection of two units of blood; the sequence of collection of the two units, control versus test, was randomly assigned. Control units were stored for six weeks, test units for eight.

RESULTS

Twelve volunteers completed collection of a test unit and eleven completed both unit collections and thereby provided analyzable data. The mean 24 hour post-reinfusion red cell survival of the test units was 64% with a range of 54 - 83%; the mean 24 hour post-reinfusion survival for the control units was 76%, range 62 - 90%. Mean hemolysis was less than 1% at end of storage in both test and control units. Red cell morphology of the control and test units was nearly identical at end of storage.

DISCUSSION / CONCLUSION

Red cell survivals at twenty-four hours post transfusion was less than 75% when the red cells were stored for eight weeks in the test preservative system. It appears the hypotonic preservative solution, AS-24, coupled with prestorage white cell removal, does not improve viability sufficiently to extend shelf life to eight weeks.

C. "Evaluation of the *in vivo* Viability of Red Blood Cells Stored for Eight Weeks in the Leukotrap® Whole Blood AS-3 System" WRAIR #591, HURRAD Log #A-7089.

BACKGROUND

Protocol #591 was designed to evaluate a second experimental system for potential eight week red cell storage. A secondary purpose was to evaluate effects of periodic mixing combined with either horizontal or vertical storage.

METHODS

The blood collection system utilized in protocol # 591 differed from the system employed in protocol # 572 in two aspects. First, the FDA licensed AS-3 replaced the experimental hypotonic AS-24 preservative solution in the test units. Second, the system was reconfigured to accomplish white cell removal during collection of the whole blood rather than after the preparation of the packed cells as was the case in protocol #572. Unfiltered AS-3 preserved cells are again used as the control. Test units were stored for 8 weeks; control units for 6 weeks.

Fifteen volunteers were enrolled in a randomized crossover trial for the first portion protocol for the collection of a test and control units; both units were not mixed routinely during storage. Six volunteers continued into the second part of the protocol which evaluated the effects of periodic mixing combined with two different orientations of the units during storage. The units collected in the second portion were thoroughly mixed at weekly intervals. One set was stored flat and horizontal on the blood refrigerator shelf and second set stored upright or vertically in a plastic holder.

RESULTS

Thirteen volunteers completed the first phase of the study which evaluated unmixed storage. Double-labeled 24 hour post-transfusion survival for the filtered AS-3 test units stored for eight weeks averaged 65% with a range of 50-82 %. AS-3 control unit 24 hour post-transfusion survival averaged 77% and ranged from 69-84% after six weeks of storage. Mean hemolysis for the test units was 0.208% (0.082-0.399%) and 0.493% (0.23-1.795%) for the controls.

All six completed the second portion of the trial; results from the mixing studies follow. Double-labeled 24 hour post-transfusion survival for the mixed AS-3 test units stored for eight weeks stored horizontally averaged 69% with a range of 61 -76%. The 24 hour post-transfusion survival for the units stored in the vertical position averaged 67% and ranged from 54 - 80%. Mean hemolysis for the units mixed during storage was 0.131% (0.091-0.171%) and 0.165% (0.047-0.231%) respectively.

DISCUSSION / CONCLUSION

Whole blood filtration with the high efficiency whole blood white cell removal filter incorporated into the Leukotrap AS-3 blood collection system does not permit eight week storage of red cells. Though hemolysis is well within the maximal allowed and some improvement in survival may have been achieved, twenty-four hour post transfusion survival was less than 75% after eight weeks of storage even when the red cells were mixed weekly and stored either upright or horizontally. The two candidate red cell collection and storage systems evaluated to date do not appear to provide the minimum 75% 24-hour post-transfusion survival required for the FDA licensure.

D. "Evaluation of the *in vitro* and *in vivo* Viability of Red Blood Cells Stored in Blood Storage Solutions CPDA-1 for 35 Days and in AS-5 for 42 Days at 1-6 C and Exposed for Short Periods to Higher Temperature" WRAIR #633, HURRAD Log #A-7818.

BACKGROUND

The objectives of this clinical trial are twofold. The first is to critically test, with *in vivo* measurements of 24 post-transfusion survival, the proposed "rule of thumb" that a day of storage at room temperature ages stored cells equivalent to approximately a week in the refrigerator for the militarily relevant storage systems CPDA-1 and AS-5. This hypothesis is based on previous work in this laboratory. Shields[16] has shown that a day at room temperature would reduce post transfusion survival of CPD preserved red cells on the twenty-first day of storage to what would be expected at day 28, the expiration date. Moore[17] did preliminary work which suggested the same would be true for CPDA-1. Additional evidence for this hypothesis was provided for the anticoagulant preservative solution CPDA-1 by the *in vitro* work of Ruddell, see attached draft manuscript at Appendix 3. In Ruddell's work, mean ATP concentrations and glucose consumption at day 25 - 28 of storage in the warmed units were equivalent to those in the unwarmed units at day 35.

