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# Prevalence of Anxiety Symptoms in Medical Students in the Inner State of São Paulo, Southeast Brazil

By Leonor de Castro Monteiro Loffredo, Rodolpho Telarolli Júnior, Jaquelini Diniz, Gabriel Orlandi de Souza, Gabriela Bombarda Barros, Luiz Carlos Lobato Miani & Thiago Eduardo Manzi

*Universidade Estadual Paulista - UNESP*

*Abstract-* *Background:* Anxiety is a global health concern among medical students. It is associated with difficulty concentrating, insomnia, and a damaged lifestyle.

*Objectives:* To describe anxiety symptoms and to assess the prevalence of anxiety in medical students.

*Design and Setting:* It was conducted a cross-sectional study in a sample of 120 first- to fourth-year medical students in the inner state of São Paulo, Brazil.

*Methods:* An anxiety symptoms formulary was applied to the medical students. There were estimated the prevalence of anxiety and the prevalence ratios according to the variables age, gender, course year, sleep, physical activity, and use of anti-anxiety medication.

*Keywords:* *mental disorders; anxiety disorders; education, medical, undergraduate; epidemiology; prevalence.*

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# Prevalence of Anxiety Symptoms in Medical Students in the Inner State of São Paulo, Southeast Brazil

Leonor de Castro Monteiro Loffredo [α], Rodolpho Telarolli Júnior [σ], Jaquelini Diniz [ρ], Gabriel Orlandi de Souza [ω], Gabriela Bombarda Barros [¥], Luiz Carlos Lobato Miani [§] & Thiago Eduardo Manzi [χ]

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*Results:* The prevalence of mild, moderate and severe anxiety was 31.7%, 30.8% and 7.5%, respectively. There was a significant association of anxiety with age ($\chi^2$ = 16.2858; p=0.001) and gender ($\chi^2$ = 11.5165; p=0.009), though it was non-significant for course year, sleep, physical activity, and regular use of anti-anxiety medication (p>0.05).

*Conclusions:* Higher rates of anxiety were seen among female and younger students. Better understanding the extent of the problem can help develop and implement interventions in order to control and prevent anxiety among students in medical education institutions.

*Keywords (MeSH):* *mental disorders; anxiety disorders; education, medical, undergraduate; epidemiology; prevalence.*

*Authors´ Keywords:* *epidemiology. prevalence. anxiety. students, medical. education, medical.*

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## I. Introduction

Anxiety is a common state of the body characterized by fear and physical stress, and when it becomes pathological, anxiety symptoms may affect everyday life. Exposure to traumatic experiences in childhood could explain the origin of anxiety symptoms[1] and, during life, can be associated with age, gender, cultural background, and economic condition.[2]

Different types of anxiety disorders that can affect daily life to a greater or lesser extent; social anxiety and generalized anxiety have an impact on social life and can make people feel discouraged and anguished.[3, 4]

The global prevalence of people suffering from anxiety was 3.6% in 2015. In the Americas, it was 5.8%, affecting 9.3% of the Brazilian population.[3]

A cross-sectional study among 40,348 medical students found a prevalence of 33.8%. It was most prevalent among those from the Middle East and Asia, with no significant association with gender and year of study.[5]

In Brazil, a study with 603 medical students attending medical school (ABC Medical School) found high anxiety scores in 20.1% and moderate anxiety in 79.9%. Still, there were not reported variations in the prevalence of anxiety symptoms by the medical school year.[6]

A multicenter study of anxiety among Brazilian medical students reported 81.7% state-anxiety and 85.6% trait-anxiety, with a significant association with female sex.[7]

Evidence shows that medical students are more likely to have anxiety than the general population, as they go through various transition periods during the course.[8]

Medical students' mental health is a global public health concern, and there is an academic movement calling for medical education institutions to promote medical students' health and well-being by addressing curricular issues and reinforcing their support network.[7, 9]



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Students' academic life routinely involves a high study load and many learning activities and, if their average sleep hours reduces, the anxiety rates increase. They may even reduce their level of physical activity with compromised cardiorespiratory and muscular fitness and behavioral changes.

This study aimed to describe anxiety symptoms and assess the prevalence of anxiety among medical students with respect to the variables: age, gender, course year, sleep, physical activity, and use of anti-anxiety medication.

## II. Methods

A cross-sectional study described the anxiety symptoms and assessed the prevalence of anxiety in medical students from a private medical school program in the inner state of São Paulo, Brazil, in 2019.

The study population comprised 677 students enrolled in a private medical education program in August 2019. For a prevalence of anxiety among medical students of 20.1% as previously reported,[6] an estimated sample size of 181students was determined with a margin of error to be no more than ±5% at the 95% confidence level. The participants were from each one of the 12 learning modules in the medical school curriculum, proportional to module size and gender. The interviews were done to all second-year students (learning modules 3 and 4); two first-year (5%), four third-year (15%), eight fourth-year (27%), 14 fifth-year (54%), and 13 sixth-year students (50%) did not show up to their scheduled interview. There were 41 losses, mostly fifth- and sixth-year students. The sample included 140 students, but it was not representative of fifth- and sixth-year students as there were only 24 participants (out of 51 drawn; 47%). The final sample consisted of 120 first- to fourth-year students (out of 489 enrolled in the medical program). All participants signed an informed consent form agreeing to participate in the study.

It was assessed anxiety symptoms, considering psychic and somatic aspects. Demographic data (age and sex) and information on course year, hours of sleep per day, hours of physical exercise per week, and use of anti-anxiety medication were also collected (Annex 1).

Psychic symptoms included worrying too much; feeling "on edge;" difficulty concentrating; trouble relaxing; fearing for the worst; being terrified; being nervous; fear of losing control; fear of dying; and being frightened. Somatic symptoms included numbness or tingling; restlessness; tremors; heat sensation; palpitations or heart racing; dizziness; loss of balance; feeling of choking; difficulty breathing; stomach discomfort, and cold sweating. The institutional ethics committee (protocol no 12353719.0.0000.5383) approved this research.

A psychologist interviewed the participants and asked them to rate their symptoms in the last 10 days. They were classified as 0,1,2,3, where the higher the score, the more intense the anxiety symptoms were. As the psychologist finished the interviews, the students were classified according to the anxiety's level as "very mild", "mild", "moderate", and "severe", respectively to the sum of scores as 0 to 9, 10 to 19, 20 to 29 and 30 and above.

First, it was conducted a pilot test with a subsample of 18 participants, and each student was interviewed twice within ten days to test the intra-examiner agreement of anxiety symptoms.

The dependent variable was anxiety severity (scores) and independent variables included:

- Age: <23 years, 24+ years;
- Gender: male, female;
- Course year: consisted of two consecutive modules - first year(M1 + M2); second year (M3 + M4); third year(M5 + M6); and fourth year(M7 + M8);
- Sleep: inadequate (<7hours per day); adequate (7 to 9 hours per day);[10]
- Physical activity: inactive (sedentary); active (1 hour or more per week) and
- Regular use of anti-anxiety medication (yes / no).

The data was pre-coded, entered into an Excel spreadsheet and analyzed using STATA Statistical Software version 11.2. It was used kappa (κ) statistic to test the intra-examiner agreement: <0.00 (none); 0.00-0.20 (slight agreement); 0.21-0.40 (fair agreement); 0.41-0.60 (moderate agreement); 0.61-0.80 (substantial agreement); and 0.81-1 (near-perfect agreement).[11] Prevalence data were presented by point (p) with respective 95% confidence intervals (95% CI) and prevalence ratios (PR). It was performed the chi-square test ($\chi^2$) to test associations of interest at a 5% significance level.

## III. Results

The sample consisted of 120 first- to fourth-year medical students, where 66.7% were female, and the mean age was 22.1 ± 4.0 years.

Regarding to the anxiety symptoms formulary, the overall intra-examiner agreement was moderate to near-perfect ($0.51 \leq \kappa \leq 0.90$). It was moderate for trouble relaxing (κ=0.55) and feeling frightened (κ=0.51). The classification was good for worrying too much (κ=0.67), feeling "on edge" (κ=0.68), difficulty concentrating (κ=0.78), being terrified (κ=0.74), being nervous (κ=0.68), fearing of losing control (κ=0.62) and fear of dying (κ=0.75). And it was excellent for tremors (κ=0.90), heat sensation (κ=0.90), fearing for the worst (κ=0.85), dizziness (κ=0.85), loss of balance (κ=0.85), stomach discomfort (κ=0.84), and cold sweating (κ=0.83).

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According to Figure 1, the prevalence of moderate and severe anxiety among medical students was 38.3%.

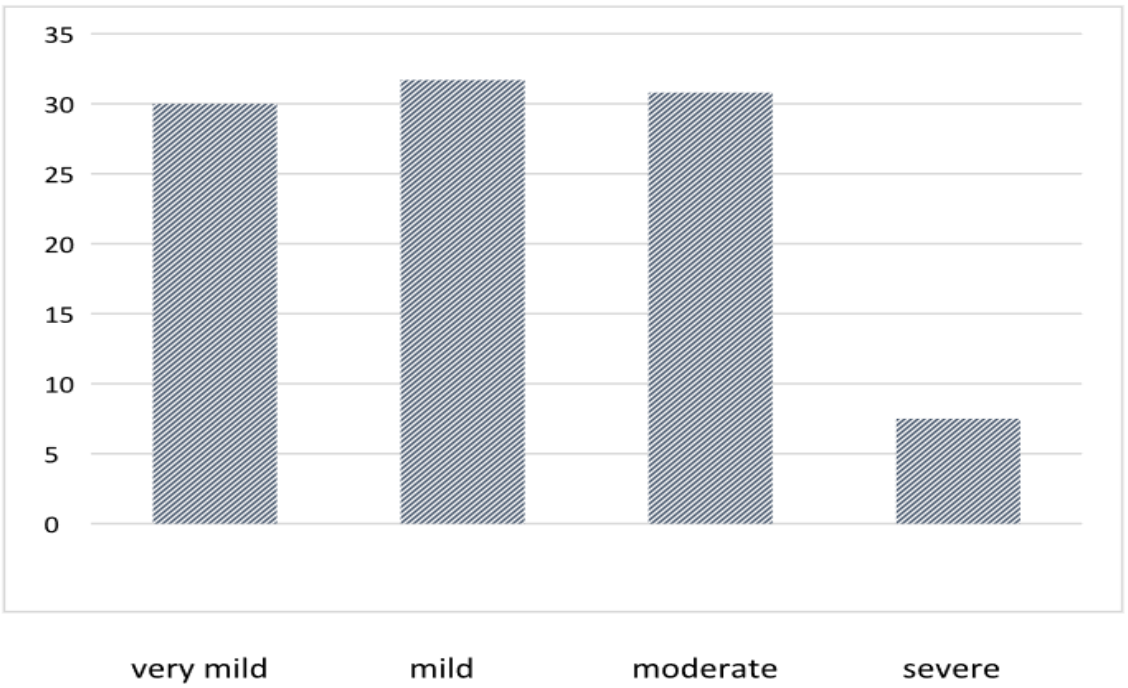



*Figure 1:* Prevalence of medical students' anxiety in a private university, São Paulo State, Brazil

Table 1 shows the prevalence of anxiety symptoms, where most students reported moderate to severe symptoms of worrying too much (59%) and being nervous (61%).

*Table 1:* Prevalence of anxiety symptoms in number (n) and in percentage (%) according to rate (0: definitely not; 1: slightly; 2: moderate; 3: severe).

| Anxiety symptom | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| | n (%) | n (%) | n (%) | n (%) |
| Worring too much | 9 (8) | 40 (33) | 47 (39) | 24 (20) |
| Feeling "on the edge" | 27 (23) | 43 (36) | 40 (33) | 10 (8) |
| Difficulty concentrating | 31 (26) | 57 (48) | 27 (23) | 5 (4) |
| Trouble relaxing | 35 (29) | 43 (36) | 32 (27) | 10 (8) |
| Fearing for the worst | 23 (19) | 48 (40) | 29 (24) | 20 (17) |
| Being terrified | 83 (69) | 27 (23) | 7 (06) | 3 (3) |
| Being nervous | 13 (11) | 34 (28) | 45 (38) | 28 (23) |
| Fear of losing control | 50 (42) | 36 (30) | 23 (19) | 11 (9) |
| Fear of dying | 94 (78) | 15 (13) | 6 (5) | 5 (4) |
| Being frightened | 70 (58) | 30 (25) | 14 (12) | 6 (5) |
| Numbness or tingling | 97 (81) | 13 (11) | 6 (5) | 4 (3) |
| Restlessness | 41 (34) | 45 (38) | 23 (9) | 11 (9) |
| Tremors | 93 (78) | 20 (17) | 7 (6) | - (0) |
| Heat sensation | 79 (66) | 21 (18) | 17 (14) | 3 (3) |
| Palpitation or racing heart | 54 (45) | 36 (30) | 22 (18) | 8 (7) |
| Dizziness | 89 (74) | 19 (16) | 11 (9) | 1 (1) |
| Loss of balance | 104 (87) | 11 (9) | 4 (3) | 1 (1) |
| Feeling of choking | 85 (71) | 26 (22) | 5 (4) | 4 (3) |
| Difficulty breathing | 100 (83) | 11 (9) | 5 (4) | 4 (3) |
| Stomach discomfort | 41 (34) | 40 (33) | 27 (23) | 12 (10) |
| Cold sweating | 67 (56) | 28 (23) | 22(18) | 3 (3) |



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*a) Anxiety severity in medical students*

Thirty-six students showed very mild (30.0%; 95%CI: 21.8%-38.2%), 38 mild (31.7%; 95% CI: 23.4-40.0), 37 moderate (30.8%; 95% CI: 22.5-39.1) and 9 severe anxiety (7.5%; 95% CI: 2.8-12.2). In our sample, 61.7% showed very mild/mild and 38.3% moderate/severe anxiety. Table 2 summarizes the association between the severity of anxiety and the study variables.

*Table 2:* Association between the severity of anxiety among medical students and the study variables.

| Variable | Verymild | | Mild | | Moderate | | Severe | | $\chi^2$ (p-value) |
|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | |
| **Age** | | | | | | | | | |
| < 23 years | 19 | 20.9 | 31 | 34.1 | 32 | 35.2 | 9 | 9.9 | 16.2858 (0.001)* |
| 24 +years | 17 | 58.6 | 7 | 24.1 | 5 | 17.2 | - | 0.0 | |
| **Gender** | | | | | | | | | |
| Female | 17 | 21.2 | 25 | 31.2 | 31 | 38.7 | 7 | 8.7 | 11.5165 (0.009)* |
| Male | 19 | 47.5 | 13 | 32.5 | 6 | 15.0 | 2 | 5.0 | |
| **Courseyear** | | | | | | | | | |
| Firstyear | 9 | 23.1 | 13 | 33.3 | 13 | 33.3 | 4 | 10.3 | 6.2693 (0.713)$^{ns}$ |
| Secondyear | 9 | 25.0 | 13 | 36.1 | 11 | 30.6 | 3 | 8.3 | |
| Thirdyear | 10 | 43.5 | 6 | 26.1 | 5 | 21.7 | 2 | 8.7 | |
| Forthyear | 8 | 36.4 | 6 | 27.3 | 8 | 36.4 | 9 | 7.5 | |
| **Sleep** | | | | | | | | | |
| Adequate | 22 | 41.5 | 14 | 26.4 | 14 | 26.4 | 3 | 5.3 | 6.0475 (0.109)$^{ns}$ |
| Inadequate | 14 | 20.9 | 24 | 35.8 | 23 | 34.3 | 6 | 8.9 | |
| **Phys. Activity** | | | | | | | | | |
| Inactive | 7 | 29.2 | 6 | 25.0 | 10 | 41.7 | 1 | 4.2 | 2.0143 (0.569)$^{ns}$ |
| Active | 29 | 30.2 | 32 | 33.3 | 27 | 28.1 | 8 | 8.3 | |
| **Anti-anxiety** | | | | | | | | | |
| No | 33 | 31.7 | 33 | 31.7 | 32 | 30.8 | 6 | 5.8 | 3.8963 (0.273)$^{ns}$ |
| Yes | 3 | 18.7 | 5 | 31.2 | 5 | 31.2 | 3 | 18.7 | |

**significant; $^{ns}$ non-significant*

Anxiety severity was significantly associated with age. The prevalence of moderate anxiety was two times higher in younger (PR = 2.0) than older participants. Severe anxiety was seen only in those aged ≤23 years.

Anxiety was significantly associated with being female, which could be explained by a greater proportion of female students showing moderate (PR = 2.6) and severe level (PR = 1.7).

Regarding year course, the association was non-significant. As for sleep (adequate or inadequate), there was a non-significant association with anxiety.

The association between anxiety severity and physical activity was non-significant. On the other hand, moderate anxiety was more prevalent among inactive (41.7%) than active students (28.1%) (PR = 1.5).

A non-significant association was seen with the use of anti-anxiety medication; however, severe anxiety was more prevalent among students on anti-anxiety medication (18.7%) than about those not taking any medication (5.8%) (PR = 3).

## IV. Discussion

This study aimed to assess anxiety among medical students from a private medical education institution in the state of São Paulo, southeast Brazil. A formulary was specifically developed for the assessment of anxiety symptoms (Annex 1, Chart) and administered by a psychologist. After the interview, the psychologist identified the most prevalent anxiety symptoms and classified the students into four groups: very mild, mild, moderate, and severe. Also, the students' demographic data and information on sleep, physical activity, and regular use of anti-anxiety medication were collected. This formulary was reliable, according to the results of the intra-examiner agreement.

The results here presented are for a random sample of first- to fourth-year students (n = 120; 27% of total students enrolled in these school years). The majority of the students from fifth- and sixth-year was not able to attend the interview, as they spend their day on clinical rotations in health care units, outpatient clinics, and hospitals and work long hours at different services with day and night shifts during the sixth year.

It was used a cross-sectional design, as in most studies of anxiety conducted among university students.[12] This methodological design does not allow to make inferences about a temporal relationship between experiencing anxiety and the variables (sleep, physical activity, and use of anti-anxiety medication) and know whether exposure is a cause or a consequence of anxiety. Researchers have argued that investigations of anxiety in medical students should use longitudinal designs to identify risk factors associated as they could provide input for developing and implementing



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management strategies in higher education institutions.[5,12]

Of 21 anxiety symptoms assessed, "worrying too much" and "being nervous" were noteworthy as most students reported them with moderate and severe anxiety. As for anxiety severity, 38 students (31.7%) showed mild, 37 (30.8%) moderate, and 9severe anxiety (7.5%). Of all, 36 (30%) showed no symptoms or very mild anxiety.

The association of anxiety with age was statistically significant; anxiety was more prevalent among younger students. A study with undergraduate medical students reported similar anxiety rates with age over the years of a medical program, despite a trend of lower prevalence of anxiety among older students.[3] It can be speculated that lower prevalence of anxiety in older students may suggest that medical students adapt and overcome adversity over time. In general, young students that have to leave their family to move to college in faraway cities or regions can have psychological disorders.

As for gender, anxiety was more prevalent among women, which is in line with the findings of prior studies.[2,7] A meta-analysis research involving 40,348 medical students found a non-significant association between anxiety and gender.[5]

About to course year, the association with anxiety was not significant, and corroborated by the findings of a prior study.[5] On the contrary, another study suggested that stress score was higher as medical students progressed through the year of study.[13] A longitudinal design could be used to investigate this relationship, as it would allow to follow up undergraduate students, and their health status over the years.[12] Transition from family to university life is critical and first-year students are of greater concern because they have to adjust to college demands and, in 2020, overcome the effects of social and physical isolation due to the current COVID-19 pandemic.

There was no association between anxiety severity and other study variables including sleep and regular physical activity. Numerous studies described sleep and physical activity as protective factors for mental health.[14] Yet, severe anxiety was more likely among those with inadequate sleep and physically inactive. It was used an exploratory approach with a binary option to assess certain variables including hours of sleep per day, physical activity, and use of anti-anxiety medication as laid out in the study methodology. Specific instruments should be used in future studies to measure these constructs using similar approaches.

Regular use of anti-anxiety medication did not show a significant association with anxiety. Still, our interpretation of these results was hindered, as it is not known the exact timing of the events, whether they experienced an episode of anxiety and started on anti-anxiety medication, or whether it was the other way around.

We recognize the limitations of this study: one of the main limitations is the non-inclusion of senior-year students in our sample as they could not attend the interviews as mentioned before. Thus, our results are for students going through the basic cycle of medical program, from year 1 to 4. Another limitation is that our findings cannot be compared to the findings of other studies, as they have used different anxiety assessment such as, the Self-Reporting Questionnaire (SQR-20) for screening common mental disorders, the World Health Organization Quality of Life, brief(WHOQOL-BREF) to assess the quality of life and the BDI to identify depressive symptoms.[12] According to Mayer,[7] besides the BDI, the STAI is a widely used inventory for clinical evaluation and research purposes of depression and anxiety and it was validated for use in the Brazilian population.

It is important to differentiate non-pathological anxiety (or social anxiety disorder, SAD) from generalized anxiety disorder (GAD) assessed in our study. While psychosocial functioning is significantly affected in GAD, and people experience intense distress and tend to worry excessively, daily life functioning is not affected in non-pathological anxiety and people can control and overcome their worries and handle more urgent matters. In our study, 30% of the students showed very low anxiety, which is considered "productive" anxiety, thus potentially boosting people's lives. Yet, 70% showed mild, moderate and severe anxiety, which could progress to GAD and affect their quality of life.

The truth is that few students with anxiety spontaneously seek help because of the stigma associated with seeking care and treatment for mental health illnesses.[8] One of the greatest challenges is that medical students tend not to seek out help because they believe their feelings of anxiety and depression are a sign of weakness.

Intense workload, lack of free time, excessive demands on oneself, social stigmatization for seeking mental health support, financial issues, and fear of ruining one's career are factors for not seeking out specialized mental care. Because of its potential to affect students' learning, quality of daily life and professional practice, either during their clinical rotations in the final third of the undergraduate program (not assessed in the present study) or after graduation, it is critical to help them. Thus, it is necessary to develop and implement interventions and activities to help students cope with the demands of a medical program and minimize potential mental health disorders. Some pedagogic strategies as portfolios, mentoring, and feedback must be planned by the educators to favor the development of emotional competencies in medical students.[15] Moreover, it is concerning that suicidal



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thoughts are prevalent among medical students and other health care students.[8] It thus reinforces the importance of interventions, as health care students and graduates have more knowledge about effective means to commit suicide. Early identification of anxiety symptoms that may develop into depressive states and suicide thoughts/attempts is critical and should be incorporated into support and care policies for successfully curbing self-destructive behaviors.

It is crucial to identify potential risk factors for emotional distress during medical training and implement prevention and health promotion policies aiming at strengthening students' resilience under conditions of stress and their emotional balance.[7] Healthy lifestyles, including healthy eating habits, adequate sleep, and physical activity can also be promoted among medical students for improved quality of life.[9]

Strengthening empathy, resilience, and one's ability to adapt well to new learning environments and situations and professional life demands is key to improve the well-being of health care and medical students. These skills are essential for overcoming daily challenges and other emerging challenges as those created by the COVID-19 pandemic worldwide.

## V. Conclusions

Anxiety is reportedly a prevalent condition among medical students as they face large amount of learning tasks, intensive workloads, and excessive demands.

There was found 31.7% prevalence of mild, 30.8% moderate and 7.5% severe anxiety in medical students. Higher rates of anxiety were seen among females and younger students.

Our findings of anxiety in Brazilian medical students provide input for the development and implementation of strategies at medical education institutions that could minimize emotional distress.

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*Authors' contributions:*

All authors contributes to the design, analysis and interpretation of the data. All authors revised the article and approved the final version.

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The Anxiety Symptoms Formulary

ANNEX 1

Name: .....

Age (years): -----Gender: .....Learning module: .....

Hours of sleep per day: .....Hours of physical exercise per week:.....

Regular use of anti-anxiety medication: () yes () no

Chart. The Anxiety Symptom Formulary

| DOMAIN | ANXIETY SYMPTOM | 0 | 1 | 2 | 3 |
|---|---|---|---|---|---|
| PSYCHIC | Worrying too much | | | | |
| | Feeling “on the edge” | | | | |
| | Difficulty concentrating | | | | |
| | Trouble relaxing | | | | |
| | Fearing for the worst | | | | |
| | Being terrified | | | | |
| | Being nervous | | | | |
| | Fear of losing control | | | | |
| | Fear of dying | | | | |
| | Being frightened | | | | |
| SOMATIC | Numbness or tingling | | | | |
| | Restlessness | | | | |
| | Tremors | | | | |
| | Heat sensation | | | | |
| | Palpitation or racing heart | | | | |
| | Dizziness | | | | |
| | Loss of balance | | | | |
| | Feeling of choking | | | | |
| | Difficulty breathing | | | | |
| | Stomach discomfort | | | | |
| | Cold sweating | | | | |



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# From Acute Pancreatitis to Pancreonekrosis during Pregnancy

By V.O. Zabolotnov, Y.V. Yakovenko, V.Y. Shatyo, O.O. Khvatova, Y.P. Sehedina & Vira Yakovenko

*Abstract-* Acute pancreatitis during pregnancy, although a rare but extremely formidable complication, is accompanied by a high level of perinatal and maternal mortality. Excessive hypertriglyceridemia is an extremely important risk factor for pancreatitis, especially during pregnancy. The secretion of cholesterol in hepatic bile increases in the second and reaches a peak in the third trimester compared with bile acids and phospholipids, which leads to a supersaturation of bile. Which determines the highest incidence of acute pancreatitis in pregnant women in the third trimester.

The acute onset, the nature of the disease and the difficulties in diagnosis in the treatment of acute pancreatitis in pregnant women significantly threaten the health of the mother and fetus.

Although most authors argue that treating acute pancreatitis during pregnancy is similar to treating non-pregnant patients, this is actually far from the truth. During pregnancy, the features of the pathogenesis of acute pancreatitis in pregnant women come to the fore, which must be taken into account when managing these patients.

*Keywords: pregnancy, acute pancreatitis, pancreatic necrosis, treatment, obstetric tactics.*

*GJMR-K Classification: NLMC Code: WI 805*




*Strictly as per the compliance and regulations of:*

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# From Acute Pancreatitis to Pancreonekrosis during Pregnancy

V.O. Zabolotnov [α], Y.V. Yakovenko [σ], V.Y. Shatyo [ρ], O.O. Khvatova [ω], Y.P. Sehedina [¥] & Vira Yakovenko [§]

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Although most authors argue that treating acute pancreatitis during pregnancy is similar to treating non-pregnant patients, this is actually far from the truth. During pregnancy, the features of the pathogenesis of acute pancreatitis in pregnant women come to the fore, which must be taken into account when managing these patients. Before medical workers (primarily obstetricians) there is a question about the advisability of preserving pregnancy, the fetus, the timing and method of abortion, and today these are extremely controversial decisions.

Management of pregnant women with acute pancreatitis is an extremely difficult task, despite the achievements of recent years, and is accompanied by high perinatal and maternal mortality, the level of which depends, first of all, on the severity of acute pancreatitis.