The second objective is to advance the understanding of the red cell storage lesion by conducting a new kind of storage study that compares the conventional and newer research laboratory measures of red cell change during storage with the clinical end point of *in vivo*

autologous radiolabled red blood cell recovery and survival. The units collected for the clinical trial will provide samples for other BRD investigators to measure red cell membrane oxidation, Annexin V binding and membrane deformability by ektacytometry. This will be the first opportunity by any research group to correlate any of these measurements with the ultimate test of red cell viability, the 24 hour post-infusion red cell survival.

METHODS

Fifteen volunteers were recruited into the AS-5 arm of the trial. Whole blood is collected into the FDA licensed anticoagulant preservative solution AS-5 and, after centrifugation, sufficient plasma is removed to achieve a target storage hematocrit of 55%. The sequence in which the unit collected for early warming, late warming or for use as a control was randomly assigned. Units designated as controls are stored continuously at 1 - 6^{o}C for 42 days. Units designated for early warming are placed in a 25^{o}C incubator for 24 hours on day 13 of storage and units designated for late warming are placed in a 25^{o}C incubator for 24 hours on day 27 of storage instead. At all other times, the warmed units are stored at 1 - 6^{o}C. Samples are collected from all units at weekly intervals plus days 13 and 27 of storage. Samples are analyzed for an array of metabolites which are used to describe the storage lesion and the membrane characteristics described above. Control units are tested for *in vivo* 24 hour post-transfusion red cell survival at the end of 42 days storage, warmed units at the end of 35 days by standard methods.[18] The timing of warming and reinfusion for the units to be collected into CPDA-1 will follow the same pattern except all time points will be shifted one week earlier.

RESULTS

All fifteen volunteers recruited for the AS-5 arm of the trial have donated the first of three units. Eleven of 15 have been reinfused with stored blood from the first unit. Four of those eleven have progressed to collection of the second unit. The trial has not progressed to a point where any of the data sets are sufficiently complete for presentation.

DISCUSSION / CONCLUSION

Insufficient data has been collected for analysis.

E. "Psoralen Sterilization of Platelet Concentrates Infected with *Orentia tsutsugamushi* "

BACKGROUND

A CRDA between the USAMRMC and the Cerus Corporation, formerly Steritech, was approved and proof of concept studies were initiated to evaluate photochemical inactivation as a process to sterilize platelet concentrates contaminated with two intracellular organisms. The first organism tested was *Orentia tsutsugamushi*, a rickettsial organism responsible for scrub typhus. The Principal Investigator, CPT Kevin Belanger, USA, a student in the Blood Bank Fellowship training program conducted this study in collaboration with LTC Daryl Kelly, WRAIR Communicable Diseases and Immunology (CD&I). The second organism utilized was the malaria parasite *Plasmodium falciparum*; these experiments were conducted at another WRAIR laboratory by MAJ Chris Ockenhouse also of CD&I. The experiments with the malaria parasite were not an part of this contract; therefore the description and results from those experiments are not included in this report.

METHODS

Platelet concentrates were inoculated with mononuclear cells infected with *O. tsutsugamushi*. The infected platelet concentrates were treated with the psoralen 4'(aminometyl)-4,5'-8-trimethylpsoralen hydrochloride (AMT) in concentrations ranging from 0.25 μg/mL to 40 μg/mL and exposed to ultraviolet light (UVA) from a light source calibrated to deliver 5.0 joules. AMT is used in the treatment for psoriasis. Platelet concentrates which received either no infected mononuclear cells, no UVA exposure, no AMT or neither UVA exposure and AMT were included as controls. The individual platelet concentrates were injected into healthy mice and observed for apparent illness. Samples were collected from the mice for Geimsa staining, detection of organism sequences by polymerase chain reaction, indirect fluorescent antibody and direct fluorescent antigen testing.

RESULTS

Animals which received incompletely treated platelet concentrates, the controls, became ill and samples collected from those animals indicated infection by *O. tsutsugamushi* by most or all of the *in vitro* tests. All mice injected with fully treated platelet concentrates remained healthy; samples collected from those animals exhibited no evidence of infection by any of the *in vitro* detection systems employed. All concentrations of the photoactive psoralen compound tested, when combined with UVA irradiation, were effective.

DISCUSSION / CONCLUSION

AMT appears to be highly effective in inactivating the intracellular organism *O. tsutsugamushi*. AMT concentrations as small as 0.25 μg/mL combined with 5.0 joule UVA light exposure rendered *O. tsutsugamushi* contaminated platelet concentrates uninfective. AMT with UVA would appear to be a potent agent for sterilizing platelet concentrates contaminated with either intracellular or extracellular microorganisms. Psoralen treatment has the potential of reducing bacterial overgrowth in platelet concentrates prepared from a collection contaminated during phlebotomy and transmission of blood-borne pathogens. Bacterial overgrowth is a significant complication in the current practice of room temperature storage of platelets. It is unknown whether AMT at the concentrations tested combined with UVA compromises platelet function.

GENERAL AND ADMINISTRATIVE

The following actions have either been initiated or refined and expanded in a continual effort to provide high quality laboratory support to the BRD.

1. Documentation: In accordance with good laboratory practices, the system for tracking, documenting and reviewing all protocols and processes, hereafter referred to as Standard Operating Procedures (SOP's), established in the initial contract period was refined. The SOPs related to the operation of the Hemoglobin Production Facility, whether in completed or draft form, were file. Additional SOPs were added as needed and the annual review completed.
2. Equipment Repair and Maintenance: The preventive maintenance program of all critical and high value equipment, established during the first contract year, was refined and expanded.

3. The contract staff identified a significant performance deficiency in a new gamma counter purchased by the government for use in the BSL. As a result of substantial data gathering effort and following immense persistence, the manufacturer of the instrument recognized the performance deficit in the entire model line, not just the specific instrument in our laboratory. The manufacturer substituted a more expensive instrument suitable for our requirements at no cost.
4. A refrigeration monitoring system was installed to provide warning in the event of refrigeration failure in any of the units containing clinical trials related specimens and materials and also the existing inventory of hemoglobin solution. The monitoring system consists of temperature probes installed in all six freezers containing hemoglobin inventory or refrigerators containing clinical trials material and a control unit which activates a telephone modem whenever the temperature of any of the refrigeration units falls outside an acceptable temperature range. The warning provided by the monitoring system is especially critical in the event of refrigeration or power failure during nights, weekends and holidays. On three occasions during this period the system alerted key individuals of a potential problem.

Results of research supported by contract staff are being prepared for publication and published in peer reviewed scientific journals. The manuscript detailing the Hemoglobin Production Facility process improvements was accepted for publication by the journal "Biologics", the journal of the International Association of Biological Standardization. Publication is scheduled for the fall of 1997. The manuscript was included in the previous annual report. An abstract of the platelet sterilization has been accepted for presentation at the 50th annual meeting of the American Association of Blood Banks 18 - 23 October 1997. The abstract will be published in a supplement to the journal TRANSFUSION. A copy of the accepted abstract is at Appendix 2. A manuscript describing the results of an *in vitro* research project completed the previous fiscal year was prepared and submitted to the journal TRANSFUSION and is currently under review. A copy of the manuscript being reviewed is at Appendix 3.

SUMMARY

The contract staff has supported the BRD by operating and maintaining the Blood Storage Laboratory to support both existing and new requirements. Staff are trained and

systems are in place which supported specific red cell survival protocols. The BRD mission has been supported.

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APPENDIX 1

LIST OF PERSONNEL

with Percent Effort of Each on Project:

(as of the end of report period)

Lloyd E. Lippert, Ph.D., SBB (ASCP), Project Manager:	100%
Claudia Derse-Anthony, MT (ASCP), Technologist:	100%
Mark Lamastra, MLT (ASCP), Technologist:	100%
Kristin Lamberger, Administrative Assistant (Part-time):	100%
Katherine Franklin, Sterile Process Assistant (Part-time/occasional):	100%

APPENDIX 2

Psoralen Sterilization of Platelet Concentrates Infected with *Orentia tsutsugamushi*

K. J. Belanger. WRAMC/SBB Program, Washington, D.C.; L. E. Lippert. WRAMC/BRD, Rockville, MD; F. Mettille. WRAMC/SBB Program, Washington, D.C.; D. J. Kelly. WRAIR/DRD, Bethesda, MD

Background: Risk of transfusion transmitted disease has been reduced by a combination of predonation screening and improved assays, but the risk is still present. This study was conducted to determine if a photochemical process could eliminate the possibility of bacterial transmission within platelet concentrates.

Study design and materials: Platelet concentrates were inoculated with mononuclear cells infected with *Orentia tsutsugamushi*. Sterilization was attempted using concentrations ranging from (.25 μg - 40μg/mL) of 4' -(aminomethyl) - 4,5',8-trimethlypsoralen hydrochloride (AMT) combined with a constant ultraviolet light (UVA) exposure of five joules. The effects of photochemical sterilization was analyzed using an *in vivo* mouse model along with in-vitro testing by PCR, IFA, DFA, and Giemsa staining.