The severe course of acute pancreatitis in pregnant women is extremely dangerous for the fetus and in all cases leads to its distress and in more than half of cases to fetal loss. The average severity of pancreatitis is also unfavorable for the fetus and is accompanied by its loss in every fourth case. If triglycerides are the cause of acute pancreatitis, emergency abortion is indicated. The management of such pregnant women requires a multidisciplinary approach to predict the course of pancreatitis, determine the tactics of pregnancy and acute pancreatitis of pregnant women, the method and timing of delivery.

*Keywords: pregnancy, acute pancreatitis, pancreatic necrosis, treatment, obstetric tactics.*

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## I. Introduction

Alexander the Great was the Macedonian king from 336 to 323 BC. It was assumed that he could have died from malaria, pneumonia, typhoid fever or West Nile fever, but recently researchers have rejected the old versions of his death. It turned out that the king of Macedonia from the Argead dynasty, the outstanding commander Alexander the Great died as a result of acute pancreatic necrosis - an acute form of pancreatic disease [1].

*Acute pancreatitis* is defined as a sudden inflammation of the pancreas that is clinically present with abdominal pain, nausea and dehydration, which is usually self-limiting, but can sometimes progress to severe illness and even death.

While acute pancreatitis accounts for nearly 1 in every 200 hospitalizations in the United States each year, fortunately, the incidence of acute pancreatitis in pregnant women is rare [2].

It is estimated that acute pancreatitis occurs in about 1 in every 10,000 pregnancies, but this rate varies by region and type of hospital [2].

Acute pancreatitis in pregnant women (APP) most often occurs in the third trimester, and, according to some authors, stones in the gallbladder are the most common cause [3,4]. This proportion is similar to previously reported studies that also show the majority of cases of stones in gallbladder appealing in the third trimester [4,5].

In the study, the majority of patients had APP onset during the third trimester (68%), but 31% also had APP in the first and second trimesters. This date is similar to previously reported studies [6,7].

Acute onset, disease progression, difficulties in diagnosis and in treatment of acute pancreatitis in pregnant women significantly threaten the health of the mother and fetus [8, 9].

In previous studies it was shown that the mortality rate was approximately 37% for a mother and 60% for a fetus, while, more recent studies show that these numbers have decreased due to improvements in diagnostics, intensive care and newborn care [7,8].

With regard to diagnosis, treatment, management of pregnancy and timing of delivery, specific guidelines for acute pancreatitis in pregnant women (APP) are still lacking. This is largely due to the low incidence rate and poor clinical data; it is also due



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to the multidisciplinarity of this condition, when medical care is provided by obstetricians, surgeons, therapists, gastroenterologists, anesthesiologists, etc. [10].

*Features of etiology and pathogenesis in pregnant women*

There are many causes of acute pancreatitis, but the two most common are:

- alcohol consumption,
- disease of the gallbladder / bile ducts [2].

It is believed that most cases of APP are caused by gallstone disease. With weight gain and hormonal changes caused by pregnancy, gallstones are more likely to form and thus travel through the common bile duct, obstructing pancreatic drainage.

Excessive hypertriglyceridemia (chylomicronemia) is also an extremely important risk factor for pancreatitis, especially during pregnancy. The secretion of cholesterol in hepatic bile increases in the second and reaches a peak in the third trimester compared to bile acids and phospholipids, which leads to bile oversaturation [11,12].

However, levels achieved are never high enough to cause acute pancreatitis. In patients with familial hypertriglyceridemia, pregnancy can significantly increase hyperlipoproteinemia and may be the cause of acute pancreatitis. Changes in triglyceride clearance of apoprotein or lipoprotein lipase metabolism have been discussed since the 1980s. Timely diagnosis and treatment of familial hypertriglyceridemia can prevent pregnancy complications [13,14].

Some genetic changes in 30-35% of cases increase the risk of acute pancreatitis [15]:

- Mutations in CFTR (cystic fibrosis transmembrane conductance regulator), SPINK (Kasal-type serine protease inhibitor) or chymotrypsin C.
- Hereditary form of pancreatitis (1% of cases) - autosomal dominant disease, heterozygous mutations in the cationic trypsinogen gene (PRSS1) gene on chromosome 7 (penetrance: - 80%) or on the serine protease inhibitor (SPINK1) gene or Pancreatic secretory trypsin inhibitor gene (PSTI), gene on chromosome 5.

Hormonal changes during pregnancy can predispose to the development of hypertriglyceridemia. When triglyceride levels get too high, oxygen cannot adequately reach the pancreas through the bloodstream and pancreatitis can develop. Of course, during pregnancy, all other causes of acute pancreatitis should be taken into account: alcohol consumption, reactions to certain medications, trauma to the pancreas (Fig. 1).

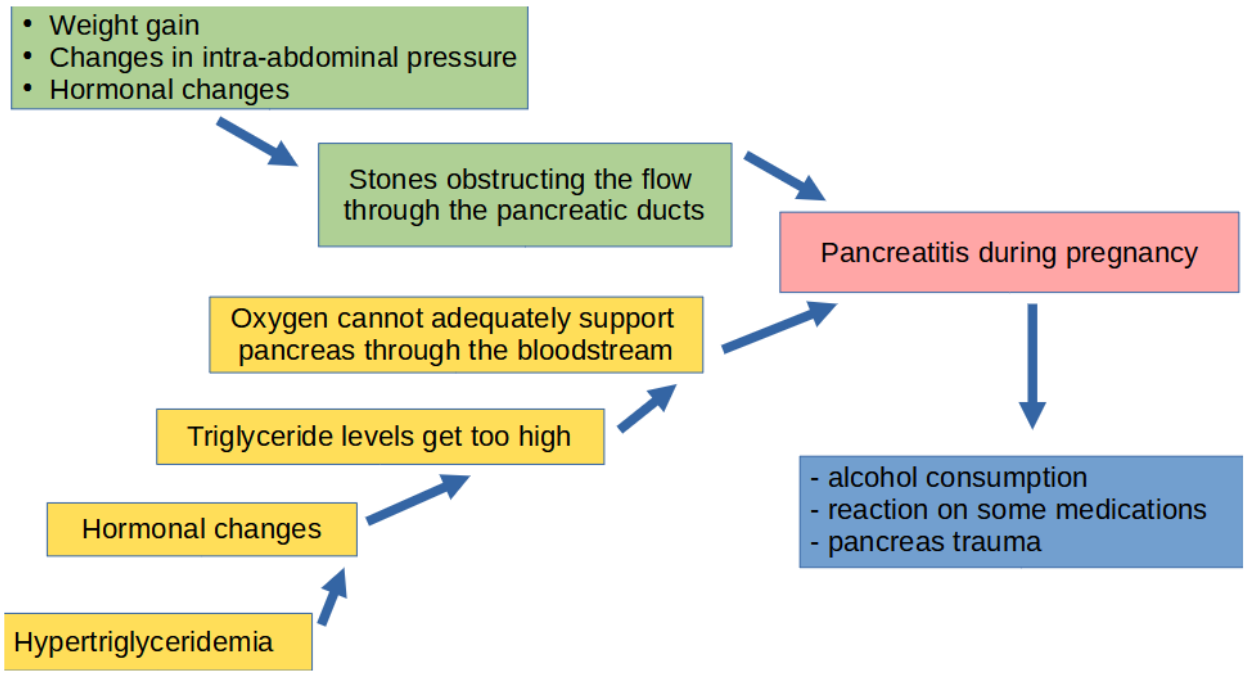



*Figure 1:* Features of acute pancreatitis in pregnant women

Determination of the diagnosis of acute pancreatitis is based on the criteria of Atlanta classification, 2013 [16]. According to it, the diagnosis of acute pancreatitis requires two of the following of three components:

- Abdominal pain corresponding to acute pancreatitis (acute onset of persistent, severe epigastric pain, often radiating to the back);
- Activity of serum lipase (or amylase activity) at a minimum of three times the upper limit of the norm;



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- Characteristic signs of acute pancreatitis on contrast computed tomography (CT) and, more rarely, magnetic resonance imaging (MRI) or transabdominal ultrasonography [16].

*Determination of types of acute pancreatitis* [16]
Acute pancreatitis can be classified into two types:

- Interstitial edematous pancreatitis
- Necrotizing pancreatitis.

To determine organ failure, three organ systems must first be assessed:

- Respiratory,
- Cardiovascular
- Renal.

This classification [5] defines three degrees of severity of the disease course:

- Mild acute pancreatitis,
- Moderately acute pancreatitis,
- Severe acute pancreatitis.

*Mild acute pancreatitis* [16]

It is characterized by the absence of organ failure and the absence of local or systemic complications. Patients with mild acute pancreatitis. Patients with mild acute pancreatitis usually do not require imaging of the pancreas, are discharged early and mortality is very rare (but possible).

*Moderate acute pancreatitis* [16]

It is characterized by the presence of temporary organ failure, local or systemic complications in the absence of permanent organ failure. An example of a symptomatic local complication is peripancreaticedema resulting in prolonged abdominal pain, leukocytosis, and fever, or which interferes with the ability to maintain oral nutrition.

An example of a symptomatic systemic complication is exacerbation of coronary artery disease or chronic lung disease caused by acute pancreatitis.

Moderate acute pancreatitis may resolve without intervention (as with temporary organ failure or with the absorption of additional fluid), or long-term specialized care may be required (as with extensive sterile necrosis without organ failure).

Mortality in acute pancreatitis of moderate severity with 15-50% is much less than in acute severe pancreatitis.

*Severe acute pancreatitis* [16]

Severe acute pancreatitis is characterized by persistent organ failure. Organ failure, which develops at an early stage, is triggered by the activation of cytokine cascades leading to SIRS - systemic inflammatory response syndrome.

When SIRS is present and persistent, there is an increased risk that pancreatitis will be complicated by persistent organ failure and the patient should be treated as if they had severe acute pancreatitis.

Persistent organ failure can be single or multiple organ failure.

Patients with persistent organ failure usually have one or more local complications.

Patients who develop persistent organ failure during the first few days of illness are at increased risk of death, and mortality is > 50%.

The development of infected necrosis in patients with persistent organ failure is associated with extremely high mortality.

The severe course of acute pancreatitis [17] is characterized by such data as:

- Signs of hypovolemia (elevated blood urea),
- Increased hematocrit,
- Increased creatinine,
- Clinical signs of pulmonary involvment, such as pleural effusion and/or infiltration, indicate that this patient may have severe acute pancreatitis.

In addition, the presence of organ failure and/or pancreatic necrosis immediately classifies acute pancreatitis as severe.

Systemic complications of acute pancreatitis during pregnancy:

- Respiratory function is impaired due to pleural effusion, atelectasis, acute pulmonary edema, or ARDS, resulting in hypoxemia and dyspnea [18,19].
- Circulatory complications are characterized by shock due to hypovolemia and/or hypotension. The main causes are retroperitoneal or peritoneal fluid loss and/or peripheral vasodilation [19].
- Cardiac complications are characterized by tachycardia and nonspecific abnormalities rather than decreased cardiac function due to a young age in a pregnant woman.
- Disorders of coagulation and especially DIC (disseminated intravascular coagulation) are very important during pregnancy, as they are accompanied by multiple organ failure and lead to a high incidence of intrauterine and maternal mortality [19].
- Renal function is impaired during severe acute pancreatitis, resulting in uremia and oliguria, either through prerenal azotemia or acute tubular necrosis [19,20].
- Metabolic complications include hypocalcemia, hyperglycemia, hypertriglyceridemia, hypoglycemia, and acid-base disorders [21].

APP can be classified for various pathogenic reasons [5].

- Acute gallstone pancreatitis is diagnosed by an elevated ALT level> 150 U/l within 48 hours from the onset of the disease, as well as by X-ray data, abdominal ultrasound and magnetic resonance cholangiopancreatography (MRCP) [5].
- Hypertriglyceridemic pancreatitis (HTGP) - after excluding gallstones, alcohol, or drug factors,



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diagnosed based on abnormal serum triglyceride levels ≥ 11.3 mmol/ l or serum triglyceride levels between 5.65 and 11.3 mmol/l.

- Idiopathic pancreatitis is diagnosed by radiological signs of pancreatitis after excluding gallstones, alcohol, hypertriglyceridemia, drug treatment, trauma, autoimmune and surgical factors [5].

*Main clinical symptoms:*

- *Extremely important:* acute pain in the upper abdomen, similar to the girdle (+ increased serum pancreatic enzyme).
- Other common symptoms:

– Fever, tachycardia, nausea, vomiting.
– Tension of the abdominal wall ("rubber belly - sensations resemble pressing on a slightly deflated rubber ball"), flatulence, paralytic incomplete (partial) intestinal obstruction, Blumberg sign symptom is doubtful or negative. With the development of peritonitis, Blumberg sign becomes positive.

One of the studies [5] indicated that abdominal pain and vomiting remained the two most predominant clinical symptoms in pregnant women with APP. Abdominal pain was localized mainly in the upper abdomen, which was observed in 86.8% of patients (105/121), while only 11.6% had lower (5.0%, 6/121) or generalized pain in abdomen (6.6%, 8/121). More than half of the patients had vomiting (73.6%, 89/121) and fever was less common (23.1%, 28/121). The tension of the muscles of the anterior abdominal wall was mainly in the upper abdomen (91%, 90/99), while only 3% was in the lower abdomen (3/99) and 6% of the entire anterior abdominal wall (6/99) [5].

Depending on the severity of the APP and the cause, the following complications may be present:

- Circulatory reaction: hypotension, shock,
- Ascites, pleural effusions (left> right),
- Jaundice (often only shortly) in the presence of common bile duct stones,
- Rare, but prognostically unfavorable signs: cyanosis of the skin around the navel - with hemorrhage into the abdominal cavity (Cullen sign) or hemorrhagic cyanotic spots on the left lateral wall of the abdomen, sometimes with a yellowish tinge (Gray-Turner sign)
- Scoring systems for determining severity, such as the Ranson Criteria (clinical prediction rules for predicting the disease and mortality risks of acute pancreatitis) are of secondary importance in practice.

Important criteria that indicate a severe course are: clinical symptoms (pain, shock symptoms), CRP increase (>120 mg/l), creatinine increase (>1.2 mg/dl) and decrease in pO2.

*Complications of APP:*

- Shock due to lack of circulating blood volume, release of vasodilators and toxic substances. Consequences: acute renal failure, pulmonary shock (ARDS), consumption coagulopathy.
- Abscess, sepsis.
- Diabetes.
- Extensive necrosis and hemorrhage (possibly gastrointestinal bleeding).
- Pseudocysts: usually after 10 to 14 days (possibly with bleeding, rupture, abscess).
- Formation of fistula, stenosis (duodenum, biliary tract, large intestine).
- Thrombosis of the portal vein and splenic vein. Result: portal hypertension with esophageal varices,
- Splenomegaly.
- Paralytic intestinal obstruction.

*Diagnostics*

- Sonography: enlarged pancreas: pancreatic head > 3cm, body > 2cm, tail >3cm, pancreatic duct >3mm; echogenicity roughly corresponds to that of a healthy liver, with age it is more intense and not always clearly limited (edema). Pathological sings: identification of *hypoechogenicity*, i.e. possibly necrosis, as well as ascites, pleural effusion, abscesses, pseudocysts, with biliary genesis, an increase in Ductus choledochus, dilated bile ducts or visible stones in the gallbladder is possible.



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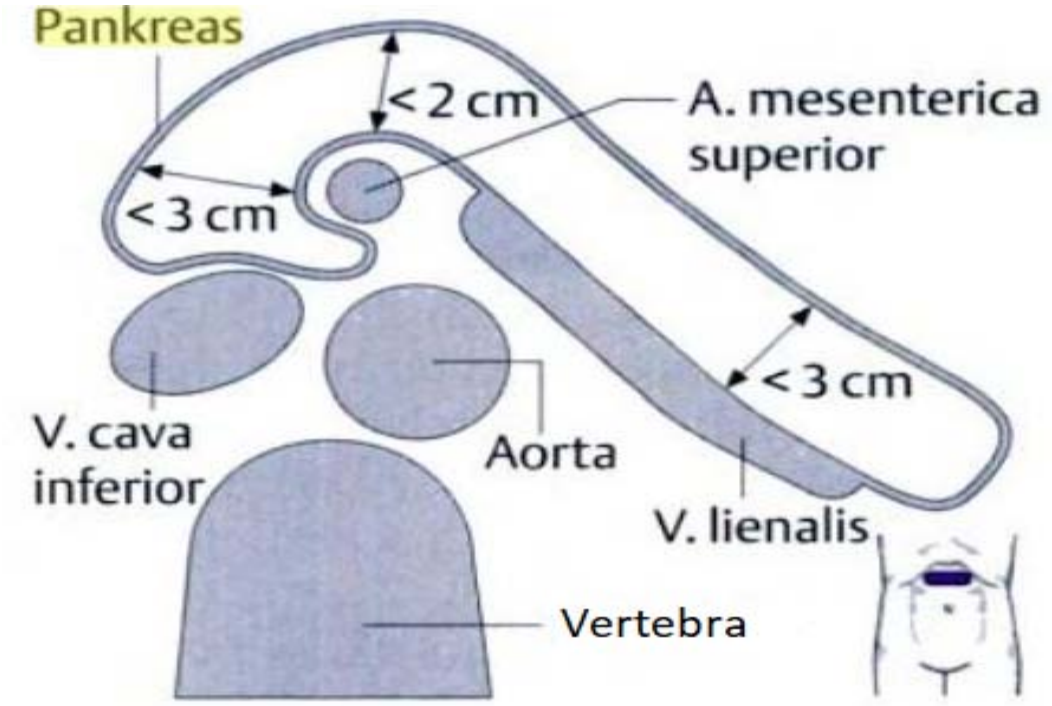



*Figure 2:* Sonography of the pancreas (normal)

- CT scan of the abdomen or endosonography: only if conventional sonography is insufficient due to the overlap of air associated with the disease (use is contraindicated, or severely limited during pregnancy).
- Chest X-ray: pleural effusion, lamellar atelectasis, high standing of the left part of diaphragm, basal pneumonia, signs of ARDS (use is limited during pregnancy).
- X-ray of abdominal cavity: level/volume of intestinal obstruction, perforation (use is limited during pregnancy).
- Endoscopic retrograde cholangiopancreatography (ERCP) (if biliary genesis is suspected): if necessary, papillotomy (use is contraindicated, extremly limited during pregnancy).
- Gastroscopy: the presence of ulcers and "stress" lesions of the mucosa
- Fine-needle puncture (under ultrasound control or CT) to collect material for microbiology, to exclude infected necrosis, (usually not earlier than 10-14 days after the onset of the disease) (use is limited, rather termination of pregnancy is indicated).
- If necessary, further search for reasons.

*Laboratory deviations and severity of APP*

Increased serum lipase and pancreatic amylase. Sensitivity of lipase> amylase; absolute values do not correlate with the severity of the disease (it is not always a reliable "marker" for controlling the course of the disease).

*Depending on the cause and course:*

- leukocytosis(leukocyte count> 10 × 109 /l)
- increase in CRP and LDH, CRP = marker of severity!
- by cholestasis an increase in AP, γGT, as well as "direct" bilirubin
- by diabetes mellitus hyperglycemia(fasting glucose ≥ 7.8mmol/l)
- hypocalcemia(serum calcium <1.75mmol/l)
- an increase in urea and creatinine.

Hypertriglyceridemiais defined as fasting serumtriglyceride level ≥11.3mmol/l.

*Signs of systemic inflammatory response syndrome (SIRS)*

*SIRS - determined by the presence of two or more criteria:*

1. Heart rate> 90 /min
2. Body temperature <36 °C or >38 °C
3. The level of leukocytes in the blood <4000 or >12000 /mm$^3$
4. Breathing rate >20/min or pCO2 art. <33 mmHg.

According to one of the studies [5], only serum calcium level correlated negatively with the severity of APP (p <0.01), which corresponds to date from non-pregnant patients with pancreatitis. Serum glucose, triglycerides, or leukocyte levels did not correlate with the severity of APP. Some of the most commonly used laboratory results were compared based on the severity of APP (Table 1). Testing serum calcium may indicate



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the severity of the disease, but more investigation is needed to confirm this [5].

*Table 1:* Laboratory indicators depending on the severity of acute pancreatitis in pregnant women Lingyu Luo (2018)

| | Mild | Moderate | Severe | *P* value |
|---|---|---|---|---|
| Number of cases | 59 | 44 | 18 | |
| Hyperglycemia | 10 (16.9) | 13 (29.5) | 7 (38.9) | 0.111 |
| Hypertriglyceredemia | 10 (16.9) | 13 (29.5) | 7 (38.9) | 0.185 |
| Hypocalcemia | 3 (5.1) | 7 (15.9) | 8 (44.4) | <0.01 |
| Increased white blood cell count | 45 (76.3) | 36 (81.8) | 15 (83.3) | 0.712 |

Program of acute pancreatitis monitoring J.M. Hahn, G.Adler 2012 [22]

| Monitoring interval | Criteria for evaluation |
|---|---|
| Several times a day | Health condition (degree of abdominal pain), palpation and auscultation of the abdomen, blood pressure, pulse, fluid balance, CVP/ultrasound control of the inferior vena cava, body temperature |
| At least daily (depending on individual parameters also more often) | Laboratory parameters:<br>• Blood count<br>• Creatinine, Na +, K +, Ca ++<br>• AP , γGT, AST<br>• Bilirubin<br>• Coagulation parameters Quick/INR, PTT)<br>• CRP and LDH<br>• protein/albumin<br>• daily sugar profile<br>• BGA<br>Abdominal sonography |
| With clinical deterioration | Rö-Грудной клетки/брюшной полости, ЭКГ Мониторинг<br>Дополнительно: КТ/МРТ или эндо-узи<br>возможно ЭРХПГ, хирургическое наблюдение<br>Rö Thorax/Abdominal, ECG Monitoring<br>Optional: CT/MRI or endo-ultrasound<br>possibly ERCP, surgical observation |

*Therapy*

Although most authors claim that the treatment of acute pancreatitis during pregnancy is similar to that in non-pregnant patients, this is actually should be taken with caution. During pregnancy, the features of the pathogenesis of APP must be taken into account when managing these patients. The medical stuff (primarily obstetricians) are faced with the question of the feasibility of maintaining the pregnancy, the fetus, the timing and method of termination of pregnancy, and today these are extremely controversial decisions. And it is no coincidence that in the scientific obstetric literature there are many articles on surgical tactics, but not on the obstetric-surgical management of patients with APP.

When assessing pregnant women with acute pancreatitis, it is proposed to answer four important questions, namely:

1) Does this patient have acute pancreatitis (diagnosis and exclusion of other causes)?
2) If it is acute pancreatitis, what is the predicted severity (mild or severe?)
3) Is there a biliary pathology?
4) 1st, 2nd or 3rdtrimester of pregnancy? This last question will determine the choice of imaging and therapy regimen [23].

*Conservative therapy*

It is very important that if a pregnant woman develops any abnormal abdominal pain symptoms, she should be admitted to the emergency department as soon as possible to assess her condition.

Initial treatment is aimed to reduce exocrine pancreatic secretion, restoring third-space fluid sequestration, and supporting the patient by providing adequate nutrition, oxygenation, analgesics, and monitoring of maternal and fetal vital functions [24].

Initial treatment for acute pancreatitis is limited to forced intravenous hydration of 250-500 ml/h of isotonic crystalloid solution, preferably lactated Ringer's



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solution, unless cardiovascular, renal, or other comorbilities are present [25]. The forces intravenous hydration is indicated within the first 12 to 24 hours.

In patients with circulatory manifestations of severe fluid loss such as hypotension and tachycardia, more aggressive hydration is recommended. Fluid requirements should be reviewed at frequent intervals over the next 48 hours by assessing blood urea levels [26].

According to SNAP-Based Recommendations (Multicenter Nutritional Study in Acute Pancreatitis was conducted in the US by the National Institutes of Health) [27]:

*Patients with symptoms of ileus should be fed parenterally !!!!*

- Nutrition improves intestinal integrity, reduces the movement of bacteria and therefore the rate of serious infections.
- Start early with a jejunaltube feeding.
- It is recommended to combine the use of enteral and parenteral nutrition, especially in the case of severe forms, since exclusively enteral nutrition cannot adequately cover the patient's energy/fluid/electrolyte costs.
- Parenteral fluid and electrolyte supply should be controlled by electrolyte balance and CVP (CVP target 4-12 cm H2O). Ultrasound control of the inferior vena cava: hypovolemie by diameter <1.8cm (norm 1.8-2 cm). Fluid requirements 2-4 l/day for mild pancreatitis, up to 10 l/day for severe pancreatitis [28]. For high fluid requirements (>4 l/day) replacement of human albumin should not be forgotten: for example, 500ml of 5% human albumin per 4l of fluid (serum albumin control).
- Caution: depending on the severity of the condition, CVP may be overestimated due to increased intra-abdominal pressure, resulting elevated diaphragm, so volume requirements may be underestimated.
- Pain manegment (regular administration, dosage based on need):
- Pethidine: s/c or i/v as an injection or in a perfuser. Single dose 50-100 mg, max.every 2-3 hours, max. 500 mg/day
- Pyritramide/Dipidolor: s/c or intravenously as an injection or in a perfuser. Single dose 15-30 mg, max.every 4 - 6 hours, max. 300 mg/day
- Epidural catheter, eg with bupivacaine (eg Carbostesin® 0.25%, 5-10 mg/h = 2-4 ml/h), with a high infusion level (Th 7-10), and good prevention of ileus.

*Advanced therapy:*

- For biliary pancreatitis - ERCP and possibly simultaneous papillotomy with stone removal.
- Parenteral nutrition. At an early stage, especially with hypertriglyceridemia, shock and sepsis, preferably without fatty solutions.
- Treatment of hyperglycemia: perfuserand, if necessary, careful blood sugar checks.
- Treatment of electrolyte imbalances. Especially important are:
- K +, in particular by insulin therapy
- Ca ++, (by Ca ++ <1.6 mmol/l (depends on protein loss, CAVE check anbinned).
- Treatment of acid-base balance disorders: in particular, compensation of metabolic acidosis.
- Antibiotic therapy by septic manifestations (based on blood cultures) is not absolute indicated by mild to moderate severity.
- Severe necrotizing pancreatitis: ciprofloxacin (eg Ciprobay® 3x200 mg/day) or imipenem (eg. Zienam® 4x500 mg/day), additionally metronidazole (egClont® 3x500 mg/day).

!!!! Antibiotics !!!! In acute pancreatitis, antibiotics are currently not usually recommended even for the stage of detection of necrosis (Aseptic!). Cochrane review found no benefit in routine antibiotic treatment [29].

- Treatment of acute renal failure: mainly due to lack of hydration. If necessary, hemodialysis or hemofiltration.
- Ensuring adequate blood gas levels. If necessary, respiratory support, inuncertaincase – generousindications for lung ventilation (NIV) as therapy for ARDS.
- Prevention and, if necessary, therapy of consumption coagulopathy.
- If signs of shock appear: start therapy immediately, as in septic shock.
- Pseudocysts (usually 10-14 days after the onset of the disease, high spontaneous regression rate of 50% within 6 weeks):
  - Ultrasound monitoring (possibly also CT)
  - If an infection is suspected: diagnostic puncturefora possible focus.
  - In exceptional cases, open surgical therapy (increased mortality).
- Surgical treatment: only if conservative therapy does not help and there is an unresolved septic focus (high mortality).
- Enteral feeding: start as early as possible in a pain-free period, low-fat food, possibly additional enzyme preparations.
- Prevention of relapse: for example, debridement of the bile ducts for calculus, abstinence from alcohol, treatment of hypertriglyceridemia or hyperparathyroidism.

Some recent reports [30] suggested a combination of intravenous infusion of heparin and



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insulin in severe cases of gestational hypertriglyceridemia caused by acute pancreatitis, which increased lipoprotein lipase activity.

In addition, although there is no clear clinical guideline, the use of plasmapheresis and hemofiltration may also be beneficial in some cases [31].

*Surgical treatment*

As for the disease itself, surgical treatment is suggested in special cases of necrotizing pancreatitis. Sterile necrosis, as well as asymptomatic local changes such as a pancreatic pseudocyst, do not require intervention regardless of size, location and/or expansion [32, 33].

In case of progression of a pseudocyst (> 6 cm) or complications (infection, compression of adjacent structures), an internal drainage and/or removal of the cyst is surgically performed [3].

Surgical treatment: only if conservative therapy does not help and there is a non-sanitized septic focus (high mortality).

*Interventional treatment of APP*

Usually, if gallstones result pancreatitis, the removal of the gallbladder is delayed until the end of the pregnancy. Often, a stent placed in the bile duct can correct the situation until operative resection is required [32]. However, if waiting until the end of pregnancy is not possible, surgical resection can usually be performed safely, especially before 28-30 weeks of gestation [34].

In stable patients with infected necrosis, surgery should be delayed for more than 4 weeks from the onset of symptoms so that the lesions can be organized into a more specific shape [26].

Treatment of stones in the biliary tract can be X-ray surgical- antegradetranshepatic access to the ducts (limited during pregnancy), as well as endoscopic retrograde access through an endoscope in duodenum. Both of these approaches can mechanically destroy the stones, then they will independently enter the intestinal lumen, or remove them. Bile duct stenting is not required in this situation.

Acute biliary pancreatitis in pregnant women requires *surgical treatment* only when there is:

1) Acute cholecystitis, without treatment success under conservative therapy,
2) Peritonitis,
3) Obstructive jaundice and severe symptoms that will disappear after surgical treatment [26].

However, in necrotizing acute biliary pancreatitis, cholecystectomy must wait until active inflammation subsides, fluid accumulations dissolve and stabilize in order to avoid contamination of necrotic tissue regardless of the severity of the disease [23].

*Obstetric procedure*

The data in Tables 2-4 provide strong evidence of the relationship between the severity of APP, hyperlipidemia, and maternal and fetal outcome [35,36].

Hyperlipidemia (hypertriglyceredemia) is an extremely dangerous symptom and is mainly combined with moderate severity and severe course of APP, leading to fetal distress and fetal loss [37,38].

*Table 2:* Course of APP by major etiology (Tang et al. 2018)

| Etiology | Mild N=23 | Moderate N=24 | Severe N=7 | Number of Pat. N=54 |
|---|---|---|---|---|
| Biliary | 9 (39.1%) | 5 (20.8%) | 0 | 14 (25.9%) |
| Hyperlipidemia | 1 (4.2%) | 14 (58.4%) | 7 (100%) | 22 (40.7%) |
| Other | 13 (56.6%) | 5 (20.8%) | 0 | 18 (33.4%) |

*Table 3:* Fetal distress and fetal loss based on etiology based on APP etiology (Tang et al. 2018)

| | Biliary (n=14) | Hyperlipidemia (n=22) | Other (n=18) | P value |
|---|---|---|---|---|
| fetal distress | 2 | 14 | 4 | <.01 |
| fetal loss | 1 | 7 | 3 | .203 |

The severe course of APP is extremely dangerous for the fetus and leads in all cases to its distress and in 60.0% of cases to fetal loss (Table 4). The moderate severity of pancreatitis is also unfavorable for the fetus and is accompanied by its loss in every fourth case (25%) [26].

If fetal distress occurs in the period of gestation from 22 to 26 weeks and there are no conditions for high professional resuscitation of the newborn (a favorable perinatal outcome is extremely doubtful), the decision on the strategy of pregnancy management and/or its termination, first of all, is made in favor of saving the health and life of the pregnant woman.



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*Table 4:* Outcome for mother and fetus by patients with APP (Tang et al. 2018)

| | Mild | Moderate | Severe | Number | |
|---|---|---|---|---|---|
| | N=23 | N=24 | N=7 | N=54 | P value |
| Spontaneous delivery on term | 20 (87.0%) | 11 (45.8%) | 0 (0.0%) | 31 (57.4%) | <.001 |
| Pregnancy termination due to maternal and/or fetal complications | 2 (8.7%) | 3 (12.5%) | 0 (0.0%) | 5 (9.26%) | >.999 |
| Premature birth | 0 (0.0%) | 7 (29.2%) | 5 (71.4%) | 12 (22.2%) | <.001 |
| Abortion (spontaneous or med. indicated) | 1 (4.3%) | 3 (12.5%) | 2 (26.6%) | 6 (11.1%) | .211 |
| Fetal distress | 2 (8.7%) | 11 (45.8%) | 7 (100%) | 20 (37.0%) | <.001 |
| Fetal loss | 1 (4.3%) | 6 (25.0%) | 4 (57.1%) | 11 (20.4%) | .007 |

If triglycerides are the cause of acute pancreatitis, urgent termination of pregnancy is indicated. Before 30 weeks of gestation, and in the presence of professional newborn resuscitation, urgent delivery is preferred, as this will lead to an immediate decrease in triglyceride levels [39,40].

When the cervix is ready for childbirth, it is advisable to deliver through the natural birth canal. During childbirth after 28 weeks (approximately at this time), monitoring of the intrauterine state of the fetus is indicated.

At the same time, the outcome of delivery largely depends on the severity of acute pancreatitis (Table 5), and does not always depend on the method of delivery. A cesarean section performed in conjunction with a surgeon allows immediate delivery of a pregnant woman with APP, increases the likelihood of a favorable perinatal outcome, creates conditions for performing the necessary amount of surgical interventions for pancreatitis, but increases the risk of septic complications. Delivery through the natural birth canal reduces the risk of purulent-septic complications, but somewhat delays the time of delivery. Therefore, the decision on the method of delivery is made by the council after considering all risk factors.

*Table 5:* Maternal and fetus intrauterine mortality bz patients with different severity of APP (Lingyu Luo, 2018)

| | Mild | Moderate | Severe |
|---|---|---|---|
| | N=23 | N=24 | N=7 |
| Total number | 59 | 44 | 18 |
| Total delivery number, n (%) | 55 | 40 | 10 |
| Pregnancy duration week | 35 | 8 | 3 |
| Cesarean section and | 18 (30.5) | 32 (72.7) | 6 (33.3) |
| Delivery on term | 2 (3.4) | 0 | 1 (5.6) |
| **Total number of deaths, n (%)** | 4 | 4 | 8 |
| **Caesarean section and death** | 0 | 2 (4.5) | 2 (11.1) |
| Premature birth | 1 (1.7) | 1 (2.3) | 0 |
| Stimulated delivery | 2 (3.4) | 1 (2.3) | 2 (11.1) |
| Natural abortion | 1 (1.7) | 0 | 0 |
| Mother's. and fetus mortality | 0 | 0 | 4 (22.2) |

During a cesarean section, the decision whether the surgical intervention due to pancreatitis should be extended or not should meet a surgeon (preferably a council of surgeons).

*Indications for immediate pregnancy termination:*

1. Moderate and severe acute pancreatitis;
2. Pancreatic necrosis (or reasonable suspicion of it);
3. The presence of organ failure - acute renal failure, ARDS
4. Hypertriglyceridemia;
5. Hypocalcemia;
6. Complications of pancreatitis: shock, consumption coagulopathy, abscess, sepsis, extensive necrosis and hemorrhage, paralytic ileus.

## II. Conclusion

The management of pregnant women with acute pancreatitis is an extremely difficult task, despite the achievements of recent years, and is accompanied by high perinatal and maternal mortality, the level of which depends, first of all, on the severity of acute pancreatitis.

The management of such pregnant women with acute pancreatitis requires a multidisciplinary approach to predict the course of pancreatitis, to determine the strategies for pregnancy management and for method and timing of delivery.



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# A Hand Gesture Recognition System for Deaf-Mute Individuals

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*Abstract-* A deaf-dumb individual always uses gestures to convey his/her ideas to others. However, it is hard for people to understand this gesture language. The purpose of the project is to develop a computer-based system to recognize 26 gestures from American Sign Language (ASL) using MATLAB, which will enable deaf-dumb individuals significantly to communicate with all other people using their natural hand gestures. The proposed system in this project is composed of five modules, which are prepared datasets for ASL which was self-collected using hand gestures from both male and female volunteers, who have alternative ages and skin color in different backgrounds and postures by an ordinary phone camera in total the dataset was 260 images preprocessing, hand segmentation, feature extraction, sign recognition, and text of sign voice conversion. Segmentation is done by converting the image to Hue-Saturation- Value (HSV) format and using color threshold APP. Blob features are extracted by using (BOF) which used the Speed up Robust Features (SURF) algorithm. Furthermore… the K- Nearest Neighbor (KNN) and Support Vector Machine (SVM) algorithms are used for gesture recognition. The Recognized gesture is converted into voice format.

*Keywords:* *bag of feature (BOF), HSV, KNN, SURF, SVM, gesture.*

*GJMR-K Classification:* *NLMC Code: WV 280*




*Strictly as per the compliance and regulations of:*




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# A Hand Gesture Recognition System for Deaf-Mute Individuals

Rugia S.T.Kamal Eldeen [α] & Dr. Ebtihal H.G.Yousif [σ]

*Abstract-* A deaf-dumb individual always uses gestures to convey his/her ideas to others. However, it is hard for people to understand this gesture language. The purpose of the project is to develop a computer-based system to recognize 26 gestures from American Sign Language (ASL) using MATLAB, which will enable deaf-dumb individuals significantly to communicate with all other people using their natural hand gestures. The proposed system in this project is composed of five modules, which are prepared datasets for ASL which was self-collected using hand gestures from both male and female volunteers, who have alternative ages and skin color in different backgrounds and postures by an ordinary phone camera in total the dataset was 260 images preprocessing, hand segmentation, feature extraction, sign recognition, and text of sign voice conversion. Segmentation is done by converting the image to Hue-Saturation- Value (HSV) format and using color threshold APP. Blob features are extracted by using (BOF) which used the Speed up Robust Features (SURF) algorithm. Furthermore… the K- Nearest Neighbor (KNN) and Support Vector Machine (SVM) algorithms are used for gesture recognition. The Recognized gesture is converted into voice format. To make the system friendlier, The Graphical User Interface (GUI) is designed to train and test the model. The implemented model demonstrates that the average accuracy from the evaluation set is (89%), whereas the average accuracy obtained from the test set is (84.6%).

*Keywords: bag of feature (BOF), HSV, KNN, SURF, SVM, gesture.*

## I. Introduction

### a) Introduction

Communication between normal and handicapped person such as deaf people, dumb people, and blind people has always been a challenging task. They found it really difficult at times to interact with normal people with their gestures, as only a very few of those are recognized by most people. Since people with hearing impairment or deaf people cannot talk like normal people so they have to depend on some sort of visual communication in most of the time. Sign Language is the primary means of communication in the deaf and dumb community [1]. The deaf people become neglected by society because ordinary people never try to learn ASL nor try to interact with the deaf people. They mostly remain uneducated and isolated. So the only way to enhancing the communication between mute people and ordinary people is recognition of sign language and converting it to the corresponding voice [2]. Sign language recognition developed in the „90s.

Research related to hand gestures classified into two parts. In the first part, electromagnetic gloves and sensors are introduced, which consist of the hand shape, movements and, orientation of the hand. These have limitations such as cost and not suitable for practical use. The second one is a computer vision-based gesture recognition system, which consists of image processing techniques, which required only a camera and computer or mobile device like phones or tablets, which are very common among people [3].

### b) American Sign Language

American Sign Language (ASL) is a complete, complex language that employs signs made by moving the hands combined with facial expressions and postures of the body. It is the primary language of many North Americans who are deaf and is one of several communications. [4]

### c) Proposed Model

The dataset used in the proposed model was collected and created by ourselves by using phone camera without perfect aligned with camera. Firstly, the system will take the dataset images and apply skin detection algorithm on it and detect the skin color pixels from it. Then it will make the image binary. Secondly for feature extraction and evaluation we are using Bag of features in category classification. It splits the image grid by grid and takes number of image patches from it. The strongest features are identified by using SURF algorithm and K-means clustering is used for vector quantization. The features or bag of words are stored in the feature vector After that for multi-class SVM (Support Vector Machine) classifier has been used for categorize training and testing set for evaluation. Thirdly, the system will take the tested image and apply skin detection algorithm on it for purpose of skin detection, using SURF algorithm to extract features and K-mean clustering for vector quantization finally it was compared with features in dataset to find the corresponding letter. This proposed model is shown in figure.1 below.

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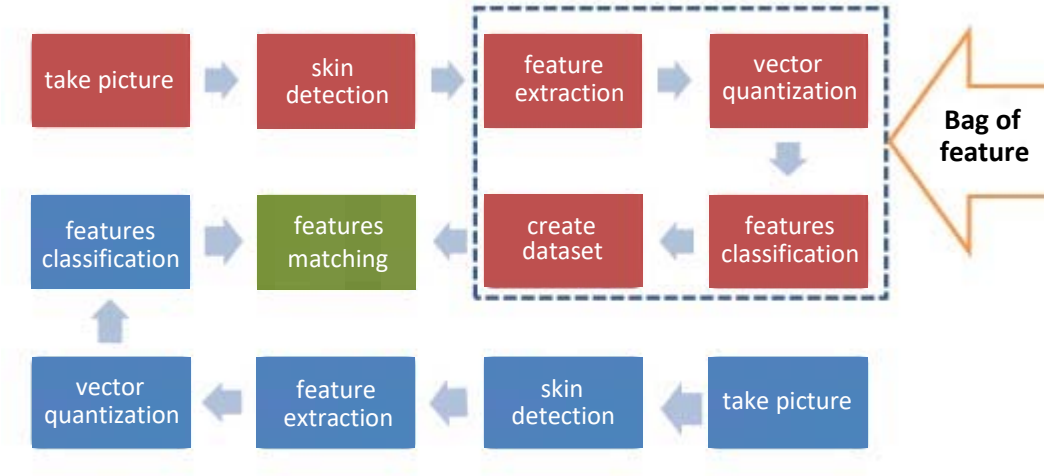



*Figure 1:* Flow Chart

*d) Bag of Feature*

The past decade has seen the rise of the Bag of Features approach in computer vision. Bag of Features (BOF) methods have been applied to image classification, object detection, image retrieval and even visual localization for robots. BOF approaches are characterized by the use of an order less collection of image features. The Bag of Features image representation is analogous. A visual vocabulary is constructed to represent the dictionary by clustering features extracted from a set of training images. The image features represent local areas of the image, just as words are local features of a document. Clustering is required so that a discrete vocabulary can be generated from millions (or billions) of local features sampled from the training data. Each feature cluster is a visual word. Given a novel image, features are detected and assigned to their nearest matching terms (cluster centers) from the visual vocabulary. The term vector is then simply the normalized histogram of the quantized features detected in the image. The steps of bag of feature shown in figure.2 below.[5]

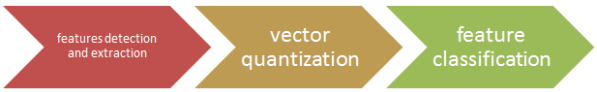



*Figure 2:* Steps of Bag of Feature

i. *Feature Detection and Descriptor*

SURF is a local feature detector and descriptor that can be used for tasks such as object recognition or registration or classification or 3D reconstruction. It is a scale and in-plane rotation invariant feature. The detector locates the interest points in the image, and the descriptor describes the features of the interest points and constructs the feature vectors of the interest points. SURF features are invariant of shifting, rotation and scaling, and partially invariant to illumination and affine transformation. [6]

Algorithm consists of four main parts:

1. Integral image generation,
2. Fast-Hessian detector (interest point detection),
3. Descriptor orientation assignment (optional),
4. Descriptor generation.

ii. *Quantization and Distance Measures*

Vector Quantization (Clustering) is used to build the visual vocabulary in Bag of Features algorithms. Nearest-neighbor assignments are used not only in the clustering of features but also in the comparison of term vectors for similarity ranking or classification. Many BOF implementations are described as using K-means cluster.

With K-means clustering process we want to minimize sum of squared Euclidean distances between points xi and their nearest cluster centers mk. [5]

$$D(x,m) = sum(cluster) - k * sum(pointiincluster) - k(x_i - m_k)^2$$

*Algorithms:*

1. Select initial centroids at random,
2. Assign each object to the cluster with the nearest centroid,
3. Compute each centroid as the mean of the objects assigned to it,
4. Repeat previous 2 steps until no change.



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iii. *Feature Classification*

Support Vector Machine (SVM) is primarily a classifier that performs classification tasks by constructing hyper planes in a multidimensional space separating cases of different class labels. According to SVM the decision boundary should be as far away from the data of both classes as possible. The linear separating hyper plane is the plane

$$w^t x + b = 0$$

## II. Literature Review

In [7], nine gestures are recognized and converted to speech and text in real-time by using MATLAB. YCbCr color transformation used for feature extraction and, PCA algorithm has been used for recognition to captured image using a web camera. In [8], PCA algorithms also have been used to recognize 26 gestures from Indian sign language, morphological filter and, outs algorithms for segmentation to get comparable accuracy. A comparison has been made between using web camera and traditional image processing techniques against the android devices and PCA algorithms the first method is more accurate (90) But it takes time and memory the second method is faster and need less memory but has low accuracy(77) [9]. In [10] does not require the background to be perfectly black to recognize sign language. Image preprocessing, calculating coordinate for feature extraction and, finally the pattern matching algorithm for classifying purpose used to recognize. In terms of machine learning-based approaches, Abdo et al. [11] employed Hidden Markov Model (HMM) for Arabic alphabet and numbers sign language recognition. This system is suitable and reliable compared with other competitive systems but the limitations of this system are required users to wear a color glove. Also, in the study by Dogic and Karli [12], sign language recognition has been applied with accuracy 84; the work has been doing with the use of digital image processing methods providing a system that teaches a multilayer neural network using a back propagation algorithm. Images processed by feature extraction methods (canny edge detector), and by masking method, the data set has been created. Training has been doing using the cross-validation method for better performance.

Video processing used to translate Real-time Arabic sign language to Arabic text. For example, the method used in [12] includes video segmentation (shot boundary detection, keyframe extraction), pattern construction and discrimination, and feature extraction; the extracted features are intensity histogram and Gray Level Co-Occurrence Matrix (GLCM). To identify English alphabetic sign language without requiring the hand to be perfectly aligned to the camera, an image processing technique (the detection of skin and marker pixels) has been used in [14]. For the purpose of segmentation, the threshold used finally to extract feature, and recognition, the coordinate calculation, color calibration and pattern matching algorithm used ,this system is easy and high accuracy but it requires users to wear the specific color band in their fingers.

## III. Methodology

*a) Data collection*

The sign language recognition is not a widely researched topic; so we did not find any dataset on any resources. Therefore, we made our dataset. We took images of hands for males and females of vary ages and with alternative positions also different in backgrounds using a camera phone and without perfectly aligned with the camera. We acquired images for 26 alphabets of American Sign Language.

We took images of 10 people's hands in alternative positions. So, in total in the dataset, we have 260 images. For each alphabet, we are getting ten pictures of ten people in different postures and different ages. as shown in figure [3]



*Figure 3:* Examples of Pictures for ASL



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*b) Skin Color Detection Algorithm*

Skin detection means detecting those pixels and regions from an image should be contain human skin tone color in a picture. The use of color information as a feature for skin detection enables fast processing and brings robustness to such application [15]

Skin color detection is applied to the input image for the detection of hand gestures. This technique is used for separating the skin-colored areas from the non-skin colored regions. The steps used in this skin color detection algorithm are shown in Figure [4]. RGB image converted to HSV color space which used because it is more convenient for research purposes. Conceptually, the HSV color space is a cone. Viewed from the circular side of the cone, the hues are represented by the angle of each color in the cone relative to the 0° line, which is traditionally assigned to be red. The saturation is represented as the distance from the center of the circle. Highly saturated colors are on the outer edge of the cone, whereas gray tones (which have no saturation) are at the very center. The brightness is determined by the colors vertical position in the cone. At the pointy end of the cone, there is no brightness, so all colors are black. At the fat end of the cone are the brightest colors. As hue varies from 0 to 1.0, the corresponding colors vary from red through yellow, green, cyan, blue, magenta, and back to red, so that there are actually red values both at 0 and 1.0. As saturation varies from 0 to 1.0, the corresponding colors (hues) vary from unsaturated (shades of gray) to fully saturated (no white component). As value, or brightness, varies from 0 to 1.0, the corresponding colors become increasingly brighter. The conversion of RGB to HSV is given by the following equations:

$$H = across \frac{\frac{1}{2}(R-G)+(R-B)}{\sqrt{(R-G)^2+(R-B)(G-B)}}$$

$$S = (1-3)\frac{\min(R,G,B)}{(R+G+B)}$$

$$V = \frac{1}{R+G+B)}$$

People have different color skin so, the value of H, S, and V are different from person to person depending on his color skin; the images in the dataset was taken from various people have alternative color skin, the value of H, S, and V were set depending on the color skin of individual, according to these values the hand was segmented from background after that the images was converted to black and white format by using color threshold APP, this process is shown in figure.5 and figure.4 below. The skin detected image is resized to 255×255 pixels for faster computing.

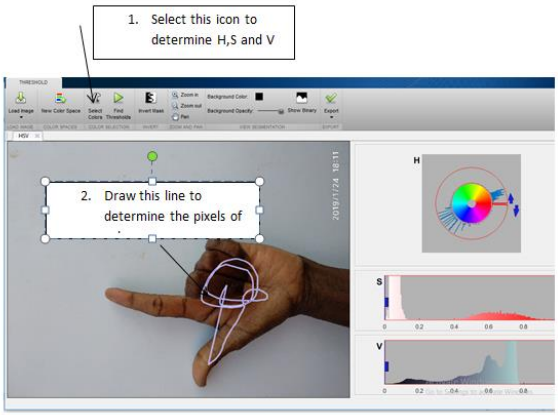



*Figure 4:* The Steps of Determine the H,S and V value for Individual



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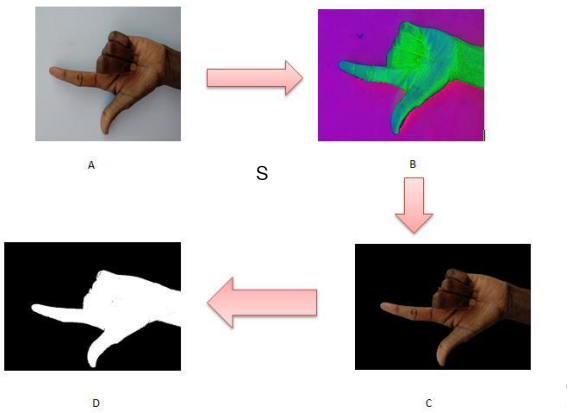



*Figure 5:* Step by step skin detection and binarization: (A) Actual image, (B) Converted to HSV, (c) segmented image, (d) converted to BW format

*c) Bag of Feature*

i. *Step 1: Setting Up Image Category Sets*

The images are Organized and partitioned into training and test subsets. The image Set function used to organize categories of images to use for training an image classifier. Images organized into categories made handling large sets of images much easier. The sets separated into training and test image subsets. In our proposed system, in sake of more accurate evaluation we took ninety percentages of images for training and the other ten percent for testing from each class randomly, as shown in Table [1]. We found the more images in the training set, the more accuracy we have. However, for technical constraints, we cannot increase the number of images more than ten in each class. Consequently, the total number of images in the training set is 260. as shown in table [2] below

*Table [1]:* Comparison between Different Percent

| Percent | Accuracy |
|---|---|
| 90 | 0.89 |
| 70 | 0.87 |
| 50 | 0.65 |
| 30 | 0.56 |

*Table [2]:* Training Sets

| | |
|---|---|
| Total Class of images | 26 |
| In one class number of images | 10 |
| Total images | 260 |
| For training the image classifier number of images | 234 |
| For testing and evaluation number of images | 26 |

ii. *Step 2: Creating Bag of Features*

A visual vocabulary or BOF created by extracting feature de-scripters from representative images of each category. The BOF object defines the features, or visual words, by using the k- means clustering algorithm on the feature descriptors extracted from training Sets. The algorithm iteratively groups the descriptors into k mutually exclusive clusters. The resulting clusters are compacted and separated by similar characteristics. Each cluster center has been represented a feature or visual word. Speeded up robust features (or SURF) detector is used that is provides



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greater scale invariance, SURF algorithm used as a detector and descriptor.

iii. *Step 3: Training an Image Classifier with Bag of Visual Words*

The Train Image Category Classifier Function returned as an image classifier. The algorithm trained a multi-class classifier using the error-correcting output codes (ECOC) framework with binary support vector machine (SVM) classifiers.

iv. *Step 4: Classifying an Image or Image Set*

Finally the Image Category Classifier predicts method is used on the tested image to determine its category. After the BOF has been used to determine the class of picture, the letter which corresponding to the image appears in the workspace,

*d) Speech Synthesizer*

Finally, the letter is converted to the voice by using Speech Synthesizer.

Speech synthesis is the artificial production of human speech. A computer system used for this purpose is called a speech synthesizer and implemented in software or hardware. A text-to- speech (TTS) system converts language text into speech; other systems render symbolic linguistic representations like phonetic transcriptions into speech [16]. In this project, we used one which is included in computer operating systems and implemented in MATLAB.

*e) Graphical user interface*

A graphical user interface (GUI) is a pictorial interface to a program. A good GUI can make programs easier to use by providing them with a consistent appearance and with intuitive controls like pushbuttons, list boxes, sliders, menus, and so forth. The GUI should behave in an understandable and predictable manner, so that a user knows what to expect when he or she performs an action. [17]

Finally, to make the system friendlier with the user it is converted to a graphical user interface was implemented using MATLAB2016 as shown in Figure [6].