Results: The Mice that received platelets treated by photochemical inactivation showed no signs of illness and tested negative for antigen and antibody production. Platelets that did not receive the full treatment regimen showed signs of illness and were positive for antigen and antibody production.

Conclusion: AMT combined with UVA light did inactivate *Orentia tsutsugamushi* contaminated platelet concentrates. Therefore it is postulated that this treatment may also be effective against other intracellular and extracellular bacteria.

The Journal of the American Association of Blood Banks Transfusion, 37, No. 9S, Sept. 1997 Supplement, p. 17S.

APPENDIX 3

Effect of 24 Hours of Storage at 25^0 Centigrade on the *in vitro* Storage Characteristics of CPDA-1 Packed Red Blood Cells

J. Ruddell, L. Lippert, J. Babcock, and J. Hess

Walter Reed Army Institute of Research, Blood Research Laboratory

The opinions expressed herein are the private views of the authors, and are not to be construed as official or as reflecting the views of the U.S. Department of the Army or of the U.S. Department of Defense.

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Running Head: Room Temperature Storage of Packed RBC

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Abstract

Background: Packed red blood cells (pRBC) warmed above 10°C are generally discarded. Little data exists on the degree of accelerated metabolism and increased hemolysis of pRBC allowed to warm. **Methods:** 24 CPDA-1 pRBC units were combined in 3 unit pools and subdivided into 2 test units and a control unit. Test units were warmed to 25°C for 24 hours either at day 6 or day 20; control units were maintained at 1-6°C. RBC and supernatant chemistries and RBC morphology were measured weekly and days prior to warming. **Results:** Warming CPDA-1 pRBC accelerated the catabolism of glucose ten-fold and produced concentrations of glucose, lactate, and ATP after 25 days of storage equivalent to unwarmed units at 35 days. Supernatant sodium and potassium concentrations corrected modestly with warming. RBC morphology normalized transiently with warming without increased hemolysis; no bacterial growth was detected. **Conclusion:** A day of 25°C storage of CPDA-1 pRBC accelerates essential metabolite breakdown equivalent to 10 days of storage at 1-6°C. It does not appear to matter whether the pRBC are warmed at day 6 or day 20. This information may be useful in determining the acceptability of blood allowed to warm above 10°C.

Key Words: temperature, blood storage, CPDA-1, emergency medicine, disaster medicine, military medicine

Introduction

The American Association of Blood Banks (AABB) and the Food and Drug Administration (FDA) have established guidelines for the storage and preservation of packed red blood cells (pRBC). According to AABB Standards G1.300 and G2.000, pRBC must be stored between 1-6°C and shipped between 1-10°C.[1] The FDA requires the same standards. 21 CFR 640.2 (e)(3) indicates that blood may not be issued unless it has been stored at 1-6°C or maintained at 1-10°C during shipping.[2] 21 CFR 640.4 (I) indicates that blood should be placed in storage at 1-6°C immediately after platelets are separated.[3] Common practice is for red blood cells stored outside those parameters to be destroyed. However, the AABB Technical Manual states "Blood exposed to temperatures above 10°C is not necessarily unsuitable for transfusion."[4] Yet there is little or no data which supports either destruction or retention for transfusion other than the reminder of an increased possibility of bacterial contamination.[5] This investigation provides data on the accelerated rate of depletion of metabolic support when red cells are stored near ambient temperature (25°C) for a prolonged period (24 hours) either early (day 6) or late (day 20) in the shelf-life of a CPDA-1 pRBC unit.

Methods and Materials

Donor Screening

Twenty-four volunteers, ten females and fourteen males, who met all AABB and the FDA blood donor criteria and whose blood was further screened and found not to have sickle hemoglobin, alloantibodies or abnormal osmotic fragility, each donated a full unit of

blood for this study. All volunteers gave their informed consent in accordance with a protocol approved by the Institutional Review Board.