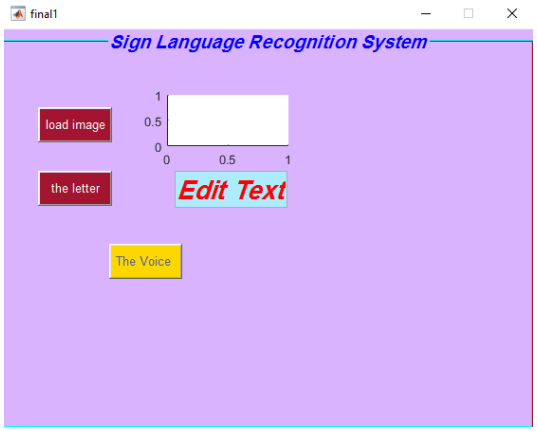



*Figure 6:* The Initial Output Window

## IV. Result

*a) Evaluating the Model*

Train set average accuracy after training the SVM classifier with the train set, we evaluated the trained classifier, on the train set and got 89 percent average accuracy.

*b) Testing the Model*

After training the classifier we tested the program on ten people, so there are 260 images, ten images in each class.

i. *Determining the Number of Images in Training Set*

When ten images have been used, we got the largest accuracy in all class (84.6). When seven images used, the accuracy lessoned in all categories (59.2). When five images used, the accuracy decreased in 17 categories and increased in 9 categories (57.7) it is clear from the above discussion, and as shown in figure [7] and figure [8], the more images in the training set, the more accuracy we have.



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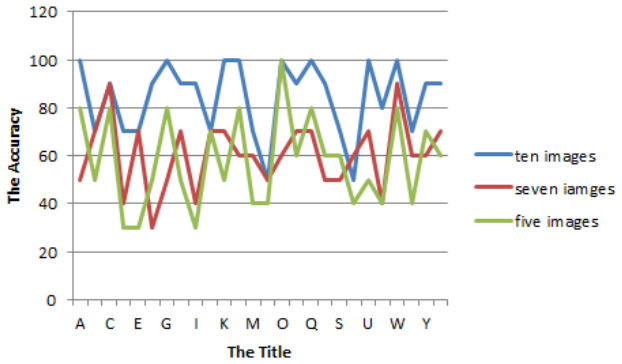



*Figure 7:* The Accuracy of Module in Line: Blue: Ten Images, Red: Seven Images, Green: Five Images

| | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| B | ✓ | ✓ | ✓ | ✓ | ✓ | × | × | ✓ | × | ✓ |
| C | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | × | ✓ | ✓ |
| D | ✓ | ✓ | × | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ |
| E | ✓ | × | × | ✓ | ✓ | ✓ | ✓ | × | ✓ | ✓ |
| F | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | × | ✓ |
| G | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| H | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| I | ✓ | ✓ | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| J | ✓ | × | × | ✓ | ✓ | ✓ | ✓ | ✓ | × | ✓ |
| K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| L | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| M | × | ✓ | × | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| N | × | ✓ | × | ✓ | ✓ | × | ✓ | ✓ | × | × |
| O | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| P | ✓ | ✓ | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Q | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| R | ✓ | ✓ | ✓ | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ |
| S | ✓ | × | ✓ | × | × | ✓ | ✓ | ✓ | ✓ | ✓ |
| T | × | ✓ | × | ✓ | ✓ | ✓ | ✓ | × | × | × |
| U | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| V | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | × | ✓ | ✓ | × |
| W | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| X | ✓ | ✓ | ✓ | × | ✓ | ✓ | ✓ | × | ✓ | × |
| Y | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Z | ✓ | × | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

*Figure 8:* Results of Each Letters in Detail

*c) Discussion*

The average accuracy for the model we received is approximately eighty-five percentages when using ten images for each class in the training set as shown in figure [9] and figure [10].



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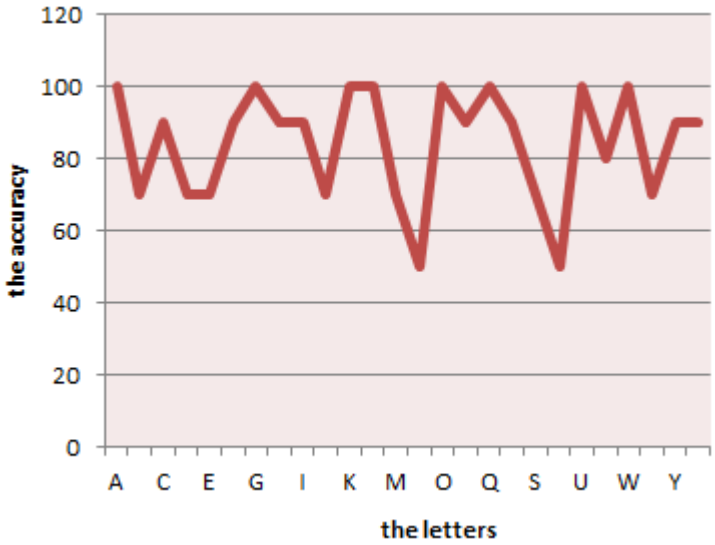



*Figure 9:* The Accuracy of the Module in Line

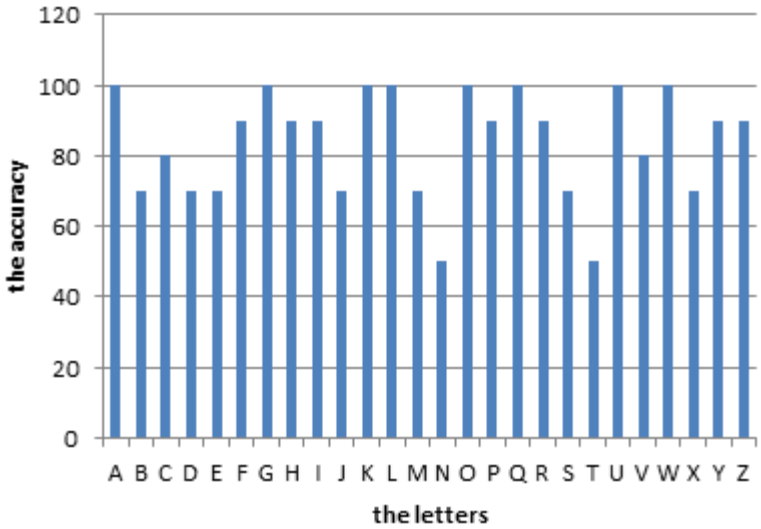



*Figure 10:* The Accuracy of the Module in Column

A, G, K, I, O, Q, U, and W have accuracy of 100% because they have distinguished shape from the other letters,

C, F, H, I, P, R, Y, and Z have accuracy of 90% because they have distinguished shape, affected by the limited resolution of phone camera beside the orientation of the image is not standard,

V has an accuracy of 80% because it has a appearance approximately close to the shape of the K letter,

B, D, E, J, M, S, and X. have accuracy 70%, because B, D, and J have an approximately similar shape and also need to perfect aligned with the camera. E, M, S, and X have more similarity to each other and the person must tries his/her the best to show the letter clear in a distinguished.

N and T have the lowest accuracy, 50% because they have almost the same shape and needs to be careful and attention from the individual to distinguish between them.



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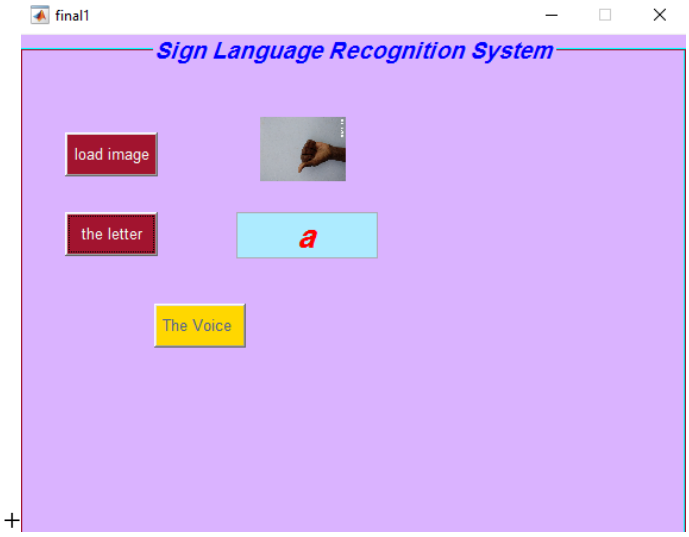



*Figure 11:* The Output of Letter a

## V. Conclusion

Communication with an ordinary person is always a challenging task for a dumb person. In this research, a system called Sign language recognition is introduced, which is an effective communication aid for a deaf person. It is convenient, comfortable and, cheap; there is no need for wearable to use the device. The system extended to aid the deaf in communication and users friendly with an accuracy of 84.6%.

## VI. Recommendations

For enhancement of this study, the database can be expanded the numbers of photo in the dataset by more than ten for every letter and take the photos for dataset from more expert people in dumb and deaf language. Also, for further future enhancement, instead of using the graphical user interface, the system can converted to application phone and connect between camera and application to make it easier and friendlier it can convert to real-time application by using video processing.

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# Assessment of Storage Related Haematological and Biochemical Changes in Blood Units

By Dr. Sirat Kaur

*Introduction-* Red blood cells are still the most widely transfused blood component worldwide and their story is intimately entwined with the history of transfusion medicine and the changes in the collection and storage of blood.[1,2] At present, the most widely used protocol for the storage of red blood cells (for up to 42 days) is the collection of blood into anticoagulant solutions (typically citrate-dextrose-phosphate); red cell concentrates are prepared by the removal of plasma and, in some cases, also leukoreduction. The product is stored at 4 ± 2° C in a slightly hypertonic additive solution, generally SAGM (sodium, adenine, glucose, mannitol, 376 mOsm/L).[1]

The British obstetrician, Braxton Hicks in 1868, experimented with a solution of phosphate of soda, but this also proved toxic. Richard Lewinsohn, in 1915, of the Mount Sinai Hospital in New York is credited with introducing sodium citrate into clinical practice as an anticoagulant.[3] In fact, a 1% solution of sodium citrate was already widely used in laboratories as an anticoagulant. This high concentration was toxic to humans but, as Lewinsohn himself recalled, `Nobody had ever followed the simple thought of carrying out experiments to ascertain whether a much smaller dose might not be sufficient' for use as an anticoagulant.

*GJMR-K Classification:* *NLMC Code: QV 180*




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# Assessment of Storage Related Haematological and Biochemical Changes in Blood Units

Dr. Sirat Kaur

## I. Introduction

Red blood cells are still the most widely transfused blood component worldwide and their story is intimately entwined with the history of transfusion medicine and the changes in the collection and storage of blood.[1,2] At present, the most widely used protocol for the storage of red blood cells (for up to 42 days) is the collection of blood into anticoagulant solutions (typically citrate-dextrose-phosphate); red cell concentrates are prepared by the removal of plasma and, in some cases, also leukoreduction. The product is stored at 4 ± 2° C in a slightly hypertonic additive solution, generally SAGM (sodium, adenine, glucose, mannitol, 376 mOsm/L).[1]

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At first, blood anti coagulated with sodium citrate was generally used within hours of donation and it was certainly not anti coagulated with a view to long-term storage. The following year, experimental work in rabbits showed that the addition of dextrose to blood stored for 2 weeks was effective in correcting anaemia after transfusion to rabbits which had been bled (Rous & Turner, 1916). Acid±citrate±dextrose (ACD) solution was adopted in the UK for anticoagulation of donated blood after a clinical review that conclusively demonstrated improved red cell survival on storage without any disturbance of acid±base balance in the recipient by Loutit & Mollison in 1943. Citrate±phosphate±dextrose (CPD) solution was subsequently adopted as the anticoagulant of choice after clinical studies were conducted using blood stored for up to 28 days.[3]

The development of blood storage systems allowed donation and transfusion to be separated in time and space.[2] This separation has permitted the regionalization of donor services with improvements in the quality and availability of blood products. The availability of storage raises the question of how long blood products can and should be stored and how long they are safe and effective. The efficacy of red blood cells was originally measured as the increment in haematocrit and safety began with typing and the effort to reduce the risk of bacterial contamination. Appreciation of a growing list of storage lesions of red blood cells has developed with increasing understanding of red blood cell physiology and experience with red blood cell transfusion. However, other than frank haemolysis, rare episodes of bacterial contamination and overgrowth, the reduction of oxygen-carrying capacity associated with the failure of some transfused cells to circulate, and the toxicity of lysophospholipids released from membrane breakdown, storage-induced lesions have not had obvious correlations with safety or efficacy.[1,2]

A brief list of the elements of the so-called “red blood cell storage lesion” includes: morphological changes, slowed metabolism with a decrease in the concentration of adenosine triphosphate (ATP), acidosis with a decrease in the concentration of 2,3-diphosphoglycerate (2,3-DPG), loss of function (usually transient) of cation pumps and consequent loss of intracellular potassium and accumulation of sodium within the cytoplasm, oxidative damage with changes to the structure of band 3 and lipid peroxidation, apoptotic changes with racemisation of membrane phospholipids and loss of parts of the membrane through vesiculation.[4-6]

Some of these changes occur within the first few hours of storage, for example, the decrease in pH or the increases in potassium and lactate; others, however, take days or weeks[5].Together, these events risk compromising the safety and efficacy of long-stored red blood cells, reducing their capacity to carry and release oxygen, promoting the release of potentially toxic intermediates (for example, free haemoglobin can act as a source of reactive oxygen species) and negatively influencing physiological rheology through the increased capacity of the red blood cells to adhere to the

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endothelium[7-8] or through their enhanced thrombogenic[9] or pro-inflammatory[10] potential.[11-13]

The most evident changes affecting red blood cells during the storage period are alterations of the cell phenotype, which varies from a smooth discoid shape to a phenotype characterised by various membrane protrusions or *spicula* (echinocyte) and finally to a spheroid-shaped cell (spheroechinocyte)[14]. The reversibility of these changes is inversely proportional to the duration of storage.

The storage lesion also involves the fluxes of sodium ions (massive entry into the cell) and potassium ions (exit from the cell), since the $Na^+/K^+$ pump is inactive at 4°C .[13] Although this is a reversible process, it takes 24 hours to restore the physiological gradients for sodium, and up to 4 days for potassium, this phenomenon means that blood stored for a prolonged period should not be used for neonates or paediatric patients, unless first washed or the potassium removed from the storage medium.[15-17]Hyperkalemia caused by massive transfusion of older red cell units with an elevated amount of extracellular potassium can result in serious cardiac complications, and possibly death in cardiac, patients. Other patients at risk of complications are neonates, massively transfused patients and those with renal failure. Cardiac arrest has been reported in patients following massive transfusion because of transient hyperkalemia. It can occur during exchange transfusions for haemolytic disease of the newborn, and the levels reached can be potentially lethal because of small intravascular blood volume.[18-20]

During storage, lactic acid accumulates in the blood bag.[21] As a result, RBC pH decreases during storage [15] and this increases phosphatase 3 enzyme activity, which results in 2,3-diphosphoglycerol (DPG) degradation. 2,3-DPG binds to deoxyhemoglobin and stabilizes it. This facilitates oxygen transportation from the lungs to tissues by oxyhemoglobin. A decrease in 2,3-DPG results in increased oxygen affinity of hemoglobin and therefore less oxygen delivery to tissue. It is clear that the storage lesions outlined above progressively occur over the duration of storage. The decrease in 2,3-DPG levels is also a reversible event, and completely normal levels can be restored within 3 days after the transfusion.[17]

Leukocyte reduction of packed Red cell units is now a common practice in the USA and Europe. Prestorage leukoreduction was not a common practice before the year 2000 in the United States. Leukoreduction was introduced to minimise risk of febrile nonhemolytic transfusion reaction[22], alloimmunization[23], transfusion-related immune modulation [24,25], and transfusion transmitted infections such as cytomegalovirus [26]. There is evidence that WBC affect the quality of stored RBC.[15,17,21]

In the present study we report an integrated overview of the biochemical and haematological processes taking place in RBC during storage. It is beyond the scope of this study to provide a proper clinical answer to the critical question on the quality of long-stored Red Blood Cells.

## II. Review of Literature

In the days before red blood cell (RBC) storage solutions, the patient and the donor had to be side by side. Blood was transfused by anastomosing their circulations or by rapid transfer with funnels or syringes before clotting intervened. Attempts to separate the donor and recipient involved collecting blood either by scarification or venesection and defibrinating it by rapid stirring or delaying coagulation using paraffined flasks. None of these methods was compatible with blood banking in the modern sense.[1]

In 1915, Rous & Turner developed the first RBC storage solution, a mixture of citrate and glucose, for storing rabbit RBC for use in a heterophile agglutination test for syphilis. They showed that this solution preserved the RBC at ice-box temperatures for up to 4 weeks, with minimal haemolysis. Moreover, the stored RBC could be injected back into the donor rabbits, which raised their haematocrit more than could be accounted for by the reticulocyte count.[1] The higher haematocrit persisted without the appearance of bilirubin in the rabbits' urine, suggesting that the returned RBC did not break down. Oswald Robertson, who worked in Rous's laboratory in 1915–1916, used Rous-Turner solution in the world's first blood bank in France during World War I.[5]

Mollison,[1] reviewing this history, pointed out that citrate, by fully anticoagulating plasma, allows the donor and recipient to be separated in space, while glucose, feeding the RBC, allows donors and recipients to be separated in time. Stable storage of RBC over distance and time meant that blood could be collected from large numbers of individuals, typed and tested for syphilis at a central location, and shipped to where it was needed with a reasonable chance of finding a recipient. Storage solutions make blood banking possible. Currently, blood components can be stored for a prolonged time. The ability to store blood for a long time revolutionized blood transfusion practices and dramatically improved the practice of medicine and surgery. The subsequent history of RBC storage solution development has been one of slow and incremental progress.[1]

Progress has been slow because the understanding of the RBC storage lesion is incomplete. Progress has been incremental because industry and the regulatory systems seemed to be able to deal with only one problem at a time, and the developmental cycle tends to be a decade long. These cycles have included heat sterilization in the 1940s, phosphate in the 1950s, plastic bags in the 1960s, adenine in the 1970s, additive solutions in the 1980s, and leucoreduction in



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the 1990s. Recently, an improved understanding of the storage lesion and the application of a well-structured developmental approach have allowed further progress to be made in designing solutions that permit the storage of RBCs for longer and under better conditions. Stable storage of RBC over distance and time meant that blood could be collected from large numbers of individuals, typed and tested for syphilis at a central location, and shipped to where it was needed with a reasonable chance of finding a recipient. Storage solutions make blood banking possible.

Robertson described using mixtures of citrate and glucose solutions and transfused blood that had been stored for as long as 26 days. However, his United States (US) Army Medical Corps superiors decided that it would be safer to collect blood into citrate only, because of the risk of bacterial contamination and overgrowth. Mixtures of sodium citrate and dextrose caramelized when the solutions were heated, whereas solutions of sodium citrate alone could be autoclaved. There is enough glucose in whole blood from healthy donors to support RBC storage in ice chests for about 5 days. Thus, the collection of whole blood into autoclaved bottles of 3·8% sodium citrate solution and 5-day blood storage on ice became the first blood bank standards. This system of blood banking was implemented in casualty clearing stations and field hospitals, supporting stable trench warfare in the spring and summer of 1918, but became logistically difficult in the autumn of 1918 during the more mobile fighting in the Ardennes Forest.

Captain Oswald Hope Robertson, a physician volunteer in the United States Army, built the world's first blood bank, on the Western Front in World War I. While others performed occasional transfusions by direct artery-to-vein anastomosis or indirect syringe or flask techniques, Robertson built a donor and transfusion service that would be recognizable today. He collected blood from previously typed "universal" donors by needle venepuncture through rubber tubes into glass bottles containing citrate and dextrose solution. He stored these bottles on ice for up to 26 days and transported them to casualty clearing stations where they were needed.[11]

He administered transfusions to wounded soldiers and proved that transfusion worked. He performed hundreds of transfusions himself and taught others who performed untold numbers more. He published descriptions of the Technique and results in the British Medical Journal in April and June 1918. His work was recognized as among the most significant medical contributions of the war. The British Government awarded him the Distinguished Service Order. After the war, Robertson went to China to work for the Rockefeller Foundation, and blood banking disappeared for almost 20 years.

At the beginning of World War II, the British Army implemented a blood system using the 5-day storage method, while Americans Elmer DeGowin and John Alsever revived the study of combined citrate and glucose solutions. Loutit & Mollison then showed that a mixed citrate and glucose solution could be autoclaved if the pH was below 5·8. Acid citrate dextrose (ACD) solution was manufactured in sterile vacuum bottles and whole blood collected into those bottles could be stored for 21 days. Blood that could be stored for 21 days could be shipped around the world, and ACD blood was the basis for building national blood systems in the British Commonwealth and USA during and after World War II[1].

Plastic blood-storage bags were first developed in the 1950s. The modern plastic blood bag was invented and made as a prototype in 1947, tested clinically in the 1950s at Harvard and used experimentally in the Korean War and finally licensed and widely introduced in the early 1960s. Plastic blood bags were developed as a replacement for glass bottles to prevent contamination, breakage and air embolism and allow the sterile manufacture of components and facilitate their separate storage. They were seen as advantageous for military logistics because of their lighter weight and resistance to breakage. The ability to manufacture connected sets of bags enabled the design of a sterilized closed-collection system that decreased the rate of bacterial contamination from experimental surfaces. By the time they became approved commercial products in the 1960s, their vein-to-bag unitary construction, and ability to exclude air bubbles reducing the chance of air embolism during pressure infusion, small volume in refrigerator storage and optical clarity were all recognized as distinct advantages. With the development of blood component manufacturing, they became indispensable.[1,2]

The plastic needed to be tolerant of the High temperatures of sterilization and Low of plasma freezing and have optical clarity to allow visual infusion. Polyvinyl chloride (PVC) copolymer, plasticized with di-2-ethylhexyl phthalate (DEHP), was found to have all of these desired properties. It reduced red cell haemolysis fourfold compared with storage in glass, resulted in reduced rates of bacterial contamination (053% in plastic containers vs. 67% in glass) and gave fewer pyrogenic reactions. Twenty-four-hour recovery in vivo was 84% for ACD anticoagulated blood in DEHP-PVC after 20-day storage and 72% after 28 days, contrasting to around 70% after 21 days in bottles. Once Component preparation started, the use of the plastic was essential, thereby making available platelets for cancer care, and plasma for fractionation. The standard plastic used in blood bag manufacture contained about 55% PVC with up to 40% w/w DEHP plasticizer. This was an advantage for military logistics because of their lighter weight and resistance to breakage. The ability to



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manufacture connected sets of bags enabled a sterilized closed-collection system that decreased the rate of bacterial contamination from experimental surfaces. By the time Bags became approved commercial products in the 1960s, their vein-to-bag unitary construction, ability to exclude air bubbles reducing the chance of air embolism during pressure infusion, small volume in refrigerator storage and optical clarity were all recognized as distinct advantages.[11]

However, from the point of view of RBC storage, the most important contribution of plastic bags was the introduction of diethylhexyl phthalate (DEHP), the plasticizer used with polyvinyl chloride (PVC).[1]

It is now known that DEHP leaches out of the plastic bag and enters the RBC membrane where it limits membrane loss by microvesiculation. This effect was large, with the reduction of haemolysis during storage being fourfold when measured each week .The net result was that RBC can be stored for twice as long in PVC bags as they could be in either glass or Plastic. Only when RBC storage was extended beyond 3 weeks and storage solutions supported RBC metabolism for longer, could this effect come to light.

The safety of DEHP in blood bag systems has always raised questions since the early 1970s, when considerable quantities of the plasticizer were found in various tissues of deceased transfusion recipients. Professional and public interest in alternative plastics for blood storage has varied over time, reflecting both environmental and health concerns. DEHP had been labelled as a toxic compound category 2 (that is, toxic effect on fertility and development) earlier in this century, the search for alternatives has been intensified, resulting in nearly complete replacement by other plasticizers and/ or materials for use in the food and toys industry. However, in medical care, the basic DEHP-PVC formulation of blood container plastic and many other disposables is still in use today despite these concerns.[11,29]

A recent International Forum by Van der Meer PF revealed that for particular patient groups, including neonates that require large volume transfusions including exchange transfusions or extracorporeal membrane oxygenation (ECMO), as well as adult patients that require frequent transfusions dialysis, cardiac or trauma patients, there is concern of high exposure to DEHP. These patients receive fresh blood products, although DEHP concern is not the prime reason for using fresh blood, but is regarded as an added benefit.[41]

Blood bags have been licensed and commercially available for over 50 years. The initial goals in their manufacture: improved sterility, protection from breakage with dropping, elimination of the air bubble and foaming with infusion, and the ability to give blood under pressure without the risk of air embolism, have all been achieved. Further, the initially unanticipated benefits of sterile component manufacturing and better component shelf life have become critical and indispensible in the blood banking industry.

Whole blood collection, the first step in blood collection and component manufacture, involves placing a needle in a donor's arm and collecting the blood by gravity drainage. A tourniquet or blood pressure cuff at 40 mm Hg facilitates this collection process without the problem of venous collapse because of the vacuum in the blood bottle with the subsequent need for venting and the resultant increased risk of bacterial contamination. Modern bags are also sized to prevent overdrawing, vis-a vis the old systems where 450 ml of blood was drawn into 63 ml of anticoagulant in an 800- or 1000- ml bottle. With the modern collection of 500 ml of whole blood into 70 ml of anticoagulant in a 600- ml bag, there is no room for significant overdrawing. Squeezing or rocking the bag during collection allows for efficient mixing of anticoagulant and blood without the risk of dropping a bottle. With the adopting protocols of overnight hold of whole blood before processing, the importance of the plasticizer in the primary collection bag may increase. Whether the early incorporation of the plasticizer into the RBC membrane related to early exposure or sustained higher temperature has an effect on either RBC recovery or haemolysis is not known. The effect of the presence of plasticizer during whole blood storage on platelet storage is as yet unknown. Red cells are separated from platelets and plasma with the primary spin in blood component processing. After separation, the storage solution is added and the unit is leucoreduced when the red cells are moved to the final storage bag[11].

The important aspects of the RBC storage bag appear to be its geometry, permeability and plasticizer. Geometry and permeability affect the rate of Carbon dioxide efflux from the bag, certainly important with the new bicarbonate containing storage solutions of Hess and Greenwalt[44] i.e EAS, Experimental Additive Solutions, but important for all RBC storage systems as the presence of Carbon dioxide (as bicarbonate) accounts for about a quarter of the buffering, even in standard RBC storage systems such as SAGM.

The plasticizer remains the most important aspect of a RBC storage bag, with DEHP reducing haemolysis by fourfold and increasing recovery by several percent. These plasticizer effects can spell the difference between licensure and rejection of a RBC storage system in the United States. Platelet storage bags have been an area of rapid development over the last decade in parallel with the development of platelet additive solutions and the development of 7-day platelet storage systems. DEHP-free bags and high permeability bags to facilitate Oxygen transport have been particular areas of focus. Nevertheless, at high platelet concentrations, most modern platelet bags still cannot

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deliver sufficient oxygen for platelet metabolic processes and this is an area where further improvement is possible. Plasma can be stored for a year at -18°C and 7 years at -65°C, but there is a serious problem with the breakage of PVC bags in transport. In military use, this breakage rate can be as high as 50%. Efforts to introduce more thermo-tolerant bags are opposed with the claim that plasma processors want recovered plasma in PVC bags because they break the bags as the least expensive way to get the plasma out. As this is clearly not compatible with current good manufacturing practice, it should not be a reason not to include more thermo-tolerant bags in blood storage systems for human transfusion use.

In summary, plastic blood bags were a major advance when they were first licensed in 1962. Their continued development has meant better storage for platelets and holds the prospect of better storage for RBCs, plasma and granulocytes as well. Phosphate leaks slowly from stored RBC. This is not clinically important during the first 2 weeks of storage when the breakdown of diphosphogly cerate (DPG) provides sufficient intracellular phosphate to support the synthesis of new adenosine 5′-triphosphate (ATP). Adding sodium phosphate to ACD reduced this phosphate loss by reducing the gradient in phosphate concentration between the inside and the outside of stored RBC. Maintaining the production of RBC ATP increased the fraction of RBC that stayed in the circulation at the end of storage. Chromium-51, produced in nuclear reactors, became available in the 1950s and improved the accuracy of measurements of the fraction of RBC that stayed in the circulation. These more precise measurements confirmed the earlier observation that RBC, which survived their first 24 h in the circulation (in vivo recovery), went on to have a normal 100- day life span. Citrate phosphate dextrose (CPD) solution, with 16 mM/l phosphate, increased the fraction of RBC recovered after 3 weeks of storage from ≈ 75% with ACD to > 79%, and even when the cells were stored for 4 weeks, the recovered fraction was only slightly lower than after storage in ACD for 3 weeks. However, the American Food and Drug Administration licensed CPD whole blood for 3-week storage, arguing that better storage was more important than longer storage.

In 1962, Nakao and colleagues described the relationship of the loss of RBC viability with their loss of ATP.[1] They showed that adenine and inosine could be added to stored RBC with the restoration of cell shape, ATP concentration and viability. Moreover, these materials could be added to the original ACD storage solution where they slowed the onset of the loss of ATP and viability in stored RBC. Subsequently, it was recognized that RBC lose adenine and adenosine to deamination reactions, and that the provision of additional adenine could extend storage and improve RBC recovery and osmotic fragility. Citrate phosphate dextrose adenine (CPDA-1) solution was developed in 1968 and shown to permit whole-blood storage for 5 weeks. However, regulatory questions about the safety of adenine, specifically the question of whether its use would lead to the development of uric acid stones, delayed the licensure of CPDA-1 in the USA for 11 years. In these 11 years, blood banks moved away from whole blood storage to the manufacture of components. Whole blood transfusion was largely replaced by the use of packed RBCs.

Zuck and colleagues, in 1981, performed the critical licensure study for CPDA-1 in both whole blood and packed cells, they found that the recovery of RBCs stored for 5 weeks as whole blood averaged 81%, whereas those stored as packed cells had a lower average recovery of 72%.Beutler & West went on to show that the tighter that CPDA-1 RBC were packed, the sooner they ran out of glucose, and the worse was the in vivo recovery. It was possible to add more glucose to the original anticoagulant storage solution, making CPDA-2, but it meant that the plasma and platelets from those donors contained large amounts of unnecessary sugar. Removing most of the plasma from CPDA-1 packed RBC also meant that the storage haematocrit was greater than 75%, making the blood viscous and difficult to administer rapidly in emergency situations. [17]

The concept of using additive solutions instead of re suspending packed RBCs in anticoagulant and plasma was first introduced by Fischer and his colleagues and rigorously explored by Beutler[45]. Beutler prepared a solution that was added in equal volume to the red cells after the plasma was removed. The solution, which was designated BAGPAM, was composed of the following ingredients in mmol/L: 101.4 sodium bicarbonate, 14.3 sodium carbonate, 1.0 sodium phosphate,1 adenine, 55 glucose, and 0.5 mannitol. In this additive 2,3-DPG was maintained at better than initial concentrations for 42 days, but ATP dropped to 20% of its initial level within 7 days. However, the effect could only be achieved if the volume of the blood was limited to 50 mL so that the volume to surface ratio would permit the diffusion of sufficient Carbon Dioxide from the bag. Mixing at least 3 times per week was also necessary. Storage of 250 and 500 mL of blood in standard bags was not satisfactory.

Plastic films with higher permeability to CO2 were considered necessary. He suggested that bags made of silicone rubber might be suitable. The bicarbonate buffering was a particularly interesting innovation. RBCs metabolize glucose to make ATP. The waste products are lactate and protons. The protons accumulate, driving down the pH poisoning further metabolism: Bicarbonate combines with the protons and, in the presence of RBC carbonic anhydrase, is converted to water and carbon dioxide. The carbon dioxide diffuses out of the plastic bag preventing the



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back reaction. This buffer system has considerable capacity. The 200 mL of RBCs in a pint of blood metabolize about 5 mmol of glucose in 6 weeks at 4~ and produce 10 mmol of lactate and of protons. The buffer capacity of the oxyhemoglobin in 200 mL of RBCs is about 13 mEq/pH unit in the range 7.2 to 6.2. Bicarbonate in physiologic concentrations in the additive solution creates pCO2 in the solution that drives the diffusion of 1 to 2 mmol of CO2 from a 600-mL PVC bag each week.

The first additive solution was SAG, named after its constituents, saline, adenine and glucose[1,38]. It consisted of 1·25 mMol adenine and 5 mMol glucose in 100 ml of normal saline. The added volume decreased the storage haematocrit of the starting packed RBC from ≈ 80% to ≈ 55%,reducing the viscosity from 40 to 10 cps (centipoise = 1 millipascal second). The added glucose supplemented the 2 mMol present in a tightly packed unit of CPD RBC, ensuring an excess of the 3 mMol that the RBC would typically consume in 5 weeks of storage. The added adenine was a threefold excess of the estimated losses by deamination, so it had a mass action effect to increase adenylate nucleotide stores. Buffy coat-reduced RBC stored for 5 weeks in SAG had a measured 24-h in vivo recovery of 83 ± 7% and the haemolysis at that time averaged 0·58 ± 0·15%. Haemolysis of RBC during storage was highly variable from donor to donor, and was the factor limiting storage beyond 5 weeks.

Hogman showed that the addition of 30 mM mannitol could reduce that haemolysis by 50% and allow storage for an additional week.[15] Mannitol works as a free radical scavenger, but also in some sense as a membrane stabilizer.

Hogman led the development of saline-adenine-glucose (SAG) as a 5-week additive solution and showed that it preserved packed RBCs better than CPDA-1.

Mannitol was later added to SAG to suppress haemolysis and produced SAG-M, which was licensed for 6-week RBC' s storage. The additive solutions AS-1 (Adsol, Baxter Healthcare, Round Lake, IL) and AS-5 (Optisol, Terumo, Somerset, NJ), widely used in the United States, are variants of the SAG-M formulation. The other additive solution in use in North America is AS-3 (Nutricel, Pall, Covina, CA). It is a combination of saline, adenine, and glucose with citrate as a membrane stabilizer and monosodium phosphate as a stimulant to ATP synthesis. The solution works as well as the SAG-M variants and consistently yields 24-hour in vivo RBC recoveries in the range of 78% to 84% after 6 weeks of storage: It is the standard RBC storage solution in Canada. The citrate appears to serve the same membrane-protective function that mannitol serves in SAGM, but also functions as an impermeable ion that balances the osmotic pressure of small ion permeable RBCs. AS-3, like the other additive solutions, is associated with 78–84% recovery and 0·4% haemolysis after 6 weeks of storage All of the currently licensed 100-mL additive solutions (AS-1,AS-3, AS-5, and SAG-M) support component manufacture and leukoreduction. The mannitol, phosphate, and citrate are tolerated by premature neonates and in massive transfusion in trauma and liver transplantation. They typically yield RBC recoveries of 80% to 84% and 0.5% hemolysis at 6 weeks and even better values at the 1- to 3-week interval after donation when most RBCs are transfused. They are inexpensive to produce, lightweight for transport, and, with a storage hematocrit of 55%, easy to infuse rapidly through 14-gauge catheters in surgery.

Additive solutions were developed to provide additional volume and nutrients for longer storage and better flow of packed RBCs .They function in the context of a blood collection and storage system. In a typical whole blood collection system, a needle is placed in an antecubital vein and drains blood into the primary collection bag containing an anticoagulant solution such as CPD. After blood collection is complete, the primary collection bag is centrifuged to sediment the RBCs, and the platelet-rich plasma is expressed for further processing. The additive solution is than added to packed RBC, or the packed RBC are added to the additive solution for separate storage as packed RBC in additive solution. This means that there is some carryover of citrate, phosphate and dextrose from the primary anticoagulant into the final storage mixture. Also, the final suspending solution volume and ingredient concentrations are determined by how much of the primary anticoagulant and plasma were expressed before addition of the additive solution. Finally, the number of RBC in the bag is a function of the donor haematocrit and the exact volume of blood collected. Thus, while an additive solution has defined components and concentrations, the concentrations of ingredients in the final suspending solution of RBC in additive solution is a function of donor characteristics and blood centre product manufacturing techniques.

Additive solutions used in some other countries, such as Circle Pack in Australia and MAP in Japan, are like AS-3 in that they depend on a higher dextrose version of the primary CPD anticoagulant, called CP2D. None of these additive solutions appears to have significant advantages over the others.

RBC are the most frequently transfused blood product. The efficiency of their storage determines the overall need for blood donors. Current 5 and 6-week RBC storage solutions have worked well, with 92% of all RBC units that met release criteria finding a recipient. There has been little demand from transfusion organizations for longer or better RBC storage. Nevertheless, there are clinical problems. Seasonal shortages of RBC occur, and local supplies occasionally run out. Questions about the safety of stored RBC arise and persist, most recently in terms of the ability of



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supernatants from stored RBC to cause transfusion-related acute lung injury (TRALI) .There are retrospective studies in trauma patients that relate increased multiple organ failure and mortality to the length of storage of the RBC transfused . Clinicians also question the flow characteristics of RBC that have lost membrane and the oxygen-delivery capacity of cells with reduced DPG.

The development of storage solutions (reviewed in Moore,1987 and Hess in 2006[1]; citrate-phosphate-dextrose (CPD) and acid-citrate-dextrose (ACD) (Weisert and Jeremic, 1973, saline-adenine-glucose (SAG) (Ambrus et al., 1975; Kreuger et al., 1975; Herve et al., 1980; Strauss et al., 1980; Peck et al., 1981, with added mannitol [15] as in the SAG-M additive commonly used , or the more recent development of chloride-free additives (Högman[15] et al., 2006) and phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAG GGM) has helped to preserve metabolic functions and reduce lipid peroxidation.

The function of red cell storage is to maintain the functionality and viability of red cells throughout the approved storage period .The difficulty that is shared by modern storage mediums, however, is that red cell functionality and viability are progressively impaired during storage by three interrelated mechanisms: altered metabolism; increased Cold-storage of red cells at 4 +/- 2 °C helps maintain red cell functionality and viability by reducing the red cell metabolic rate. For each one degree drop in storage temperature, there is approximately a 10% decrease in red cell metabolic rate, and at 4 °C, the metabolic rate is ten times lower than at 25 °C. As metabolic activity does not completely cease when red cells are stored, glucose or dextrose are added to storage mediums to allow red cells to continue glycolysis and thus produce sufficient ATP, 2,3- DPG and NADH to maintain adequate functionality and viability during storage. Continued glycolysis, however, results in the accumulation of its primary by-product, lactic acid, in the supernatant. The resulting acidosis inhibits glycolysis via a negative feedback loop, which results in a progressive reduction in ATP, 2,3-DPG and NADH levels . By the sixth week of storage, lactate levels in the supernatant are increased by several fold, the pH is below 6.5, the ATP and NADH contents are substantially reduced, and 2,3-DPG content is depleted. Reduced ATP levels impair all red cell metabolic activities including glycolysis itself (as ATP is needed for the initial steps of glycolysis, creating a vicious cycle), formation of cytosolic antioxidants (thus reducing antioxidant defences), and maintenance of membrane integrity (thereby reducing red cell deformability and promoting alterations in their discoid shape) . Although there exists a direct relationship between reduced ATP levels and red cell viability, the overall role of ATP depletion in the 'storage lesion' seems to be limited. Depletion of 2,3-DPG reduces oxygen delivery, but levels are rapidly replenished after transfusion. The most important contribution to the storage lesion is likely to be the depletion of reduced NADH, which impairs the conversion of methaemoglobin back to oxyhaemoglobin within the red cell, thereby aggravating oxidative stress.

Another effect of cold-storage is that it impairs the exchange of sodium and potassium across the red cell membrane by the sodium/potassium adenosine triphosphatase (Na+ /K+ ATPase) pump.[13,43] Under normal circumstances, the concentration of potassium and sodium inside the red cell are maintained at approximately 90 mM and 5 mM, respectively, whereas outside, they are approximately 5 and 140 mM, respectively. By the sixth week of storage, red cell potassium content is decreased by about 40% and sodium content is increased by about 300%. At the same time, the potassium content in the supernatant of the stored units is markedly increased, which may lead to hyperkalemia after transfusion. Increased intracellular sodium content affects cell volume and shape, such that the mean corpuscular volume of stored red cells is increased after 3 weeks of storage. This effect is more pronounced in older red cells, impairing their deformability and viability.

During storage, RBCs lose potassium, DPG, ATP stores, lipids and membrane, while becoming more rigid and demonstrating reduced oxygen off-loading. Stored units become more acidotic and the suspending fluid has higher concentrations of free haemoglobin and biologically active lipids, and contains greater quantities of negatively charged micro vesicles with pro-inflammatory and procoagulant activity.[95] The fraction of stored RBC that circulates when re infused decreases with the duration of storage in each of the liquid storage systems. These changes occurring with storage are collectively known as the RBC storage lesion. Some, such as the potassium loss, are recognized to be secondary to the changes in metabolic activity with cooling. Others, like the loss of DPG and reduced glycolytic activity, are related to decreasing pH. RBCs have an intrinsic program of cell death that is held in check by normal concentrations of RBC ATP. Normal ATP concentrations are necessary to prevent calcium-induced membrane loss by microvesiculation and for active transport of negatively charged phospholipids, specifically phosphotidyl serine, from the outer to the inner surface of the RBC membrane to prevent RBC clearance from the circulation by macrophages.

There are 2 major kinds of RBC-storage studies. One group, usually called in vitro studies, is composed of those in which the RBCs are stored and studied periodically using biochemical and morphologic measures. In the other group, in vivo studies, all or a portion of the RBCs are infused into volunteers for measuring RBC recovery or survival. This difference between in vitro and in vivo studies is important because each has important strengths but also practical limits and sources of error.



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The appropriate sequential use of these studies dates to the first RBC-storage studies, which were published by Rous and Turner in 1916.[2] In the first of the back-to-back articles, they described how rabbit RBCs could be stored in a citrate and dextrose solution for 4 weeks with minimal hemolysis. In the second, they showed that these stored RBCs could be infused back into the donor rabbits with a sustained increment in the hematocrit that could not be explained by a concomitant reticulocytosis and was not associated with bilirubinuria. These two simple but elegant studies provided the scientific basis for Robertson's successful use of stored human blood a year later. In vitro studies typically precede in vivo studies. Human-use committees recognize that drawing blood for an ex vivo study, even in 500-mL amounts, is associated with minimal risk to the donors when conventional blood bank standards and procedures are followed. On the other hand, reinfusion of stored blood exposes recipients to the risks of novel solution ingredients, unexpected contaminants, blood breakdown products, and RBC radionuclide labels, as well as the infectious and hemolytic risks of transfusion. Thus, in vivo studies are of greater than minimal risk and require scientific, medical, and radiation physics oversight.

In vitro studies, because of their safety and lower expense, serve both as scientific test beds for ideas about improving storage and as demonstrations of process safety by showing that the methods used do not lead to RBC breakdown or contamination. In vitro studies of RBC storage are complicated because drawing a unit of blood from each of several volunteers does not create uniform material for study. The RBC fraction of donor blood varies from 38% to 50 % or more so that the volume of RBCs in a donated unit may vary by 60 mL. With higher RBC concentrations, nutrients are consumed, and waste products accumulate sooner. The metabolic rates of RBCs vary considerably from donor to donor, and the propensity of RBCs to haemolyse under storage conditions varies even more. Allocating blood from a single donor into smaller storage containers raises the question of how well the conditions in the small containers mimic those in conventional storage systems. Collecting large numbers of units in the hope of statistical averaging rapidly becomes unwieldy.

Performing long-term in vitro RBC-storage studies requires sampling the cells and suspending fluid during storage, making measurements, and comparing those measures to some standard. Closed plastic bags can be sampled by sterile tube splicing to attach sample pouches or by equivalent procedures. Measures of many RBC analytes are automated, but measures of ATP and 2,3-DPG concentrations, haemolysis, and RBC morphology are not. These measures require technical proficiency. The only absolute standard for in vitro storage studies is that the percentage of RBC that haemolyse during storage should be less than the upper limit set by regulatory agencies for product licensure, 1% in the United States and 0.8% in Europe. The rule of thumb that the RBC ATP concentration should exceed 2/xmol/g Hb is probably necessary to predict good in vivo recovery but does not ensure it. In vivo studies have the advantage of clear standards.

In 1984, the American Association of Blood Banks sponsored a working party that published methods for measuring RBC recovery and survival using 51-Cr as a radioactive label. Regulatory agencies, such as the US Food and Drug Administration, have a history of requesting studies of a certain size for product licensure. It is unlikely that these agencies will license products in the future that work less well or are tested less rigorously than those already approved. Thus, the standards that at least 75% of stored RBCs must circulate for at least 24 hours after reinfusion and that hemolysis should be less than 1% should be viewed as minimums.

In vivo studies have the disadvantages of the need for a stable population of volunteers, the large variability in the tolerance for storage of RBC from different individuals, and the errors intrinsic to the measurement of RBC recovery using radioactive labeling. Stable populations of volunteers are necessary because the time from recruitment to the end of survival measurements in a single-arm study can be 4 to 6 months or more. A 2-armed crossover study with a radiation washout period can take a year. If laboratory errors or accidents occur, such as the breaking of a blood bag or failure of a volunteer's participation, a portion of a study may be lost. Schedules are critical. Once the blood is drawn, a 70-day storage study needs to have the blood reinfused and post reinfusion samples drawn on specific dates. Social emergencies for staff and volunteers occur. Natural emergencies, including hurricanes, blizzards, and earthquakes, are uncommon but occur frequently enough to disrupt RBC-storage studies that are continuing over a period of years. Losing volunteers is serious because in any modest-sized cohort, a few volunteers will have RBC that store poorly.

These differences may be related to inosine triphosphate pyrophosphohydrolase deficiency or other genetic polymorphisms. The difference between RBC recovery in individuals whose RBCs store well and those whose cells store poorly are typically of the order of 90% versus 60%, with a range in the fraction of cells lost that is typically 400%. The distribution is skewed to the upper end, and only a few individuals' RBCs have recovery percentages toward the lower end of the range. Because these individuals are not recognized beforehand, their loss or overrepresentation in a diminished volunteer cohort can lead to falsely rejecting a useful solution or accepting a bad solution and possibly years of additional work. Finally, the actual

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measurements of 24-hour in vivo RBC recovery are only accurate to within a few percentage points. The errors come from volumetric errors in small fluid sample handling, errors in estimation of the RBC fraction using the micro hematocrit method, and Poisson errors in radiation counting. The error in an individual measure of RBC recovery is thus of the order of 3% to 4%.

Even before the recognition of programmed cell death pathways in erythrocytes, Meryman[15] and colleagues had shown that RBC could be stored for many months in solutions that maintained their ATP concentrations. These findings meant that the limited shelf life of conventionally stored RBC was a result of the conditions of storage, not an intrinsic limit imposed by the cells. Hogman[15] and colleagues showed that rejuvenation of ATP concentrations at the end of conventional storage, by restoring pH and adenylate stores, improved in vivo recovery, reducing the number of cells that failed to circulate by > 50%. Thus, the removal of cells from the circulation did not mean that the cells had died, just that they were reversibly marked for removal from the circulation.

In 1999, Hogman & Meryman[15] reviewed the many factors affecting RBC storage and post-transfusion survival and function. Information on more esoteric aspects of storage, such as the intermingled effects of hypo tonicity and low chloride concentration, can be found in their publication. However, rather than correcting the storage lesion after it had occurred, it appeared to be more useful to maintain the ATP concentration and try to prevent the loss of membrane and viability during storage. Several groups tried to increase the duration of storage by forcing ATP production.

Walker and colleagues added pathway of glycolysis, in an additive solution called PAGGS-M (phosphate, adenine, glucose, guanosine, saline, and mannitol).It provided 7 weeks of RBC storage with a recovery of 74%. Hogman and colleagues tried to increase the storage pH by reducing the amount of CPD anticoagulant and using a neutral additive solution with the glucose autoclaved in a separate pouch.[15] This 1/2CPD/ErythroSol could store RBC for 7 weeks with 78% recovery. The solution was commercialized as ErythoSol by Baxter Healthcare in Europe and licensed on the basis of a small clinical trial that showed 78% recovery after 7 weeks of storage. To allow terminal sterilization by autoclaving, they separated the phosphate from the glucose into different parts of the bag assembly.

Greenwalt and colleagues developed an increased-volume alkaline additive solution that led to higher concentrations of ATP for longer periods during storage and showed that RBC in vivo recovery could be extended to 8 weeks with membrane loss reduced [44].

Walker et al, sought to improve storage with additional nutrient support. They added guanine and phosphate to SAG-M to make PAGGS-mannitol, which was commercialized by Maco Pharma in Europe. [88]

Babcock and colleagues showed that the hypotonic solution failed to produce better in vivo recoveries than the hypertonic AS-3. More importantly, the studies showed that leukoreduction reduced hemolysis during storage, a finding that had been reported before. The measured effect was a 50% reduction in hemolysis even though the leukoreduced cells were stored for 8 weeks and the controls for 6 weeks. At the same time, Greenwalt and colleagues conducted a study of a 200-mL additive solution containing glycerol that yielded 73% recovery after 9 weeks of storage compared with 60% recovery in AS-1 split-sample controls (n = 7). The high concentrations of glycerol would probably render the solution unsafe in massive transfusion situations, but the suggestion that RBC might be stored for 8 weeks was tantalizing.[11]

Hess & Greenwalt then conducted a systematic exploration of storage conditions that maximized RBC ATP content while minimizing haemolysis and membrane loss.[11] As part of the plan of development, these additive solutions contained only ingredients that were already approved for blood storage or large-volume parenteral use. They determined the guanosine as a source of ribose, in an attempt to drive the distal Embden–Meyerhof pathway of glycolysis, in an additive solution called PAGGS-M (phosphate, adenine, glucose, guanosine, saline, and mannitol). It provided 7 weeks of RBC storage with a recovery of 74%.

They determined the effects of additive solution pH and volume, and of phosphate, sodium, chloride and mannitol concentrations. Glycolysis slows as pH falls. Collecting whole blood into acidic CPD normally reduces its pH from ≈ 7·35 to ≈ 7·1. Adding an acidic additive solution reduces the pH further, to ≈ 7·0. However, if the pH of the additive solution is raised to 8·5 by adding disodium phosphate, then the resulting pH of the RBC suspending fluid can be raised to ≥ 7·2 at the beginning of storage. However, if the pH is raised above 7·2, then DPG is produced and consumes all of the intracellular phosphate, leading to a decrease in the ATP content that limits storage time and RBC quality. A pH of 7·2 at the beginning of storage means that ATP production exceeds its use for several weeks, leading to increased content. As pH decreases and metabolism slows, RBC ATP concentrations reach a maximum higher and later than usual, resulting in a longer time that the ATP concentration exceeds the critical values that are necessary to suppress microvesiculation and phosphotidyl serine exposure.

Several recent publications describe high pH storage solutions that preserve DPG content for many weeks. ATP concentrations are reduced with some of these solutions because the pH is too high, but Hogman and colleagues have found the point where DPG is maintained and ATP is synthesized in excess of



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immediate use. Normally, the storage life of RBC is determined by the length of time it takes the cells to produce enough lactic acid and protons to reduce the pH to 6·5 where low ATP production no longer supports cell viability. This time is largely determined by the rate of production of protons and the buffer capacity of the suspension. This buffer capacity can be increased by adding sodium bicarbonate to the additive solution. In solution, bicarbonate combines with a proton to produce carbonic acid, which is converted to Carbon dioxide and water by RBC carbonic anhydrase. The $CO_2$ then diffuses out through the plastic bag, effectively removing protons from the solution and slowing the rate of pH fall. The combination of using alkaline additive solutions and bicarbonate buffering can effectively double the metabolic capacity of the storage system and allow RBC to be stored for longer and under better conditions. The historical requirement to acidify blood storage solutions so that glucose does not caramelize, can be by-passed by separating the sugar from the alkaline ingredients in different parts of the blood-collection set. These developments mean that RBC can be stored for longer and under better conditions. Longer duration of storage will reduce losses from outdating and improve the efficiency of autologous blood collection. Better storage conditions means better cell flow characteristics from reduced membrane loss and improved efficacy from better in vivo recovery at all times during storage. Eight- and perhaps 9-week storage should cost no more than storage for RBC units at present, but will provide increased utility for general care and emergencies. Under a variety of storage conditions, RBC metabolism starts briskly and slows as pH falls. As a general observation, the lower limit of pH for RBC storage is about 6.2, when it is measured at 37∼ as estimated from the asymptote of the pH curve of many samples of stored blood stored in a variety of systems.

Wallas CH, in 1979, reported that storage of red cells for three weeks at 4 degrees centigrade under blood bank conditions resulted in a rise in intracellular Na+ and a fall in intracellular K+ with concomitant opposite changes in Na+ and K+ levels in the suspending plasma. A decline in red blood cell ATP during the storage period did not appear to be contributing to the changes. Increasing red blood cell ATP to levels 2 to 3 times normal did not prevent the cation changes from occurring[1].

During refrigerated storage of RBC units, RBCs undergo progressive biochemical and morphological changes, referred to as the RBC storage lesion, as mentioned earlier. Many of the changes are the consequence of oxidative stress, leading to the generation of reactive oxygen species, altered proteins and lipids, loss of membrane and cell constituents in the form of shed micro particles, changes to the RBC cytoskeleton resulting in RBC shape change and increased cell rigidity. These changes share some features of normal RBC aging and/or apoptosis, as well as changes seen in certain diseases that affect RBCs, including Thalassemia, sickle cell anaemia and malaria. However, a notable difference is that, unlike *in vivo* circulating RBCs, stored RBCs are also exposed to the cell debris that accumulates in the suspension fluid (supernatant) during storage, which may contain reactive constituents, such as denatured, aggregated or oxidised proteins and lipids.