Packed Red Blood Cells

Four hundred fifty milliliters of blood was collected into a CPDA-1 double bag collection system (Fenwal, Baxter Health Care Corporation, Deerfield, IL, Code 4R6210) and held at ambient room temperature before processing. Packed cells were prepared within 2 hours of collection of the whole blood by centrifugation 5000 x g for 5 minutes at room temperature and removal of plasma to achieve an average hematocrit of 67 %, range of 66 - 68 %. Three units of packed cells of the same ABO group were pooled by serial sterile connections to a 1.0 L transfer bag (Fenwal Division, Baxter Health Care Corporation, Deerfield, IL, Code 4R2032). The contents were mixed and divided into three equal aliquots by weight into 400 mL satellite bags (PL 146) from a CPDA-1 quadruple bag collection system (Fenwal Division, Baxter Health Care Corporation, Deerfield, IL, Code 4R6423P) connected by use of a sterile connecting device (Terumo Medical Corporation, Elkton, MD, SCD312 Sterile Tubing Welder) to the pooling bag. The pooled packed cells were mixed by gently inverting the transfer bag along its long axis a minimum of 15 times. All transfers to or from the pooling bag were made by gravity. The three aliquots from each pool, the day 6 early warmed and day 20 late warmed test units and the control unit, were placed in refrigerated storage (1-6°C) within approximately 4 hours of collection and stored continuously at 1-6°C in a monitored refrigerator except during the planned warming periods.

Sampling

Samples were taken at the time of pooling (day zero) from each of the eight pools; all other samples were taken from the 24 individual units. A sampling site coupler (Fenwal Division, Baxter Health Care Corporation, Deerfield, IL, Code 4C2405) was used to collect samples from the pool at day zero and from the divided units on day 35. An alternate method was used to collect the intervening samples on days 6, 7, 14, 20, 21, and 28. Briefly, a length of integrally attached tubing was filled with mixed blood and clamped approximately 2 inches from the distal end. The distal end was cut with scissors and the forceps were released while maintaining light pressure on the blood bag. The required amount of sample was expelled, the forceps reclamped, the tubing heat sealed proximal to the forceps and the tubing restripped and reclamped. All sampling was performed in a biological safety cabinet (Nuaire, Model # NU 408FM-400, Plymouth, MN) .

Incubation of Test Units

On day 6, all units from each pool were sampled; units labeled for early warming were removed from refrigerated storage and placed in a 25°C incubator for 24 hours. On day 7, at the end of the incubation, all units were sampled and placed back in refrigerated storage. On day 20 the same processes for sampling and storage were applied to the designated units. On day 21, at the end of the incubation, all units were again sampled and placed back in refrigerated storage.

Testing

The pH of the pRBC was measured with a clinical blood gas analyzer (CIBA-Corning 855 Blood Gas Analyzer, Medfield, MA). The hemoglobin and hematocrit were measured with a clinical hematology analyzer (Baker Hematology Cell Counter System Series 9000, Allentown, PA); plasma Na+ and K+ were determined using ion-specific electrodes (Roche Diagnostic Systems Cobas Fara, Nutley, NJ) all in accordance with manufacturer's directions. Whole blood glucose, total ATP and lactate levels were obtained from deproteinized samples. Briefly, proteins in whole blood samples were precipitated with 12% perchloric acid on ice and the supernatant harvested after centrifuging at 2700 x g for 10 minutes. The portion of the supernatant used for glucose and ATP testing was adjusted to pH 8-9 with solid $KHCO_3$. The remainder of the deproteinized sample was used for lactate testing and was not pH adjusted. Glucose levels were determined with a clinical chemistry analyzer (Roche Diagnostic Systems Cobas Fara, Nutley, NJ). Total ATP was assayed enzymatically using a commercially available test kit (Sigma Diagnostics, kit # 35-B, St Louis, MO). ATP levels were reported as micromoles per gram of hemoglobin. The lactate levels were measured with a commercially available quantitative lactate kit (Sigma Diagnostics, #228, St Louis, MO) adapted to a clinical chemistry analyzer (Roche Diagnostic Systems Cobas Fara, Nutley, NJ).

The red cell morphology score was determined according to the method of Usry, Moore, and Manalo.[6] Briefly, the red cells were fixed with gluteraldehye, examined microscopically and categorized into one of six morphological types, ranging from diskocyte to spherocyte. After at least 200 cells were categorized, the number of each morphological type was multiplied by a weighting factor, ranging from 1.0 for the diskocyte

to 0.0 for the spherocyte; the summed weighted score was divided by the number of cells scored and reported as a percent.

The plasma or supernatant hemoglobin was measured spectrophotometrically at 540 nm using the method of Moore, Ledford, and Merydith which adapts the classical cyanmethemoglobin method to the lower levels of hemoglobin found in the plasma or supernatant of stored blood.[7] The plasma Hgb level was divided by the total Hgb level and multiplied by 100 to determine percent hemolysis using the formula:

$$\% \text{ Hemolysis} = (\text{Supernatant Hgb in g/dL} / \text{Total Hgb in g/dL}) \times 100$$

Sterility

The sterility of all aliquots was assessed in two ways. The first was by visual comparison of a filled tubing segment, prepared soon after collection, with the contents of the bag for any color changes or appearance of bubbles on all sampling days. Second, a commercially available blood culture system (Septi-Chek Blood Culture Bottle and Slide, Becton Dickinson Microbiology Systems, Cockeysville, MD) was used at day 35 of storage to determine if bacterial growth was present.