Beutler and West C in 1983, studied storage of red cell concentrates for 42 and 49 days.CPD-A2 is a modified CPD blood preservative with adenine, containing 1 1/2 times as much glucose as CPD. Units (450 ml) of blood from 21 normal donors were collected in CPD-A2 in plastic bags and held at room temperature for 8 hr. An 80%red cell concentrate was prepared and this was stored for 42 or 49 days at 4 degrees C, with the containers in either a standing or lying position. Measurements of glucose consumption, red cell ATP, and 2,3-DPG and of plasma haemoglobin, pH, Na+, and K+ were performed on all samples. After 42 days but not after 49 days of storage, red cells in concentrates stored in the lying position had consumed more glucose and had a higher post storage pH than did cells stored in the standing position. The post storage 24 hr viability of red cells stored for 42 days averaged 83.6%, with all units exceeding 70% viability. At 49 days the average viability was 69.1%. Although the average viability of cells stored in the lying position for 42 days was higher than that of concentrates stored standing, the difference was not statistically significant at the 5% level. The plasma haemoglobin level showed a weak correlation with viability of stored cells. Red cell ATP levels were correlated with viability only at 42 days and not at 49 days storage. Concentrates of red cell collected in CPD-A2 manifested fully satisfactory viability for 42 days. At 49 days storage the results of viability studies were borderline. High plasma haemoglobin values are observed at both 42 and 49 days' storage and may limit the usefulness of red cell concentrates stored for prolonged periods of time.[17]

Hess JR reviewed, that better RBC storage can prevent membrane loss and preserve the secretion of adenosine 5'-triphosphate (ATP) in response to deformation[11]. Better RBC storage may also reduce the formation of pro inflammatory membrane breakdown products that lead to transfusion-related acute lung injury and the systemic inflammatory response syndrome. To improve RBC storage, it was attempted to maximize the production of ATP by the manipulation of pH and to minimize membrane loss through the use of membrane protectants and the manipulation of tonicity. Increasing the initial storage pH led to successively higher RBC ATP concentrations until pH 7.2 was reached, when the synthesis of 2,3-diphophoglycerate was initiated at the expense of ATP. Synthesis of ATP

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could be maintained by buffering the fall of pH with increased storage solution volume or the addition of bicarbonate. Maintaining RBC ATP turns out to be an important way of preventing membrane microvesiculation or blebbing, as does the manipulation of tonicity and the addition of mannitol. It is possible to store RBC in experimental additive solutions containing only saline, adenine, glucose, mannitol, sodium bicarbonate, and disodium phosphate for 11 weeks or longer with little loss of membrane and high in vivo recovery.

As observed by Heaton et al[31] and later by Sawant[32] there are many variables in the preparation of blood components influencing the final quality. These include the duration and force of centrifugation, the separation procedure, the configuration of blood bag sets and composition of anticoagulants and storage solutions. Therefore there is no doubt that blood components from different manufacturers differ in parameters that characterize their quality. An extractable plasticizer like DEHP improves RBC storage by reducing hemolysis and membrane loss by microvesicles.

In a study conducted by Mukherjee S et al at Postgraduate Institute of Medical Science and Research, Chandigarh[33], Twenty five units each of whole blood (CPDA-1 RBC, SAGM RBC) were selected for serial biochemical parameter assessment after each fulfilled the quality criteria of volume and haematocrit. These units were tested serially for supernatant potassium, *p*H, lactate, haemoglobin, glucose and red cell 2,3 diphosphoglycerate (2,3 DPG) up to 21 days of storage.

Within each group of RBC, rise in mean concentration of potassium, lactate and plasma haemoglobin from day 1 to 21 of storage was significant in CPDA-1 RBC having the highest levels at day 21. From day 3 to 21, SAGM RBC had higher mean *p*H value than CPDA-1 RBC though this difference was not statistically significant. SAGM RBC had highest mean glucose concentration during storage than other two types of red cell preparations ($P<0.005$). Within each group, fall in mean 2,3 DPG concentration from day 1 to 7 was significant ($P<0.05$). A positive correlation existed between mean plasma potassium and haemoglobin in all three types of red cells (r=0.726, 0.419, 0.605 for CPDA-1 RBC, SAGM RBC and whole blood respectively, $P<0.005$) The main concerns for stored RBCs are ex vivo storage lesions that undermine red cell functions and may affect the metabolic status of the *in vivo* milieu of the neonatal recipients. Additional possible risks are also from the additives like glucose, mannitol which are present in red cells in large amount. However, when repeated top up transfusions are required, each fresh unit increases the donor exposure for neonate and its subsequent risks of developing transfusion transmitted diseases. In the present study significant rise in supernatant $K^+$ was seen in the three RBC preparations on storage similar to other studies.

In a study by Strauss[40], the supernatant plasma level after 42 days of RBC storage in additive solution rose to 50 meq/litre. However, the actual dose of bio available $K^+$ transfused (ionic $K^+$ in the volume of extracellular fluid) during small volume transfusion is very low. It has been estimated that the $K^+$ concentration of CPDA-1 RBC at haematocrit of 70 per cent at 35 days of storage (permitted shelf life) will be around 70-80 meq/litre. The transfusion dose in a neonate is 15 ml/kg and in a one kg neonate only 0.3 to 0.4 meq $K^+$ will be infused. This dose is even smaller than the usual daily requirement of 2-3 meq/kg. However, this rationale will not apply to large volume transfusions (>25 ml/kg) such as for exchange transfusions.

As expected, there was a significant positive correlation between plasma $K^+$ and haemoglobin levels in all the three RBC preparations and was in agreement with earlier studies. The plasma haemoglobin values were higher in CPDA-1 RBC as compared to leukoreduced SAGM RBC. Possible explanation for less hemolysis in SAGM RBC was due to presence of membrane stabilizers such as mannitol or citrate in the additive solutions. The degree of haemolysis was well below 0.8 per cent, the permissible value at the end of shelf life of all RBC preparations.

Glucose is the main source of energy for red cell metabolism via glycolytic pathway. In blood bags the glucose concentration is limited and as glucose is utilized, there is a concomitant ATP (adenosine triphosphate) depletion and decrease in red cell viability. As observed by Mukherjee et al, a fall in glucose on storage is there in all the three RBC preparations, but SAGM RBC had significantly higher glucose concentration than whole blood. The highest glucose concentration in SAGM RBC was due to additional 900 mg dextrose present in 100 ml of additive solution. This helps to prolong the shelf life of RBCs by ATP generation through glycolytic pathway.

Lactate, the end product of anaerobic metabolism of red cells increases during storage. The glucose utilization and lactate production were negatively correlated in all the red cell preparations with CPDA-1 RBC having highest lactate concentration on day 21. Possible explanation may be due to less quantity of adenine and other nutrients present in CPDA-1 RBCs for ATP generation as compared to SAGM RBC or whole blood. *p*H is an important marker of RBC metabolism during storage which slows as *p*H falls. A mathematical deduction of the *p*H curve of many samples of stored blood in various storage solutions revealed a lower limit of *p*H 6.2 below which RBCs had decreased ATP generation. Though ATP measurement was not done in the study, by day 21, CPDA-1 RBC, SAGM RBC and whole blood mean *p*H was above the lower limit of *p*H threshold of 6.2, thus ATP generation



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would most likely be persisting in all the red cell preparations.

Beutler[1 7] found 2,3 DPG to be totally depleted from RBCs by 21 days of storage. Although the levels decline during storage, they increase rapidly after transfusion in the recipient. This regeneration of 2,3 DPG is also supported by a study which observed that red cells stored for 3 wk were as efficacious as erythrocytes of 3.5 h of storage in reversing neurocognitive deficit of acute anemia[17]. The p50 of transfused adult RBCs increases rapidly as compared to low p50 values of preterm infant's endogenous red cells due to high foetal Hb concentration. Moreover, cord blood has undetectable 2,3 DPG level which along with high foetal Hb concentration cause difficulty in offloading oxygen to the tissues. Thus, transfusing the stored adult RBCs to preterm infants still has an advantage over endogenously produced infant's own RBCs.

The three red cell preparations tested revealed changes within acceptable limits of safety till 21 days of storage. Though, whole blood had least biochemical alterations followed by SAGM RBC, but for transfusing the same amount of blood (15 ml/kg) for correcting anaemia, CPDA-1 or SAGM RBC are preferred over whole blood because of higher post-transfusion haemoglobin increment by RBCs as compared to whole blood. Additional benefits like pre-storage leucoreduction and better inventory management are possible with RBC preparations and various adverse effects of fresh whole blood, both immunological and cytomegalovirus transmission can be minimized. Though, CPDA-1 RBCs had highest degree of alterations, these changes need to be considered in light of their effect on neonatal top-up transfusions.

White blood cells collected with whole blood break down in the cold, releasing proteases and lipases that damage RBC during storage. White blood cell removal (i.e. leucoreduction), by buffy coat removal or leucofiltration, can prevent this damage and improve RBC recovery and reduce haemolysis. Studies done where RBC recovery was compared directly between leucoreduced and non-leucoreduced RBC stored in the same system, leucoreduction increased RBC recovery by 4% and reduced haemolysis from about 0·40% to 0·25% at 6 weeks. Leucoreduction is now universally practiced in several European countries and Canada, and is widely used in the USA. While leucoreduction is justified by its contributions to blood safety, its contribution to blood efficacy, through improved RBC recovery for every patient, is also important.

Leucocytes found in red blood cellular allogeneic products are seldom of therapeutic benefit to the patient but are known to escalate the rate of cellular damage and to cause adverse transfusion reactions in recipients. These adverse reactions include alloimmunization to human leucocyte antigens (HLA), nonhaemolytic febrile transfusion reaction (NHFTR), transfusion-associated lung injury (TRALI), and immunomodulatory effects which include possible postoperation infection, postoperative mortality, or cancer recurrence[41,42]

Leucocytes may also be regarded as the vector of infectious pathogens for instance Epstein Barr virus, cytomegalovirus and human T-lymphotropic virus I/II. It has been established that B-lymphocytes are vectors for the prions causing variant Creutzfeldt-Jakob disease. It has been reported that using leukocyte reduced RBC reduces the incidence of multi organ failure in patients having vascular or oncological surgery and decreases hospital stay by 2, 4 days as well as mortality in patients having gastrointestinal oncology surgery. The average reduction of 2, 4 days per patient would significantly cut costs of a national hospital. British haemovigilance evidence demonstrates that using filtered RBC components reduce the frequency of transfusion-associated graft versus host disease.

It should be noted, however, that only using leucoreduced RBC to prevent TA-GvHD is not recommended as the RBC used for transfusion should be filtered and irradiated to prevent this serious and often fatal disease.

According to the Matzinger's 'danger' theory,[96] normal autologous leucocytes alone are unable to stimulate their own immune system. However humoral factors accumulated during blood storage may provoke inflammatory/immunomodulatory effects, even if they are autologous. Against this background, Tasaki T and Ohto H carried out a study to clarify the significance of pre-storage leucoreduction for autologous blood, especially in terms of cytokine movement[75]. No differences were observed in postoperative inflammatory responses or infection rates, persuading us that these measures are clinically unnecessary for autologous blood. However, this result does not deny the transfusion-related immunomodulation (TRIM) effect, as not all factors associated with TRIM are removed by this method, and the mechanism would be more complex in allogeneic blood.

According to the 'danger' theory, the 'foreignness 'of a pathogen is not the important feature that triggers an immune response, which is more connected with danger/alarm signals from injured cells, such as those exposed to pathogens, toxins, medical damage and so forth. Clinical consequences such as enhanced survival of renal allografts suggest that TRIM does exist. But the clinical effect following allogeneic blood transfusion may differ, because the ability to induce alarm signals from stressed or injured tissue differs among stimulators. Nevertheless, autologous or leucoreduced blood would be desirable for blood transfusion in order to prevent TRIM induced by allogenic blood.

The filters used for leucocyte depletion are readily available and filtration of RBCC may be prepared

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at the patient's bedside during transfusion, before storage (in-line filtration) or after the buffy-coat layer and plasma have been removed (prestorage or 24-hour expiry product). Leucocyte depletion by filtration is best performed in the processing laboratory of the transfusion services as this maintains better quality assurance. It is advisable to filter the blood soon after collection and/or processing as granulocytes fragment and degranulate during storage, which may cause a NHFTR or the antigen-presenting cells presenting major histocompatibility complex (MHC) classes I and II antigens, leading to alloimmunization. It has been reported that leukocyte antibodies associated with TRALI are possibly targeted at HLA antigens (class II) and neutrophil alloantigens. In antibody-mediated TRALI, the antibody causing TRALI in a patient is usually recognised in multiparous female donors, but these donors cannot be excluded as this would reduce the donor-pool substantially .The FDA recommends that a filtered unit of blood contains less than $5 \times 10^6$ of white blood cells (WBC) and a retention of approximately 85% of the original RBC. Patients are stimulated to produce antibodies against the transfused histocompatibility antigens when the WBC exceed the 5-log count and thus to prevent primary alloimmunization, the FDA has stipulated this rule. They also suggest that quality control testing be done on 1% of filtered units, of which 100% should not have more than $5 \times 10^6$ WBC.

Bedside filtration should be the last option to use as adequate quality control procedures cannot be completed on bedside leucocyte reduction filters. Bedside filtration requires a slow flow rate which reduces the filter performance. The filters that are currently used in most blood transfusion establishments provide a 3-log leucocyte depletion.

Leukoreduction, which was introduced in the UK in the late 1990s, primarily to reduce the transmission of viruses, decreased hemolysis as observed by Williamson et al.,1999 and the oxidative damage and calcium-related stress of stored RBCs [28]. Nonetheless, stored RBCs still deteriorate during storage in ways that are not fully understood [29] and this "storage lesion" has been implicated in the poor outcome, post-transfusion, of certain categories of patients studied by Wang et al[29]. Prestorage leucoreduction has been shown to improve the quality of stored blood components. By removing leucocytes before they can disintegrate, the release of enzymes, cytokines and intracellular components is strongly diminished, as is the generation of leucocyte fragments. Consequently, and according to experimental and clinical data, removing white blood cells (WBC) prior to storage may reduce the incidence of non-haemolytic febrile transfusion reactions, human leucocyte antigen (HLA) alloimmunization, refractoriness to platelet transfusion and the transmission of infectious agents.

The potential harm during storage includes scavengers of nitric oxide (NO) and inhibitors of NO generation that may result in lack of vascular relaxation (eg, hemoglobin, both free and in microparticles,[14] alterations in RBCs themselves,[15] and asymmetric dimethylarginine that may inhibit NO synthase[16];microparticles and RBCs themselves that may have procoagulant effects[18-21] and/or other effects on innate and/or adaptive immunity and also physiology.[22,23]

The free and nontransferrin bound iron that may promote growth of siderophilic bacteria and clearance of damaged RBCs that may result in activation of innate immunity[27,31]. There is the generation of bioactive lipids that prime neutrophils and may contribute to transfusion-related acute lung injury,[32,34] and cytokines and chemokines, especially in units that are not filter leukoreduced.

M. Wadhwa[34] has studied the effects of elevated levels of proinflammatory cytokines in the plasma of stored cellular blood components that may be responsible for some transfusion reactions noted in recipients of these components[42]. Little information is available on the role of residual leucocytes and/or platelets on accumulation of cytokines in stored red cell products prepared by different conventional methods. Some reports have highlighted the accumulation of leucocyte derived cytokines in stored RCCs but none so far has focused on the role of platelet derived cytokines in RCCs since platelets are present in abundance in some red cell products. In the study, they have therefore quantified cytokines of both leukocyte and platelet origin in RCCs prepared by two different procedures and examined the effectiveness of pre storage and post storage filtration strategies in reducing the generation of cytokines in supernatants of stored concentrates. There is a progressive increase of IL-8 in the supernatant plasma of stored plasma reduced Red Cell Concentrates.

Stack et al, in 1995, showed higher IL-8 levels ranging from 63 to 1610 pg/ml in day 42 AS-1 red cell units. In contrast, lower levels of 0±280 pg/ml of IL-8 at days 2 and 3 of storage were detected in the same units. In another study, Shanwell et al,1997, described low level generation of IL-1b, IL-6, IL-8 and TNF-a in stored RCCs. Storage of red cell units at 4°C rather than 22°C as used for platelet concentrates may account for the reduced cytokine generation noted with RCCs. In addition to IL-8, RANTES and TGF-b1 were detected in plasma reduced RCCs[98]. These cytokines are mainly stored within the cytoplasmic alpha granules of platelets and may be released during the preparation and storage of these concentrates. Indeed, the levels of these cytokines were lower early during storage than the later storage phase of plasma reduced Red cell concentrates. As for platelet concentrates, leukocyte reduction by filtration prior to storage prevented cytokine



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generation in red cell units. Filtration prevented not only accumulation of IL-8 but also of RANTES and TGF-b1. This is because filtration also reduced the platelet content of the plasma reduced red cell units. In addition, pre-storage filtration appeared to be better than post storage filtration. Rot A, in 1992, studied that RANTES is mainly a monocyte chemoattractant but can also attract various T-cell subtypes. Moreover, RANTES can chemoattract eosinophils and cause them to degranulate, and also chemoattract basophils and induce histamine release.[12,97]

These activities suggest that RANTES may have a role in inflammation and transfusion reactions. In addition to RANTES, TGF-b1 is also present in plasma reduced RCCs. Previously, high amounts of this cytokine have been detected in patients following platelet transfusion. In the in vivo context however, it is difficult to predict the role of TGF-b1 as it can function in a stimulatory or inhibitory capacity depending on the target cell type (resting/activated state) and the immediate extracellular milieu. Within an inflammatory site, TGF-b can chemo attract, recruit and/or activate immature leukocytes, induce secretion of several inflammatory mediators from monocytes(e.g., IL-1, IL-6, TGF-b itself, prostaglandins) and consequently trigger or even possibly amplify inflammatory responses. In contrast, TGF-b can inhibit activated monocytes, activated T-cells and impair cytokine synthesis and related functions. Additionally, TGF-b can inhibit the generation of cytotoxic T-cells, lymphokine activated killer cell activity, natural killer cell activity, proliferation and differentiation of B-cells and also the expansion and functional responses of haemopietic cells. Some cytokines, such as TGF-b, may mediate at least some of the adverse effects of red cell transfusions either by acting by themselves or by inducing their own secretion and/or that of other cytokines from different target cell types or in synergy with other cytokines to cause amplification of biological events relevant to the manifestation of adverse clinical events. The effects of transfusing cytokine contaminated blood components may vary depending on the biological activities of the cytokine(s), the frequency and/or dosage and also the clinical state of the recipient(s). The amounts of cytokines incapable of causing adverse effects in healthy individuals may, in some instances, trigger reactions in patients whose immune system is affected by the underlying disease, immunosuppressive therapy and other factors. Wadhwa et al have studied that the buffy coat depleted method seems to be better as it results in a low cytokine content because of depleting RCCs of both residual plasma and leucocyte/platelet content relative to plasma reduced method. Pre storage red cell filtration which removes both leucocytes and platelets appears to be a good strategy in terms of reducing cytokine levels in stored RCCs. In general, therefore, as cytokines elicit immunoregulatory and/or inflammatory responses, the best strategy should be to prevent their accumulation in blood components. The importance of these in vitro findings can only be substantiated by data obtained from large clinical trials in well-defined cohort(s) of patients in which RCCs produced by depletion of buffy-coat are compared with RCCs prepared by pre storage filtration of Red cell concentrates.

Numerous studies have aimed to resolve the controversy around the presumed deleterious effects of stored blood on patient outcome. Most have focused on four patient populations: critical care patients, trauma patients, cardiac surgery patients, and neonates, and with the exception of the extremely ambitious study in neonates described later (the ARIPI trial), the majority of these studies have critical flaws. Most reports are small size (underpowered), single-centre, and retrospective. In many, no adjustments for confounding parameters were performed, arbitrary thresholds for fresh *versus* aged RBCs were used, and/or subjects were transfused with units of mixed storage times (for both control and study groups). Also, different storage media were used, there is no documentation of transfusion protocol, and various outcomes or endpoints have been evaluated.

*a) Intensive care unit patients*

Despite recent improvements in the policy of RBC transfusion to intensive care unit (ICU) patients, the prevalence of RBC administration to this patient population remains significant. For example, a Scottish prospective study found that as many as 40% of patients admitted to ICU was transfused, with a haemoglobin threshold of 7.8 g $dl^{-1}$ in the absence of haemorrhage.[44] Moreover, critical care patients are more likely to develop complications resulting from RBC transfusion as a result of their inflammatory state, impaired tissue oxygen demand–supply status, and perturbations of their microcirculation. It is, therefore, not surprising that intensive care patients were the first group to be evaluated regarding effects of RBC storage time on oxygen delivery and outcome.

One of the earliest reports suggesting a possible correlation between RBC storage age and mortality involved 31 patients admitted to a single ICU during 1992 with a diagnosis of severe sepsis.[46] The authors retrospectively examined risk factors for increased mortality by comparing the clinical characteristics of the 12 survivors with the 19 non-survivors. The data confirmed that the mean age of transfused RBCs was the only parameter to influence the relative risk of mortality. This study, however, did not adjust for confounding factors. Another early study from that decade, which was a prospective, controlled study enrolling 23 critically ill, mechanically ventilated patients, attempted to evaluate the effect of stored RBCs on oxygen uptake, and availability to the splanchnic bed. It demonstrated that 3 units of stored RBCs failed to raise



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systemic oxygen uptake as measured by indirect calorimetry.[47,48] Moreover, the investigators found a decrease in splanchnic oxygen availability that correlated with the age of the stored RBC units, that is, administration of older units reduced gastric intramucosal pH. The authors concluded that stored RBC administration not only failed in its main role of increasing oxygen delivery, but rather augmented end-organ ischaemia. A study on neurosurgical intensive care patients that enrolled 102 traumatic brain injury patients reported that unlike fresh RBCs, stored RBCs stored >19 days did not increase brain oxygenation. A recent, noteworthy multi-centre, prospective observational study enrolled 757 intensive care patients from 47 Australian and New Zealand ICUs. In this study, patients received a mix of RBC units of different ages, and the analysis used the age of the oldest RBC unit administered as representative of age of RBCs transfused for statistical assessment of effect. Accordingly, the data showed a significant increase in ICU length of stay and in-hospital mortality in patients receiving old RBCs (median age 17.6 days, range 12.9–24.0) compared with fresher RBCs (median age 7.5 days, range 5.7–9.0). This effect was seen after adjustment for several confounding factors, including severity of illness (APACHE III score), number of transfusions, fresh-frozen plasma and platelet transfusions, leucodepletion status, pre-ICU transfusions, pre-transfusion haemoglobin concentration, and cardiac surgery.[50]

Several other studies were not conclusive or failed to demonstrate a significant deleterious effect of old blood on oxygenation and flow parameters. In a prospective, single-centre, observational study conducted in 35 patients with severe sepsis and septic shock, Sakr and colleagues did not find any impact of storage time on sublingual microvascular perfusion. In neurosurgical ICU patients, Smith and colleagues examined the ability of stored RBCs to increase brain tissue oxygen supply. In three-quarters of the 35 evaluated patients, there was an elevation in brain oxygen partial pressure, whereas in the others, a disturbing decrease in brain oxygenation was documented. In this small cohort, no correlation was demonstrated between RBC storage age and changes in brain oxygenation. It should be noted, however, that patients received a varying number of packed RBCs (from 1 to 6 units) for diverse indications, with the main indication being a haemoglobin $<10$ g $dl^{-1}$. Yet, another study in anaemic critically ill patients (double-blind, randomized) that compared the effect of RBC transfusion on tonometric indexes when RBC units were stored $<5$ *vs* $>20$ days failed to show any significant differences between the study groups.

Recently, a randomized controlled trial (RCT) enrolling 100 mechanically ventilated ICU patients failed to show any impact of storage duration of transfused RBCs (≤5 days in the study group *vs* standard care in controls) on pulmonary gas exchange (as reflected in the $Pa_{O_2}/FI_{O_2}$ ratio) and on immune and coagulation status.[51,52] Negative results regarding the effects of storage duration on organ injury (acute lung injury, deep vein thrombosis, stroke, and myocardial ischaemia), nosocomial infection, mortality, and/or on length of stay in the ICU or hospital were also reported by several other investigators in both retrospective, prospective observational, prospective nested case–control, or randomized controlled studies[53].

*b) Neonatal ICU patients*

The most comprehensive trial published to date involved premature, very low-birth-weight neonates, The ARIPI trial a prospective[54], double-blind, randomized, controlled, multi-centre study, aimed to determine whether fresh RBC units transfused to premature neonates produce a better outcome than old RBC units. Three hundred and seventy-seven premature very-low-birth-weight (<1250 g) neonates from six Canadian neonatal ICUs that needed at least one RBC unit were randomized to either receive fresh units (<7 days old, mean 5.1 days) or older units (>7 days old, according to local blood-bank standard practice, mean 14.6 days). This 'old' RBCs in the ARIPI trial were not that old (mean 14.6 days of storage). Moreover, there was no predefined protocolized transfusion threshold, and each hospital adhered to its own standard practice. In general, the practice in the ARIPI study appears to follow a liberal transfusion strategy. It is, therefore, likely that the enrolled preterm infant did not reach a critical degree of anaemia necessary to unmask potential harmful effects of prolonged RBC storage on oxygen delivery. Finally, 7.5% of neonates in the fresh RBC group were actually transfused with 'old' blood but were analysed according to the 'intension to treat' approach. All these limitations should not dismiss the results of this large-scale study, but might discourage one from generalizing these results to patients of other age groups, from other countries with different blood-bank practices, with other illnesses, different transfusion thresholds, or with larger amounts of RBC units required.

*c) Trauma patients*

Trauma patients are frequently treated with allogeneic blood transfusion, either during the resuscitation phase or the first day of management. It is, therefore, not surprising that many investigators have studied the relationship between blood storage duration and outcome in this population.

An early publication[55] in this area was a small, retrospective, case–control single-centre study from 1999 that compared trauma victims that developed multi-organ failure (MOF) with a control group that did not develop MOF. Only patients who received 6–20 units



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of RBCs were included. After multivariate analysis, the authors found that predicting factors for developing MOF were higher mean age of transfused blood and absolute number of transfused units older than 14 or 21 days. They concluded that trauma patients should preferably receive fresh RBCs (<14 days of storage), since the age of transfused erythrocytes was an independent risk factor for developing MOF.

In order to overcome the problem inherent in most studies, in which a mixture of old and fresh units is administered to trauma patients, Weinberg and colleagues[56] compared mortality in 1637 patients receiving exclusively fresh (≤14 days) or old (>14 days) RBC units during the first day after major trauma. Although they did not demonstrate a difference in mortality compared with patients receiving only 1 or 2 units of RBCs, in patients receiving >3 units, mortality was significantly higher in patients transfused with old RBCs.

Except for one prospective single-centre study[57], all other studies reporting harmful effects from transfusion of 'old' RBC units to trauma patients[57-63] were retrospective, single-centre studies in which transfusion protocols (for RBCs and other blood components) were not standardized, and in most studies, a mixture of old and fresh units was administered. The cut-off point for the definition of old RBCs was 14 days of storage, and evaluated outcomes were organ injury (renal failure and pneumonia), length of stay, and/or mortality.

*d) Cardiac surgery patients*

Cardiac surgery provides a good opportunity for investigation, since this is often an elective procedure with detailed databases collected for many years, for which many patients are transfused with RBCs, many remain on mechanical ventilation in the early postoperative period, and postoperative morbidity is relatively high.

The only available prospective study showing the effect of RBC storage duration on outcome was carried out by Leal-Noval and colleagues,[64] who enrolled nearly 800 patients undergoing cardiac surgery (mostly CABG, valve surgery, or both) in a single centre. Patients with early (<48 h) mortality or ICU discharge and those with early signs of infection were excluded. There was no correlation of RBC age with length of stay, prolonged ventilation, or perioperative myocardial infarction. There was an elevated risk of postoperative pneumonia for blood stored >28 days. These results correlate with two additional studies: an earlier report by Vamvakas and Carven,[65] who demonstrated in a single-centre retrospective study of 416 CABG patients an increased rate of pneumonia and/or infection in patients transfused with older RBCs, and a second more recent retrospective multi-centre study that enrolled 1748 patients undergoing CABG or valve surgery in which a higher risk of severe postoperative infections was documented in patients transfused exclusively with RBCs stored ≥14 days.[66]

Although retrospective and from a single centre, the largest trial conducted to date in surgical cardiac patients got the attention of the media, the public, and the US Department of Health and Human Service, published in 2008 by Koch[67] and colleagues. The authors evaluated 6002 patients who received nearly 20 000 units of RBCs during cardiac surgery (both CABG and valve surgeries). Only patients who received exclusively 'old' (>14 days of storage) or 'fresh' (<14 days of storage) blood were included in the study, reducing the confounding effect of patients administered a mixture of both old and fresh blood. Patients who were transfused with exclusively older RBCs suffered higher in-hospital mortality, 1 yr mortality, renal failure, and sepsis, and had prolonged mechanical ventilation compared with those who received exclusively fresh RBCs. Moreover, transfusion of older RBCs was independently associated with an increased risk-adjusted rate of a composite of serious adverse events (25.9% *vs* 22.4%, $P=0.001$). Despite the critical limitations of this study, the impact that this report created pushed in part the initiation of several randomized controlled studies, the results of which are yet to be published.

Several other studies have not confirmed a positive correlation between RBC age and adverse postoperative events. A group from Australia[68] reviewed the records of 670 patients undergoing cardiac surgery (CABG, valve, or both) for early mortality, renal failure, pneumonia, ICU length of stay, and prolonged mechanical ventilation. None of these end-points was found to correlate with either mean or maximal age of RBCs transfused, although there was a clear association of all these end-points with the number of units transfused. These negative results have been confirmed by other single-centre retrospective studies that enrolled a larger number of patients.

The enormous amount of both experimental and clinical data suggests, but does not prove, deleterious effects of transfusion of RBCs after longer storage duration. However, the quality of the evidence, as summarized in this review, is much too poor to make recommendations to change current transfusion practice. Currently, several studies are comparing the effects of different blood storage durations on patient outcomes in randomized clinical trials.

The RECESS study (Red Cell Storage Duration Study) is a multi-centre, prospective, partially blinded, RCT that aims to enrol 1434 patients from more than 25 medical centres in the USA, undergoing mid-sternotomy cardiac surgery. Participants are being allocated to receive either exclusively 'fresh' units of RBCs (≤10 days of storage) or 'older' units (≥21 days of storage). Patients are enrolled into the study only if the blood

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service is capable of supplying units of both arms described, meaning that patients in the control ('older' RBCs) group are actually being treated according to common practice (i.e. they would get the oldest matching blood available at that time even if not in the study). [69]

The ABLE study (age of blood evaluation) is another multi-centre, prospective, double-blind, RCT being conducted in Canada. It will enrol just over 2500 severely ill ICU patients from more than 20 hospitals, randomized to receive either exclusively fresh (≤7 days of storage) RBC units or 'older' RBC units of uncontrolled length of storage (control, common practice group). This trial is unique in its control group, where there is no threshold for the age of RBCs administered. This issue might minimize differences between the study and control groups with regard to age of transfused RBCs, but simulates 'real-life' situations in a better fashion.[70]

The TRANSFUSE trial (STandaRd Issue TrANsfusion versuS Fresher red blood cell Use in intenSive care), a randomized, multi-centre controlled study from Australia, is intended to enrol 5000 non-cardiac ICU patients and, much like the ABLE study, randomize them to receive either the youngest RBC unit available (study group) or the oldest unit available (control, standard care group). This study is again unique in not pre-setting the age of the RBC unit in either group. The protocol chosen specified only that the unit transfused should either be the freshest or oldest one available, according to the study arm. This protocol best reflects real clinical life, although it might blur the difference among groups.

These three large-scale clinical trials are still in different stages of progress, and will shed light on this controversial issue. Although limited to specific patient populations, data from these studies are expected to have a major impact on the clinical practice of transfusion medicine.

In Vamvakas meta-analysis[70], only observational studies that presented adjusted results and had adequate adjustment for confounding factors were assessed. The author concluded that no adverse effect could be attributed to transfusion of 'old' units, and pointed out that current data do not support conducting further trials with an aim to answer the equipoise in opinions. An updated meta-analysis from the same author[71], published a year later, strengthens these conclusions. The latest meta-analysis by Wang and colleagues, published in 2013, reported contradictory conclusions. This meta-analysis included more studies, and thus more patients in total and in each subgroup than the previous one, and used different enrolment criteria. The authors reviewed more than 400,000 patients from 21 reports, mostly cardiac surgical and ICU patients. The results showed increased mortality among patients receiving older erythrocytes. Subgroup analysis of these trials indicated that the increased risk was not restricted to a particular type of patient, size of trial, or amount of blood transfused. The authors calculated that 97 patients who receive exclusively 'fresh' RBCs will result in one life saved.

The ongoing discussion and debate, as reflected in these two meta-analyses, and the many reviews published in recent years, introduced RBC storage duration as a new parameter that cannot be ignored.

In recent years, the scope of clinical concern regarding transfusion of stored RBCs has widened from traditional issues of replacing lost RBCs with stored RBCs that could deliver oxygen to peripheral tissues, to concerns regarding the accumulation of toxicological entities in stored RBCs that could lead to medical sequel upon transfusion. Much like the difficulties in studying tissue oxygenation by RBCs, analyzing sequel of transfusing stored RBCs is a very challenging process. Retrospective studies have been useful, but give a wide variety of results, with a large number of studies showing worse medical outcomes as a function of RBC storage time, others showing no effect, and some reporting worse outcomes with fresh RBCs. Although of great potential utility from the standpoint of using existing medical data to generate observational hypotheses, retrospective studies inevitably suffer a series of intrinsic biases. For this reason, several prospective randomized clinical trials (RCTs) were launched, three of which have now reported no difference between groups receiving fresher vs older blood. The Age of Red Blood Cells in Premature Infants (ARIPI) study detected no difference in fresher versus older RBC units in very low-birth-weight infants, whereas the Red Cell Storage Duration Study (RECESS) reported no difference in cardiac surgery patients. Likewise, the Age of Blood Evaluation (ABLE) trial recently reported no difference in intensive care unit patients. Formal publication of the results of RECESS and ABLE (outside of abstract form) has not yet occurred and additional trials remain under way.

RCTs are of tremendous use in helping to resolve this important issue. Although it is inevitable that some challenges to methodologies and analysis will be raised, these trials nevertheless provide very reassuring data indicating that large effects are not present in the particular patient populations that have been studied, with the RBC storage methodologies being used, and with the definitions of old vs fresh blood that were used. However, an additional concern remains, due to the tremendous number of transfused patients. Conversely, this means that large and clinically relevant improvements would need to be made to RBC storage conditions before any new RBC products could be demonstrated to be superior to current products based upon RCTs.



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The ARIPI, RECESS, and ABLE trials described above bring substantial intellectual comfort that within the context of current practice and standard of care, there are not large effects between groups receiving "fresher" versus "older" stored RBCs. Nevertheless, these RCTs must likewise be interpreted with their own caveats, including the limited power to detect small effects (even below 20% depending upon the nature of the storage lesion),[72] the fact that few patients in these trials received multiple units of blood at outdate but, at some frequency, that such patients likely exist in real practice, and that just as animal biology may not reflect human biology, the clinical susceptibilities of the patients in the existing RCTs may not reflect susceptibilities in other patient populations.

Thus, both the animal studies and the human RCTs are compelling and essential parts of an evolving landscape of data, and must be given the intellectual influence and weight they are due, without over inferring or generalizing to unobserved situations to which they may not apply.

Primary metrics that have historically guided development and refinement of RBC storage conditions have focused on RBC cellular integrity. RBCs that haemolyse in the storage bag are clearly no longer viable as a therapy for anaemia; thus, the US Food and Drug Administration (FDA) requires that on average, haemolysis in the bag be <1%. However, just because an RBC is intact does not mean it will circulate upon transfusion. Accordingly, the number of RBCs that remain in circulation 24 hours post transfusion has been a primary criteria for the conditions and length of RBC storage that the FDA will approve (there must be a mean "24-hour recovery" of at least 75% with a standard deviation of <9% and with a 1-sided 95% lower confidence limit for the population proportion of successes of >70%). The cut off of 75% is based upon historical expert opinion, but is nevertheless an arbitrary metric. The rational basis for the 24-hour recovery is that the removal of irrevocably damaged RBCs appears to occur within this 24-hour window and RBCs which are still circulating 24 hours post transfusion have a normal RBC circulatory lifespan.

It seems a fair statement that RBCs which do not circulate cannot deliver oxygen; thus, using 24-hour recoveries is a meaningful metric. However, even the word "circulate" requires certain biological scrutiny in this context. It might seem a reasonable conclusion that if a labelled RBC is injected IV and 24 hours later is recovered from a peripheral blood draw, it has been circulating. It might also seem a reasonable conclusion that if 75% of transfused RBCs are recovered 24 hours after infusion, then 25% of the transfused RBCs are no longer circulating. However, both of these assumptions have undergone a critical evaluation. With regards to the first notion, it has been rightly pointed out that the flow dynamics of large vessels are profoundly different than issues of the microvasculature of capillary beds. As the vast majority of oxygen exchange occurs in the microvasculature embedded in organ parenchyma, it is the actual behaviour of RBCs in microcirculation that likely affects oxygen delivery capacity. It is for this reason that a great deal of study has gone into the rheological properties of RBCs, using a variety of instruments that measure RBC flexibility, the ability to bend and contort, and the ability to navigate artificial microvasculature with a variety of properties meant to model capillaries. Indeed, stored RBCs show progressively diminished flexibility and ability to perform the contortions necessary for microcirculation[73-76] and even show negative effects upon circulation itself[75]; although studies to the contrary have also been reported.[76] Moreover, transfusion of RBCs stored for longer periods has a statistically significant inverse correlation with both thenar muscle tissue oxygen saturation and perfused capillary vascular density in trauma patients. With regard to the assumption that RBCs that are no longer detected in venous blood are no longer circulating, it is important to note that some transfused RBCs may not be detected in peripheral circulation until over 24 hours post transfusion, presumably due to some manner of adhesion or sequestration.[77-78]

Regrettably, just because an RBC can circulate (even through the microvasculature), does not mean that it is in fact capable of delivering oxygen with normal efficiency. Indeed, there are data to indicate that stored RBCs may not optimally deliver oxygen until they have "recovered" after a period of time circulating in the recipient. This issue remains a matter of some dispute, with evidence on both sides[79]. However, what the experimental data do demonstrate is that RBCs are capable of a phenotype in which they circulate but have decreased efficiency of oxygen delivery. Thus, this must remain a concern in guiding development of RBC storage conditions and has led to the view by some that stored RBCs do not function well. An argument that is often forwarded to the contrary is that many trauma patients who have had their entire blood volume replaced with stored RBCs survive, and patients requiring exchange transfusion do better clinically, indicating that stored RBCs can function to a reasonable extent. This seems undeniably true, and is a credit to the efficacy of transfusing stored RBCs. However, the fact that patients are routinely transfused with stored RBCs and many have good outcomes cannot logically justify the claim that stored RBCs function optimally nor does it demonstrate that stored RBCs are not damaging to patients in some ways.

Early characterization of the storage lesion focused on a number of parameters for which there was a rational basis to assume that they would reflect RBC function, including: (1) metabolic changes (eg, adenosine triphosphate [ATP], 2,3-diphosphoglycerate,

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etc), (2) enzymatic changes, (3) changes in rheological properties, and (4) physiologic changes (eg, shape change, membrane remodeling), which have been recently reviewed.[80,81] Oxidative changes have also been thoroughly characterized, have been shown to correlate in many ways and have been posited as playing a causal role in the RBC storage lesion.[80,81] However, although it seems a reasonable prediction that some of the above metrics would predict how stored RBCs would perform after transfusion, such is regrettably not the case.

A major challenge to the field has been that although the above measures do reflect observable alterations in RBCs during storage, they have only an "asymmetric correlation" to the ability of RBCs to survive storage (as measured by 24-hour recoveries). In other words, RBCs with extreme changes can be shown to have poor 24-hour recoveries; however, none or a small amount of change does not necessarily predict good 24-hour recoveries. Such is even the case for ATP levels, which have been a mainstay measure of RBC storage quality and have guided the addition of adenine to RBC storage solutions. Indeed, ATP at the end of storage has been the best predictor of 24-hour RBC recoveries; however, even ATP has only a 40% correlation with 24-hour RBC recoveries (with a mean 75% to 80% recovery).[82] This poor correlation may be in part due to intrinsic difficulties in measurements of high accuracy, but may likewise reflect a lack of biological correlation.[83] The lack of correlation between in vitro measures and in vivo circulation raises the serious concern that current metrics may be untethered to relevant biology.

In more recent decades, the concept of in vivo RBC function has evolved well beyond the issue of oxygen delivery. The current paradigms now include a role of RBCs as not only a vehicle of oxygen delivery, but also as both an agent of $CO_2$ removal, a sensor of tissue oxygenation, and a regulator of biological processes extrinsic to the RBC (example, vascular tone through NO biology). Thus, additional metrics of RBC storage have been added to the above-mentioned details, including distinguishing haemoglobin outside of intact RBCs (eg, either free or in the form of microparticles that may scavenge NO), the amount of S-nitrosylation of hemoglobin itself (SNO-Hg),[84] adhesion of RBCs to endothelial beds,[85] effects of stored RBCs on models of vascular tone (isolated aortic rings),[86] and in vivo measures of how stored RBCs affect vascular tone, both in animals and directly in humans.[87]However, as with the long list of more traditional measures, the clinical meaning of these indications remains largely undetermined (and in some cases controversial) and the ability to predict RBC function is unclear.

*e) Arrival of the "omics generation"*

The era of "omics" biology has now made its way to the study of the RBC storage lesion.[88] As in many fields, the application of "big data" platforms (eg, proteomics, lipomics, glycomics, and metabolomics) has led to a progression toward a seemingly encyclopedic list of the changes that take place to RBCs over 42 days of storage. Genomics of RBC storage is a bit more abstract, as mature RBCs do not have nuclei and do not express gene products; however, it is well known that there is substantial variation in RBC storage across donors and it has also been reported that some characteristics of RBC storage are heritable traits in humans. The generation of such large quantities of data are of great potential, and have led to the ability to rapidly characterize storage biology to greater depths. These studies have detected entire panoply of new molecular changes that correlate with RBC age, as well as confirming historical observations. Importantly, such data have also allowed systems biology approaches to RBC storage biology, which will likely be of great scientific utility moving forward. However, like the older measures, it is as yet unclear which, if any, of the new measurements correlate with the in vivo behavior of the RBCs. Thus, for the time being (at least), the generation of large volumes of new data has only exacerbated the central problem of which variables to study. Thus, a downside to the new data are that given the thousands of changes RBCs undergo during storage, and the experimental difficulty (or impossibility) of changing and testing a given variable in isolation from other variables, the actual significance of any of the observed changes remains unassessed by experimental methodologies and thus remains somewhat obscure.

So, the question is raised, how will the field move forward in sorting out the proverbial wheat from the chaff? One pathway is through experimental modification of storage solutions that give a better RBC product, as assessed by traditional metrics, if not better medical outcomes. Analysis of what variables correlate with a better RBC storage solution, and which do not, will not isolate individual changes, but will nevertheless help to establish the functional significance of at least different groups of change. Controlled animal experimentation will also be of use in helping to isolate pathways, and will certainly provide a much greater degree of experimental control than can be ethically or practically achieved in humans, with the decidedly negative bedfellow that animal RBC biology will differ from human RBC biology, to an as yet unknown extent.[89] Thus, animal studies will likely find their greatest utility in sorting through the multiplicity of known changes in discovery-phase searches that will then allow more focused hypothesis testing in humans. Of central importance will be the combination of omics analysis with units of RBCs that are subsequently transfused into humans, and at the very least subjected



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to traditional measures of in the bag hemolysis and 24-hour recoveries. First steps to this approach have been taken in platelet storage biology, and have isolated initial candidates for changes that predict posttransfusion performance.[90-93]Similar approaches to RBC storage are likely to bear similar fruits, and seem a logical next step.

It is clear that certain storage lesion–based changes correlate with poor RBC performance (eg, hemolysis, extreme changes in morphology [echinospherocytosis], high levels of protein and lipid oxidation, loss of sialic acid, cross-linking of hemoglobin to itself and other proteins, clustering of band 3, etc). The significance of such changes may be somewhat exaggerated in the context of confusing the distinction between that which can occur and that which clearly does occur. Just as one example, the observation that transfused RBCs clear rapidly if first treated with neuraminidase (which removes terminal sialic acids from glycans) does indicate that if sialic acids are lost then RBCs will clear; however, this does not equate to establishing that the degree of spontaneous sialic acid loss during storage is sufficient to result in RBC clearance. This is an issue that afflicts essentially all of the proposed mechanisms of post storage clearance of damaged/aged RBCs. Unless and until a variable can be reversed in isolation from other variables, and the effects of such reversal can be assessed in vivo, then the requirement of a given pathway cannot be firmly tested. Such manoeuvres are technically challenging, if not impossible, given the massive number of changes that are known to take place during RBC storage, and present a substantial challenge even in tractable animal models, let alone the technical and ethical challenges of human studies. Moreover, even if such experimental sophistication were achievable, it can only answer questions of what is "required" but cannot exclude what is "involved" due to issues of biological redundancy. Indeed, the lack of clear understanding that has been perpetuated by this obstacle is not limited to clearance of RBCs post storage; rather, it seems a fair statement that discrete answers to the question of how RBCs are cleared in vivo as a part of normal RBC senescence remain undetermined. As with RBC storage, multiple pathways have been proposed and demonstrated as being possible, but identification of those that are clearly involved and/or essential has been elusive.

An additional concern to the issue of sorting out the storage lesion and its effects on RBC efficacy and/or toxicity is the large number of approved RBC products and the myriad medical conditions for which stored RBCs are transfused. RBCs may be anticoagulated in CPD and CP2D (and for apheresis in ACD); units may also be supplemented with a variety of additive solutions (eg, SAGM, ADSOL-1, ADSOL-3, ADSOL-5, ADSOL-7, [SOLX], etc); the different components of these solutions have been recently reviewed. However, in general, the solutions differ in concentrations of NaCl, $NaHCO_3$, $Na_2HPO_4$, citric acid, sodium citrate, adenine, guanosine, glucose, and mannitol.[88] Moreover, RBC units may be leukoreduced or not, depending upon country and region. Finally, there are additional prestorage variables (eg, time at room temperature after collection, irradiation, apheresis vs whole blood, etc) that have not been included in many studies, but for which there is a good faith basis to suggest a role in biology.[88] Indeed, differences in storage solutions in Europe ,Canada and the United States have been posited as a potential cause for different results of analysis of patient outcomes as a function of transfusing stored RBCs.[88] Perhaps ever more confounding is the wide variety of medical indications for which RBCs are given, and how the different, and at times opposing, path physiologies may confound observations of RBC efficacy. For instance, it has been observed that stored RBCs develop a "procoagulant activity." If such activity correlates to the promotion of clotting in vivo, then such procoagulant activity may simultaneously be therapeutic to a patient suffering hemorrhage and lethal to a patient suffering thrombosis. This is both a concern as far as adjusting medical practice to individual patient needs, but also for the validity of clinical trials in which patients with diseases of different pathologies are included together in groups assessing medical outcomes. In the above context, the general overarching question as stated, "Is older blood bad for you?", appears to be oversimplified to the point of being neither testable nor applicable, and more focused questions must be asked. In the field of blood product collection and transfusion, the notion of "process control" is well established and in place to variable degrees in different settings. That is to say that there is an attempt to ensure that all products are collected and distributed within the context of certain controlled parameters. In other words, blood products are all collected and treated the same, and attempts are made to sample a small number of units, from which one extrapolates a general understanding of the quality of a large number of products, which are not themselves directly tested. However, neither the scientific nor technical notion of "product control" has yet been developed or implemented. That is to say, release criteria from the blood bank (for individual units) is limited to testing negative for select pathogens, the recipient not having a detectable alloantibody against the donor unit, the unit meeting process control, and passing a visual inspection.

Of the panoply of changes that RBCs are known to undergo during storage, none is used as a quality control measure at the time of RBC release, on a unit-per-unit basis. This is in large part because the field has yet to identify storage lesion measures known to predict efficacy or toxicity. However, should such factors be described, then the application to individual units is not necessarily farfetched; in the recent past (prior to electronic cross-match), essentially every unit that was

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transfused was cross-matched to the individual recipient.

In a recent study conducted in a Tertiary care hospital in Uttar Pradesh by Baliar S. and Jaiswal M., who studied CPDA-1 blood bags over a period of 28 days the following conclusions were drawn (i) low baseline potassium blood bags might be preferred for transfusion in cases demanding a low potassium load and (ii) coordinating the 'first-in-first-out' (FIFO) policy with 'early release of blood-bags with high initial potassium' might be helpful in improving the release of suitable blood units from blood-banks.[34]

The association between transfusion of stored blood units having high K levels and cardiac dysfunction has been well demonstrated [18,19,35]. We attempt to study possible relationships between K concentrations and levels of some commonly used laboratory parameters in CPDA-1 blood bags and SAGM bags by serially examining them. This might be helpful in making optimum use of this limited resource and providing better treatment-care. Different models and immune response read-outs have been used to study the effect of allogenic WBC's, which makes comparison of the results difficult. Some studies have used whole blood assays, whilst others have used isolated WBC populations (i.e. neutrophils, mononuclear cells or T lymphocytes). Prestorage leucocyte reduction of RBC units appears to mitigate some WBC responses, but not others. Normal allogeneic mononuclear cells can be induced to release cytokines by supernatant from leucocyte-reduced RBC units, whilst cytokine release by allogeneic neutrophils appears to be mitigated. The predominance of a proinflammatory versus an immunosuppressive cytokine response may also be influenced by whether or not the RBC unit was prestorage leucocyte-reduced. Supernatant from leucocyte-reduced RBC units has recently been shown to induce regulatory T cells, and this effect was not related to storage duration of the RBC unit .Together these results suggest a complex and dynamic interplay of effects. All of these *in vitro* experiments have been performed using allogeneic "responder" WBCs from normal healthy donors. The response of WBCs from transfusion recipients with co-existing morbidities that modulate their immune and/or coagulation or vascular systems is not known and adds further dimensions to the complexity of the potential effects of Transfusion Related Immunomodulation.[34-36]

Hemolysis during processing can occur due to various reasons. Delay between collection and processing, rapid anticoagulation, high centrifugation speed, rapid addition of additive solution to packed red cells and variation in the quality of blood storage bags all contribute to hemolysis. It has been shown that mechanical stress from centrifugation or agitation can cause greater increase in the lysis of red cell units if they are stored as packed red cells or stored for longer period (near expiry date). Mechanically stressed red cell units show progressively greater signs of red cell damage as the storage period increases. This is because it accelerates the adverse effects of storage which can lead to hemolysis. The red cell units stored for longer periods are more susceptible to mechanical trauma.[13] Mechanical hemolysis is caused by the turbulent shear stress (Reynolds stress) which can be caused by sudden acceleration or deceleration during centrifugation, edges of a kinked tube, partially opened transfer tube closures, during stripping of blood into partially opened blood collection bags. Vigorous shaking of the blood bag during mixing with additive solution may cause damage to older fragile red cells. Manually raising the pressure in the blood bag to increase the rate of blood flow through leucocyte reduction filter can also damage the red cells. Low shear stress ranging 1000-1500 dyne/cm$^2$ during gravity filtration through leucocyte filters usually is not sufficient to cause lysis of red cells. Traumatic lysis is more likely in red cell concentrates with higher hematocritl.[22]

Sawant et al studied the effects of processing and storage on red cell hemolysis. They collected the red cell units in single blood bag, triple blood bag and, top and bottom blood bag system. They found that the hemolysis increases in red cell units with increase in storage period ($p < 0.001$). However, the bemolysis in all the red cell units was much less than the permissible limit of 0.8% (European guideline) at the end of 42 days of storage. Their study also showed that red cell units in SAGM (saline-adenlne-glucose-mannitol) had a higher degree of hemolysis than those in Adsol. This increase in hemolysis may be attributed to the preparation technique, in which the additive solution (SAGM) was added to the packed red cells after separation of the platelet rich plasma. Sudden exposure of red cells to additive solutions can result in damage to the red cells due to change in osmolarity.[23] Sowemimo-Coker showed that at plasma hemoglobin of 40 mg/dl, the supernatant colour appeared visibly red to unaided eyes which is much below 1% hemolysis. Hence, visual assessment of hemolysis tends to overestimate the hemolysis in red cell.[2,88]

Oxidoreductive imbalance is a major cause of excessive haemolysis in *in vitro* conditions. Leucocytes and blood platelets present in red blood cell concentrates (RBCs) are one of the sources of free radicals, which have a significant effect on the status of stored erythrocytes. Nędzi, M. et al studied RBC concentrates units obtained from 10 whole-blood units that were split into two equal units, one of which was leucoreduced on the day of donation.[94] Both units were stored for 35 days. The following markers of oxido reductive balance were measured on day 0 (donation day) and on storage days 7, 14, 21 and 35 i.e concentration of malondialdehyde (MDA) and activities of antioxidant barrier components, that is superoxide



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dismutase, glutathione peroxidase and glutathione reductase.

Lipid peroxidation in leucodepleted units (LRBC) was slower than that in non-leucodepleted ones. The analysis of LRBC revealed statistically significant decrease in concentrations of MDA. The activities of superoxide dismutase, glutathione peroxidase and glutathione reductase were higher throughout the storage period as compared to non-leucoreduced RBC. Statistically significant differences between RBC and LRBC units were noted throughout the storage in the activity of lactate dehydrogenase, and concentrations of $K^+$ ions and free haemoglobin. Leucoreduction of RBC before storage helps to preserve the activity of antioxidant barrier enzymes in stored RBCs and significantly improves the quality of stored red blood cell components.