Data Analysis

One-way analysis of variance (ANOVA) was used to analyze the data. Two comparisons were made. First, the day 35 means of all parameters for all three groups were compared. In the second comparison, the day 28 means of the test groups were compared to the day 35 mean of the control group for each parameter. Comparisons with probabilities less than 0.05 were considered statistically significant.

Results

Under all conditions of storage, the extracellular glucose concentration decreased with time. During 35 days of storage at 1-6°C, the mean glucose concentration declined from 391 ± 4 to 72 ± 13 mg/dL, averaging a decline of about 9.1 mg/dL per day (Figure 1, A). The glucose concentration declined at the much greater mean rate of 90 mg/dL per day on the days of 25°C storage, 111 ± 5 mg/dL on day 6 and 70 ± 2 mg/dL on day 20. This caused the glucose concentrations to be significantly lower in the warm-stored units at all time points after warm storage. Increases in plasma lactate and decreases in pH followed the changes in glucose concentration (Figures 1, B and C).

The ATP concentrations showed a more complex pattern, initially increasing during the first week of storage and decreasing thereafter (Figure 1, D). The rates of decrease in ATP concentration were observed to be greater in the week following warming when compared to the continuously refrigerated control. Thus, the unwarmed control units had a significantly higher mean ATP concentration at day 35 (2.4±0.1 μmol/g Hgb) than the warmed units had at either days 28 (1.9±.1 μmol/g Hgb, $p= 0.012$), or day 35 (1.4±0.1 μmol/g Hgb, $p < 10^{-7}$). One warm stored unit had a concentration of ATP of 1.4 μmoles/g Hgb on day 28, all others were 1.7 μmoles/g Hgb or greater.

Mean supernatant plasma hemoglobin concentrations appeared to increase after warming, especially in the units warmed at day 20, but the differences between the warmed and control units never achieved statistical significance (Figure 1, E). Hemolysis never exceeded 1%. Small increases in extracellular sodium and decreases in extracellular potassium that occurred during the warmed days persisted through most of storage (Figures 1, F and G).

Warming led to significant improvement in RBC morphology scores during the 24 hours of warming, but the improvement was not maintained in subsequent weeks (Figure 1, H). At day 35 the morphology scores of the three groups were not different. A comparison of the relative numbers of the morphologic forms of RBCs seen before and after warming at day 20 showed the reappearance of greater numbers of less deformed RBCs and disappearance of the more spiculated echinocytes. There was no corresponding increase in supernatant hemoglobin to suggest lysis.

Visual examination of all pools in all groups revealed no abnormalities which might have indicated bacterial contamination. Broth cultures of all units begun on day 35 and examined for four days thereafter showed no bacterial growth.

Discussion

Liquid storage systems for pRBCs are licensed for their ability to preserve cells at 1-6°C. Efforts to assure the quality of these red cell products have led to standards and regulations that require that blood be stored in continuously monitored refrigerators at 1-6°C and transported on wet ice so as not to rise above 10°C (21 CFR 640.2 (e) (1)[2] and AABB Standards G1.300 and G2.000).[1] A rule of thumb has evolved that blood maintained outside of a refrigerator for longer than 30 minutes should be discarded because the temperature will have risen above 10°C. The net effect of these rules is to assure that each unit of blood has been handled in a highly standardized way and one that has been demonstrated to assure acceptable viability and circulation post-transfusion by generally accepted criteria.

Rigid enforcement of these standards, in the face of the knowledge that "Exposure to temperatures above 10°C does not necessarily render blood unsuitable for transfusion," [4] may lead to several undesirable effects. First, units of blood are regularly discarded for potentially insignificant deviations from the standards. This has led to the proliferation of devices to measure the temperature of blood units outside of the refrigerator for short periods. Second, the reluctance to remove blood from monitored refrigeration because of concerns about potential violation of the standards sometimes compromises the availability of blood at scenes of emergency care, even in major hospitals. Finally, for military and civil emergency situations there is no information available for the medical directors of blood services about the safety implications for recipients of blood stored in current storage solutions under nonstandard conditions.

Refrigeration preserves red cells because glycolysis and other metabolic processes are slower at lower temperatures. At 37°C red cells consume glucose at rates of .014-.028 $\mu g/hr/10^6$ RBCs[8], equivalent to decreases of 7-14 mg/dL/hr in the extracellular glucose concentration of pRBCs stored in CPDA-1 at a hematocrit of 67%. Thus pRBCs stored in CPDA-1 at body temperature would be expected to consume all the available glucose in 1-3 days. Cooling to 1-6°C reduces the rate of extracellular glucose concentration consumption to the observed 9 mg/dL/day and allows storage for 35 days. Intermediate temperatures should be associated with intermediate rates of glucose consumption and intermediate periods of effective storage.

We performed this work to quantitate the acceleration in consumption of glucose and the resulting changes in cellular ATP concentration that occur with increased storage temperature. Our inspiration was the work of Shields[9] who in 1970 showed that for whole

blood stored in ACD, a day at 22 to 25°C reduced RBC recovery measured with 51-Cr by an amount equivalent to about a week of 1-6°C storage. Because of the large interdonor variation in blood storage characteristics, Shields was only able to demonstrate statistically significant differences in recovery at times beyond the conventional expiration of the units at 21 days. In our study, measuring the metabolite concentrations in aliquots of pooled units allowed accurate measure of differences resulting from changes in storage conditions. However, pooling precludes autologous RBC recovery and survival measurements, and estimates of the clinical significance of this work depend on the implications that can be drawn from the cellular ATP concentrations and morphologic measures [10].

The relationship of cellular ATP concentrations to a 24 hour RBC recovery of 75% is probably better understood for CPDA-1 than for any other storage solution. First, in the CPDA-1 licensing study[11], the mean ATP concentration was 1.93 μmol/g Hb. Second, Beutler and West[12] have shown that removing glucose from CPDA-1 units by removing plasma reduces 24 hour post-transfusion survival. Third, adding more glucose and adenine to CPDA-1, as done with the preservative solution CPDA-2[13], produces a solution that allows red cells to be stored for a week longer with equivalent recovery and survival. Thus, survival of RBCs stored in CPDA-1 is glucose and ATP limited, and is most highly correlated with maintaining ATP concentrations above a value of about 2 μmol/g Hb[11] .

The morphologic changes in the RBCs that we observed with warming and recooling were striking. During storage in the cold, RBCs slowly evolve from biconcave discs to sharply spiculated echinocytes. These changes revert with warming, but following recooling their reappearance is accelerated. Thus, the cellular injury that underlies the morphologic changes has both reversible and irreversible aspects. Electron micrographs of

microvesicles budding from the tips of echinocytic spicules[14] suggest a relationship between RBC shape change and the loss of RBC membrane area critical for survival. Warming may provide a model for studying the mechanism of these events.

We have described the effects of a specific time and temperature stress on the performance of the CPDA-1 storage system. Interpolation can serve as a basis for estimates of the effects of stresses of shorter duration or lower temperatures. Thus if the catabolism of glucose and ATP is a linear function of the time of exposure to the temperature stress, then a single two hour exposure to 25°C might be expected to reduce the storage life of a unit of CPDA-1 RBCs by a day. Smaller variances from the current standard of practice are unlikely to have any measurable influence on the quality of the product. Finally, if called upon to provide blood in emergencies, the study provides some information to guide medical directors in their difficult decisions of how far to attempt to take advantage of the performance characteristics of the storage system in their attempts to maximize the availability of blood to save lives.

We have described the effects of a specific time and temperature stress on the performance of a storage system. These measures, although the numbers are small, can serve as a basis for estimates of the effects of stresses of shorter duration or lower temperatures. Thus, if the catabolism of glucose and ATP is a linear function of the time of exposure to the temperature stress, then a single two hour exposure to 25°C might be expected to reduce the storage life of a unit of CPDA-1 red cells by a day. Second, the study provides a model for measuring the robustness of storage systems and for determining under what circumstances a measurable effect will be achieved. Based on the results of our study, small variances from the current standard of practice are unlikely to have measurable

influence on the quality of the product. Finally, if called upon to provide blood in emergencies, the study provides some information to guide medical directors in their difficult decisions of how far to attempt to take advantage of the performance characteristics of the storage system in their attempts to maximize the availability of blood to save lives.

The authors are not advocating the abandonment of current standards for red blood cell storage. Those standards provide excellent assurance of the physiologic quality of stored cells as well as protection against the uncommon event of bacterial contamination. While we saw no evidence of bacterial contamination with broth culturing of all 24 units, even in the face of repeated sampling that began in the first week of storage, data from a small number of units collected in a research setting cannot be applied to clinical blood bank practice.[15,16] Any attempt to take advantage of the performance characteristics of current storage systems through nonstandard usage must be accompanied by heightened vigilance.

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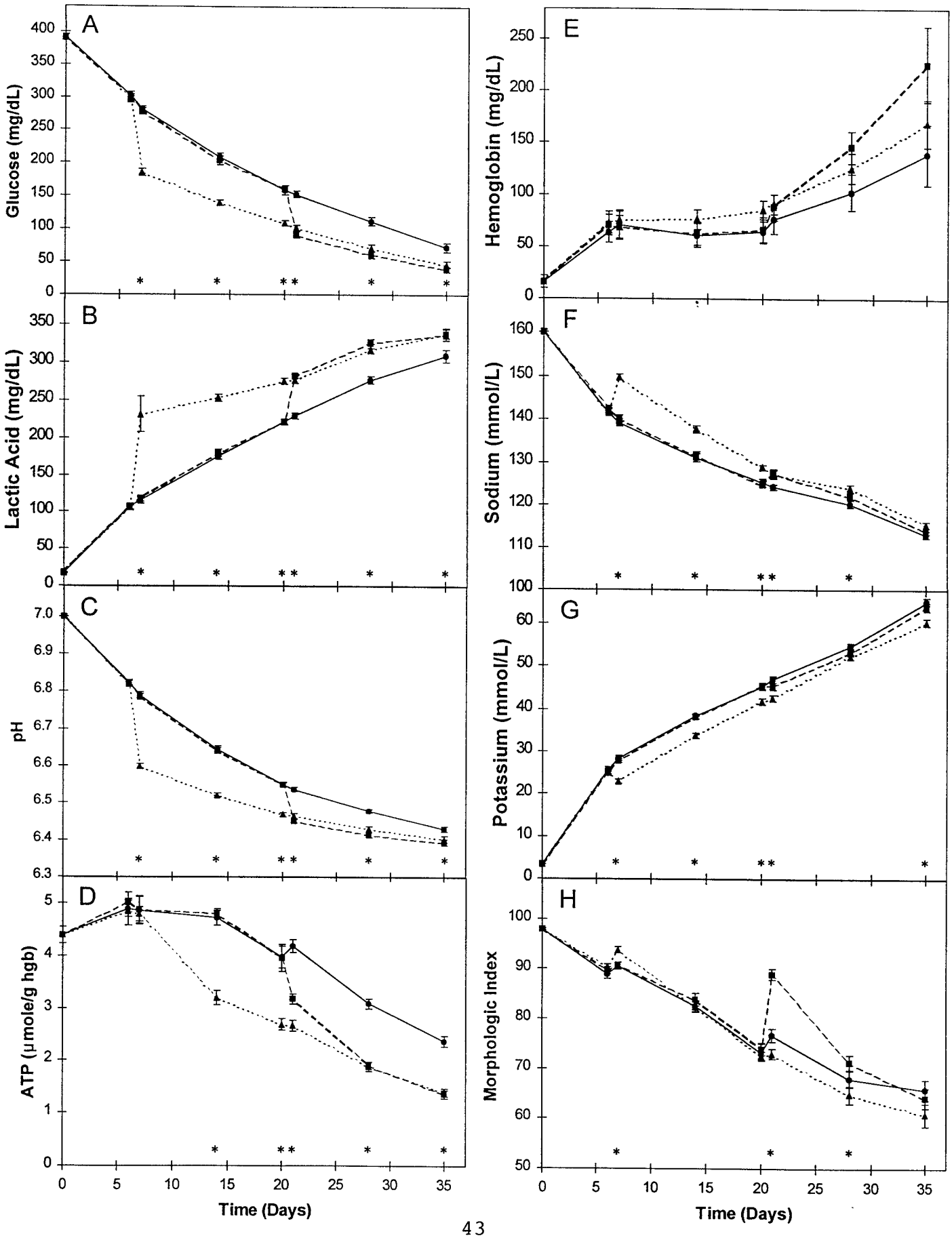
FIGURE LEGEND:

Fig.1. Concentrations of metabolites, metabolic by-products, supernatant hemoglobin, supernatant electrolytes and the morphologic index of CPDA-1 pRBCs either warmed at 25^{0}C for 24 hours at day 6 (·····▲·····), day 20 (- - -■- - -), or stored continuously at 1-6^{0}C (—●—). The asterisks (*) identify the time points where differences in group means could not be due to chance alone at a probability of $p = 0.05$. Error bars depict SEM.


A
Glucose (mg/dL)
B
Lactic Acid (mg/dL)
C
pH
D
ATP (µmole/g hgb)
E
Hemoglobin (mg/dL)
F
Sodium (mmol/L)
G
Potassium (mmol/L)
H
Morphologic Index
Time (Days)
Time (Days)