Thus, how are we to answer the question of "What are the established and theoretical factors to consider in assessing the red cell storage lesion?" As detailed here and as reviewed extensively elsewhere, there is a well-defined list of factors that are traditional measures of the storage lesion, and a panoply of new and evolving measures, as the omics era makes its way to RBC storage. As of yet, no components of the storage lesion have been identified that adequately or accurately predict 24-hour recoveries. Moreover, although RBC circulation remains a necessary property, it is unclear that 24-hour recoveries predict efficacy beyond eliminating lack thereof due to nonviable RBCs. Because some metrics can predict blood that has lost its integrity, in vitro measures remain a guiding factor in development and refinement of new storage solutions. Nevertheless, the lack of an in vitro metric known to correlate strongly with how well RBCs will circulate post transfusion, and the lack of knowledge as to whether 24-hour recoveries correlate to medical outcomes, remains a major obstacle. The field has access to the ability to carry out human trials and studies, use animal models, and harness systems biology of RBC storage. Future refinement of methodologies and metrics in the field will necessitate combining these approaches with a distinct focus on identifying the changes that correlate and/or are causally associated with increased efficacy and decrease in untoward effects, with a mindfull consideration of different patient characteristics and clinical situations.

The identification of distinct components of the storage lesion with such predictive qualities will not only guide clinical care, but also provide a rational basis for rapid storage system development and refinement. In the meantime, the lack of such metrics remains a major challenge to blood storage research.

## III. Aims & Objectives

1. To determine changes in biochemical parameters:

Electrolytes ($Na^+$, $K^+$, $Cl^-$) pH, LDH and Hemogram on day 0,1, 7, 14, 21, 28 in stored blood units.

## IV. Material and Methods

The present study was conducted in the Department of Immunohaematology and Blood Transfusion in collaboration with the Department of Biochemistry and Department of Pathology, Dayanand Medical College and Hospital, Ludhiana. The study was undertaken after approval by Institute Ethics Committee, and after obtaining written informed consent from each donor enrolled in the study. In this study, a total of 106 blood units were collected in house (blood bank) and in blood donation camps. These bags were examined for various parameters during 28 days of storage period. The study was conducted from details about the blood donation and processing were recorded in the proforma. Samples representative of the bag contents were obtained on the specified days and were analyzed for biochemical and haematological parameters. The results obtained were recorded in the proforma.

*a) Study design- The study was prospective*

i. Blood Collection- Blood collection was done strictly as per the guidelines prescribed by Directorate General Health Services. The prescribed departmental protocols for donor selection and phlebotomy were followed.

1. Donor selection

Donors belonging to the age group 18 to 60 years with weight more than 45 kg and hemoglobin more than 12.5 gm/dl were selected. At the time of blood collection, the donors were recruited taking into account the stock of different blood groups in the inventory. Donors with blood groups which were sufficient in stock were preferred for recruitment to avoid shortage of blood units. Detailed donor medical history and physical examination of the donor was undertaken. Only those donors found fit for blood donation were selected for the phlebotomy procedure. Single CPDA bags with 350ml capacity were used for blood collection from donors weighing more than 45 kgs. Triple CPD + SAGM bags, Quadruple CPD + SAGM bags, Integral bags CPD + SAGM with 450 ml capacity and Leukoreduction filter were used to collect blood from blood donors weighing more than 60 kg.

2. Phlebotomy

*a) Equipment and materials*

a. Blood bags- Blood collection was done in 100 blood bags. Three types of blood storage bags were used:



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- Single CPDA bag (Terumo Penpol) – 17 outdoor and in-house collection
- Triple CPD + SAGM bag (Fresenius Kabi) - 58 in-house collection
- Quadruple CPD + SAGM bag with integral leucoreduction filter (Fresenius Kabi ): 17 in-house collection
- Integral CPD + SAGM bag with integral leucoreduction filter (Fresenius Kabi) (3-4 log reduction): 14 in house collection.

b. Sphygmomanometer
c. Fresenius Hemocare automatic biomixer (Hemolight Plus) which were regularly calibrated and validated by known weights.
d. Terumo Penpol tube sealer (XS 1000 T) .
e. Sterile cotton swabs and band-aids
f. Methylated spirit, povidine iodine solution (10%)

*b) Method*

a. Hands were washed with soap and water.
b. An appropriate blood bag was selected for blood collection. It was then inspected for any leakage or any discoloration.
c. A unique registration number was given to the donor and registration labels were put on the bag, form and pilot tubes.
d. Identity of the donor was established and the bag was kept on the, biomixer below the level of donor arm and the tubing of bag was fitted in the biomixer. Then the biomixer was set to the requisite volume according to the type of bag.
e. The sphygmomanometer cuff was tied above the cubital fossa of the donor arm and the cuff pressure was raised to 50-60 mmHg.
f. A firm and prominent vein was chosen for venepuncture in the ante cubital area of arm.
g. The cuff pressure was released and the proposed site of venepuncture was cleaned with methylated spirit in an area of 4-5 cm moving outwards in concentric spiral manner and it was allowed to dry.
h. 10 % Povidine Iodine was applied in the same manner as described above.
i. Again cleaned the area with methylated spirit in the same manner as described above.
j. The cuff pressure was inflated to 50-60mm Hg and the donor was asked to squeeze the ball in hand and the phlebotomy was performed.
k. The donor was kept under constant observation.
l. After collection of the requisite volume of blood in the blood bag, the biomixer gave an alarm and clamps the tubing of the bag. After this the cuff was deflated followed by the needle removal.
m. A sterile cotton swab was placed at the site of venepuncture and pressure was applied so as to prevent the oozing of blood. The donor was asked to continue lie down on the couch for 8-10 minutes.
n. The procedure was completed by pressing the end button of the biomixer which releases the clamped tubing of the bag.
o. The unique identification numbers on the pilot tubes and the blood bag were matched and checked. Blood samples were then withdrawn from the bag into the pilot tubes and the tubing of the bag was sealed by the tube sealer.
p. After the 8-10 minutes of rest, donor was taken to the refreshment room. Band-aid was applied at the venepuncture site when the oozing stopped.

3. Transportation- The blood collected in single blood bags at camp were stored and transported in blood transport boxes (Insignia). These were electricity/battery operated portable blood transport boxes equipped with compressor to maintain the temperature at 4°C or 22°C selected by key control panel. The blood bags were packed in the blood transport box in accordance with the storage capacity, so as to avoid overloading. The lid of the blood transport box was securely closed. It was ascertained that the temperature during transport did not exceed 10°C. The blood transport boxes were equipped with digital display for monitoring the temperature inside the transport box. The temperature was monitored during blood donation camp and transportation, and on arrival at the blood bank.

ii. Blood processing- was done according to the guidelines prescribed by Directorate General Health Services.

Principle - Blood constituents differ in size and density and sediment at different rates when centrifugal force is applied. When whole blood, a mixture of cellular components suspended in plasma and anticoagulant is centrifuged, the red cells settle at the bottom of the blood bag because they have the highest density (have a greater mass more than the other components). Being less dense, the white cells and platelets do not settle quickly, and remain in suspension longer. As centrifugation continues, the white cells sediment above the red cells, and finally the platelets form a layer above the white cells and leave the suspending fluid (plasma plus anticoagulant) at the top.

*a) Equipment and materials*

a. Refrigerated centrifuge (RC)- 6000i Heraeus refrigerated centrifuge was used for centrifugation of blood bags and component preparation. All the RCs were regularly- calibrated and validated for speed, temperature and time. The optimal speed and time for centrifugation was determined to get packed red cells of desired hematocrit and maximum yield of platelets.
b. Two pan mechanical balance- Calibration was done at regular intervals with known weights.



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c. Manual plasma expressor (Yorco YSI- 156)
d. Fenwal OptiSeal tube sealer (XS 1000 T)
e. Plastic clamps.
f. Plastic balancing weights

*b) Procedure*

1. Single CPDA bags- Single CPDA bags were not subjected to any further processing.
2. Triple CPD + SAGM bags- After a holding period of 2 hours at 20°C to 24°C, triple CPD + SAGM bags were processed as follows:

i. *Primary centrifugation*

a. After the 2 hours holding period, the triple CPD + SAGM bags were placed in the plastic inserts of RC with satellite bags facing outwards so that they act as shock absorber during centrifugation. The opposite plastic inserts with the triple bags were balanced accurately using the two pan mechanical balance with plastic balancing weights and then placed in diagonally opposite buckets of RC. These bags were then subjected to soft spin in the RC. The settings of the RC for soft spin were:

Centrifuge speed= 1000 rpm

Acceleration=8

Deceleration=0

Time= 10 mm

Temperature= 22°C.

b. After centrifugation, the plastic insert containing the triple bag was removed from the bucket of RC. The primary bag was removed from the plastic insert with minimal disturbance and was placed in the plasma expressor. The spring of the plasma expressor was gently released and the plate of plasma expressor was allowed to come in contact with bag.

c. The tubing between the primary bag and plasma storage bag was clamped temporarily. The seal of the primary bag was broken and the supernatant (platelet rich plasma-PRP) was allowed to flow into the platelet storage bag.

d. The tubing between the primary bag and platelet bag along with satellite bag was sealed by the tube sealer and the primary bag with packed red cells was separated and stored in blood storage refrigerator (BSR) at 2°C to 6°C temperature.

ii. *Secondary centrifugation*

a. The PRP along with the satellite bag was placed in the plastic insert of RC with satellite bag facing outward. The opposite plastic inserts with the PRP bags were balanced accurately using the two pan mechanical balance using plastic balancing weights and then placed in diagonally opposite buckets of RC.

b. PRP was subjected to heavy spin in RC. The settings in RC for heavy spin

Centrifuge speed=3500 rpm

Acceleration=8

Deceleration=8

were:

Time= 10 min

Temperature=22°C

c. PRP was then placed in plasma expressor and the supernatant (platelet poor plasma) was allowed to enter into the plasma storage bag. Around 50-70ml of plasma was left behind in the platelet bag and the tubing between both the bags was sealed by the tube sealer and separated.

d. The platelet concentrate bags were kept undisturbed with label side down for about 1 hour. They were then stored at 20°C to 24°C in the platelet agitator incubator.

e. The platelet poor plasma (fresh frozen plasma) was stored at minus 80°C in the deep freezer and subsequently shifted to minus 40°C deep freezer for storage.

3. Quadruple and Integral CPD + SAGM bags with integral leucoreduction filter - After a holding period of 2 hours at 20°C to 24°C, the bags were processed as follows.

i. Primary centrifugation

a. After the 2 hours holding period, the quadruple CPD + SAGM bags and Integral Bags with integral leucoreduction filter were placed in the plastic inserts of RC with satellite bags facing outwards so that they can act as shock absorber during centrifugation. The opposite plastic inserts with the quadruple bags were balanced accurately using the two pan mechanical balance with plastic balancing weights and then placed in diagonally opposite buckets of RC. These bags were then subjected to soft spin in the RC. The settings of the RC for soft spin were:

Centrifuge speed=I 000 rpm

Acceleration--8

Deceleration--0

Time=10min

Temperature=22°C.

b. After centrifugation, the plastic insert containing the quadruple bag was removed from the bucket of RC. The primary bag was removed from the plastic insert with minimal disturbance and was placed in the plasma expressor. The spring of the plasma expressor was gently released and the plate of plasma expressor was allowed to come in contact with bag.



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c. The tubing between the primary bag and plasma storage bag was clamped temporarily. The seal of the primary bag was broken and the supernatant (platelet rich plasma-PRP) was allowed to flow into the platelet storage bag.
d. The tubing between the primary bag along with the, SAGM containing satellite bag and the platelet bag along with the plasma satellite bag was sealed by tube sealer.
e. The SAGM containing satellite was then hung with primary bag kept at a lower level. The seal of SAGM bag was then broken and SAGM solution was allowed to flow through the leucoreduction filter into the primary bag for priming of the filter.
f. After all the SAGM solution entered into the primary bag, the primary bag containing red cells was mixed gently by inversion with SAGM solution and hung at a height with satellite bag at a lower level. The packed red cells were slowly allowed to flow through the leucoreduction filter into the satellite bag under the effect of gravity.
g. After the transfer of red cells into the satellite bag from the primary bag, the tubing between them was, sealed using tube sealer and both bags were separated. Packed red cells were stored at 2°C to 6°C in BSR and the primary bag was discarded.

ii. Secondary centrifugation.- All the steps of secondary centrifugation were followed in a similar manner as for triple CPD + SAGM bags.

iii. *Storage*- All the packed red cell units were stored at 2 to 6°C in BSR with continuous temperature monitoring. The BSRs have continuous temperature monitoring with graph recording and audio alarm for any deviation in temperature from the range of 2°C to 6°C.

*iv. Sampling*

Samples were collected from single bag on the day of collection (day 0) and then on day 7, day 14 and day 28. Samples from the triple bag and quadruple bag were collected before processing (day 0) and after processing (day 1) and subsequently on day 7, day 14 and day 28. The sampling technique used was validated by different observers.

*Equipment and materials*

a. Tube stripper (J. Mitra)
b. Laboratory centrifuge (REMI R-8C)

*Stripping*

a. The stripper was applied at the distal end of the blood bag tubing and gradually drawn towards the bag so as to empty the contents of the tubing into the bag.
b. Keeping the stripper still applied, the bag was inverted 2-3 times and then the stripper was released to allow the tubing to fill with blood.
c. Again the bag tubing was stripped so as to empty the tube contents into the bag. This was repeated 3 times so that a representative sample of the bag contents could be obtained from the bag tubing.
d. The representative samples from the blood bag which were obtained on specific days were analyzed for haematocrit and total hemoglobin using fully automated cell counter.
e. The blood sample was then centrifuged at 1000 rpm for 10 minutes and the supernatant plasma was then removed into the plastic storage vials. Then the vials were labeled and stored at minus 20 °C deep freezer.
f. The plasma samples were processed within 1 month of storage. At the time of evaluation, the samples were appropriately thawed and analyzed for plasma hemoglobin and plasma potassium level.

*Measurement of parameters*- The samples obtained were evaluated for the following parameters.

1. *Haemoglobin, Haematocrit and Total Leucocyte count:* of the blood samples were measured using fully automated cell counter (Coulter LH 750 Haematology Analyser). The cell counter is based on both the electrical and optical techniques. Cell counting and sizing is based on the detection and measurement of changes in electrical impedance (resistance) produced by a particle as it passes through a small aperture The application of light scatter enables a single cell passing across the laser light beam to reflect and scatter the light The patterns of scatter are measured at various angles and this provides information about cell structure, shape, and reflectivity. Total hemoglobin is estimated by cell counter using the cyanmethemoglobin method and the haematocrit is obtained as a product of the red cell count and mean corpuscular volume (MCV).
2. *Plasma Sodium, Potassium and Chloride*- The plasma samples obtained from blood bags stored at minus 20°C in deep freezer were appropriately thawed and sent to the Department of Biochemistry for evaluation of electrolytes. The samples were evaluated using instrument ABL800 FLEX Radiometer.

Reference Electrode is used in the measurement of electrolytes and pH. It maintains a stable fixed potential against which other potential differences can be measured.

*a) Serum Sodium levels*

*Test method:* Ion Selective electrode (ISE)

The Na electrode is an ion selective electrode whose sensing element is a $Na^+$ sensitive ceramic pin contained in the tip of jacket. The electrolyte has a constant and known concentration of sodium ions.



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When a sample is brought in contact with the electrode, a potential develops across the ceramic pin. The potential depends on difference between the sodium (more precisely, activity) in the electrolyte and the sample. If the $cNa^+$ in both solutions is the same, the potential across the electrolyte tip will be 0 volt.

The total potential cross the electrode chain is a sum of the potential differences at each of the elements in the chain, all but one of which is known and constant. The unknown potential difference across the ion selective membrane or pin is then the difference between the measured total potential and the sum of the known potentials.

$$E_{sample} = E_{tot} - (E_{ref} + E_{MJ} + E_E)$$

*Where:*

| Symbol | Potential | Element |
|---|---|---|
| $E_{sample}$ | Unknown, dependent on sample composition | Ion selective membrane (or) pin junction separating the sample and electrode. |
| $E_{tot}$ | Measured by voltmeter | Total potential |
| $E_{ref}$ | Known and constant when the Ag/AgCl wire is immersed in the electrolyte solution. | Ag/AgCl electrode/ electrolyte solution.(Reference electrode) |
| $E_{MJ}$ | Known and constant, independent of sample composition. | Membrane junction between the electrolyte solution in the reference electrode and the sample |
| $E_E$ | Known and constant when the Ag/AgCl wire is immersed in the electrolyte solution | Ag/AgCl electrode/ inner buffer solution.(Electrolyte electrode) |

The potential difference at the membrane or pin in the electrolyte electrodes can be expressed by the Nernst equation:

$$E_{sample} = E_0 + 2.3\ RT/nF \log \alpha_{Na^+}$$

Where:

$E_0$ = Standard electrode potential
R = Gas Constant (8.3143 J x $K^{-1}mol^{-1}$)
T = Absolute temperature (310.15 K at $37^0$C)
n = Charge on ion
F = Faraday constant (96487 coulomb x $mol^{-1}$)
$\alpha_{Na^+}$ = Activity of sodium ion
Reference range: 136-148 mmol/L

*b) Serum potassium levels*

*Test method:* Ion Selective electrode (ISE)

The $K^+$ electrode is an ion selective electrode whose sensing element is a PVC membrane containing a potassium- neutral ion carrier. The ion sensitive membrane is covered with a cellophane membrane in order to protect it from the samples. The electrolyte has a constant and known concentration of potassium ions. When a sample is brought in contact with the electrode, a potential develops across the PVC and cellophane membranes. The potential depends on difference between the potassium (more precisely, activity) in the electrolyte and the sample. If the $cK^+$ in both solutions is the same, the potential across the electrolyte tip will be 0 volt. The total potential cross the electrode chain is a sum of the potential differences at each of the elements in the chain, all but one of which is known and constant. The unknown potential difference across the ion selective membrane or pin is then the difference between the measured total potential and the sum of the known potentials.

$$E_{sample} = E_{tot} - (E_{ref} + E_{MJ} + E_E)$$

*Where:*

| Symbol | Potential | Element |
|---|---|---|
| $E_{sample}$ | Unknown, dependent on sample composition | Ion selective membrane (or) pin junction separating the sample and electrode. |
| $E_{tot}$ | Measured by voltmeter | Total potential |
| $E_{ref}$ | Known and constant when the Ag/AgCl wire is immersed in the electrolyte solution. | Ag/AgCl electrode/ electrolyte solution.(Reference electrode) |
| $E_{MJ}$ | Known and constant, independent of sample composition. | Membrane junction between the electrolyte solution in the reference electrode and the sample |
| $E_E$ | Known and constant when the Ag/AgCl wire is immersed in the electrolyte solution | Ag/AgCl electrode/ inner buffer solution.(Electrolyte electrode) |

The potential difference at the membrane or pin in the electrolyte electrodes can be expressed by the Nernst equation:

$$E_{sample} = E_0 + 2.3\ RT/nF \log \alpha_{K^+}$$

Where:

$E_0$ = Standard electrode potential
R = Gas Constant (8.3143 J x $K^{-1}mol^{-1}$)
T = Absolute temperature (310.15 K at $37^0$C)
n = Charge on ion



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F = Faraday constant (96487 coulomb x $mol^{-1}$)
$\alpha_{K^+}$ = Activity of potassium ion
Reference range: 3.5- 5.0 mmol/L

*c) Serum Chloride levels*

*Test method:* Ion Selective electrode (ISE)

The $Cl^-$ is an ion selective electrode whose sensing element is a PVC membrane containing a chloride ion carrier. The ion sensitive membrane is covered with a cellophane membrane in order to protect it from the samples. The electrolyte has a constant and known concentration of chloride ions. When a sample is brought in contact with the electrode, a potential develops across the PVC and cellophane membranes. The potential depends on difference between the chloride (more precisely, activity) in the electrolyte and the sample. If the $cCl^-$ in both solutions is the same, the potential across the electrolyte tip will be 0 volt.

The total potential cross the electrode chain is a sum of the potential differences at each of the elements in the chain, all but one of which is known and constant. The unknown potential difference across the ion selective membrane or pin is then the difference between the measured total potential and the sum of the known potentials.

$$E_{sample} = E_{tot} - (E_{ref} + E_{MJ} + E_E)$$

*Where*

| Symbol | Potential | Element |
|---|---|---|
| $E_{sample}$ | Unknown, dependent on sample composition | Ion selective membrane (or) pin junction separating the sample and electrode. |
| $E_{tot}$ | Measured by voltmeter | Total potential |
| $E_{ref}$ | Known and constant when the Ag/AgCl wire is immersed in the electrolyte solution. | Ag/AgCl electrode/ electrolyte solution. (Reference electrode) |
| $E_{MJ}$ | Known and constant, independent of sample composition. | Membrane junction between the electrolyte solution in the reference electrode and the sample |
| $E_E$ | Known and constant when the Ag/AgCl wire is immersed in the electrolyte solution | Ag/AgCl electrode/ inner buffer solution. (Electrolyte electrode) |

The potential difference at the membrane or pin in the electrolyte electrodes can be expressed by the Nernst equation:

$$E_{sample} = E_0 + 2.3\ RT/nF \log \alpha\ Cl^-$$

Where:

$E_0$ = Standard electrode potential
R = Gas Constant (8.3143 J x $K^{-1}mol^{-1}$)
T = Absolute temperature (310.15 K at $37^0$C)
n = Charge on ion
F = Faraday constant (96487 coulomb x $mol^{-1}$)
$\alpha\ Cl^-$ = Activity of chloride ion
Reference range: 96 – 106 mmol/L

1. pH Estimation: Measurement is based on the potentiometric measuring principle. The pH electrode is a pH sensitive glass electrode. The pH sensitive glass membrane is located at the tip and seals the inner buffer solution with a constant and known pH.
2. LDH Estimation: Lactate Dehydrogenase catalyses the conversion of L Lactate to Pyuruvate.$NAD^+$ is reduced to NADH in the process. The initial rate of NADH formation is directly proportional to the catalytic LDH activity. It is determined photo metrically measuring the increase in absorbance. The equipment used was Cobas 6000.
3. Haemolysis: Was assessed by visual inspection of blood bags, carefully, before and after processing.

## V. Statistical Analysis

After all the parameters of the samples had been obtained, data was subjected to Statistical analysis. Means, Standard Deviation and .95 Confidence Intervals were calculated for each of the parameters. To study the difference in the mean values of the parameters of each bag type, over days of storage from mean baseline value, One way Analysis Of Variance followed by F test (significance level .001) was applied. Significant F ratios were followed by Fischer's least significant difference method (protected t test) at .05 level of significance for testing null hypothesis of no significant difference between baseline mean value and values obtained on different days. Error bars indicate 0.95% confidence intervals. All data was analyzed using SPSS Version 21 (SPSS Inc., Chicago, IL, USA). Graphical representations were done using Mat Lab.

## VI. Ethical Considerations

According to guidelines set up by ICMR (1994) and Helsinki declaration (modified 2000). The following will be adhered to in all donors enrolled in the study.

1. The donors involved in the research project will be volunteers and informed participants.
2. Each donor will be adequately informed of the aims, methods, the anticipated benefits and the potential risks of the study and the discomfort it may entail to him/her and the remedies thereof.



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3. Every precaution will be taken to respect the privacy of the donors, the confidentiality of the patient's information and to minimize the impact of the study on his/her physical and mental integrity and his/her personality.
4. The donor will be given the right to abstain from participation in the study or to withdraw consent to participate at any time of the study.
5. Due care and caution will be taken all stages of the research to ensure that the donor or patient is put to the minimum risk, suffer from no irreversible adverse effects and generally benefit from and by the research.
6. Written informed consent is obtained from all the persons included in the study.
7. The investigations involved were usually undertaken as routine workup and as such no major ethical issue was involved in the study.

## VII. Observations and Results

The present study was a prospective study, conducted over a period of one year, in which a total of 106 red cell units collected from voluntary and replacement donors were studied for haematological and biochemical changes over a period of 28 days in the Department of Immunohaematology and Blood Transfusion, Dayanand Medical College and Hospital, Ludhiana, Punjab.

Blood collection was done in four types of blood bags. The types and number stored blood bags which were studied are as follows:

1. Single CPDA bags - 17
2. Triple CPD-SAGM bags – 58
3. Quadruple CPD-SAGM bags- 17
4. Integral CPD-SAGM bags with integral Leukoreduction filter(3-4 log leukoreduction)- 14

*a) Donor demographic Data*

Blood was collected from donors ranging from age group of 18-60 years of age. The donors were screened as per DGHS criteria. The collection and processing was all done in the Department Of Immunohaematology and Blood Transfusion, Dayanand Medical College Ludhiana.

The stored red cell units were assessed for various quality parameters by obtaining sample representatives of the bags on day 0 of collection, day 1 after processing to packed Red cells and on subsequent days 7, day 14, day 21 and day 28 from Triple, Quadruple and Integral Bags.

For Single Bags, Sample representatives were collected on day 0, and subsequently on days 7,14,21 and 28, since these bags stored whole blood and involved no processing.

Samples were obtained in EDTA Vials to assess the haematological parameters and in Plain vials for Biochemical parameters. The blood sample were centrifuged at 1000 rpm for 10 minutes and supernatant plasma was removed for processing and testing.

Visual inspection of the plasma indicated haemolysis. Pinkish discoloration was indicative of hemolysis taking place. It was observed in three triple CPD+SAGM Bags, on day 1, after processing.

Measurement of parameters:

Plasma Sodium

Plasma Potassium

Plasma Chloride

pH

LDH

Total Leukocyte Count

Haemoglobin

Haematocrit

*Table – 1:* Plasma levels of Sodium (in mmol $L^{-1}$) in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| **SINGLE (n=17)** | | | | | | | |
| 144.59±3.26 | | 136.35±10.47 | 132.71 ± 9.21 | 128.65 ± 8.11 | 127.47 ± 6.67 | 12.84 | <.001 |
| **INTEGRAL** (n=14) | | | | | | | |
| 144.35±2.64 | 149.57±5.10 | 143.14 ± 7.39 | 132.36 ± 9.0 | 131.36±10.88 | 126.43±12.51 | 16.31 | <.001 |
| **TRIPLE** (n=54) | | | | | | | |
| 145.52±5.75 | 138.12±12.86 | 136.06±14.57 | 125.79±11.24 | 117.44±12.69 | 119.54±11.76 | 43.42 | <.001 |
| **QUADRUPLE** (n=17) | | | | | | | |
| 141.71±4.82 | 140.06±12.24 | 135.94±11.36 | 125.59±13.73 | 121.47±14.41 | 118.88±15.68 | 10.59 | <.001 |

*Note: Values are represented as mean ± SD.*



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*Effect of processing and storage on Plasma Sodium levels:*

For all types of bags, there was a statistically significant ($p<.001$) difference in Mean sodium values on different days of storage (Table 1). There was a progressive decrease in mean sodium values with increasing days of storage ( Figure 1)

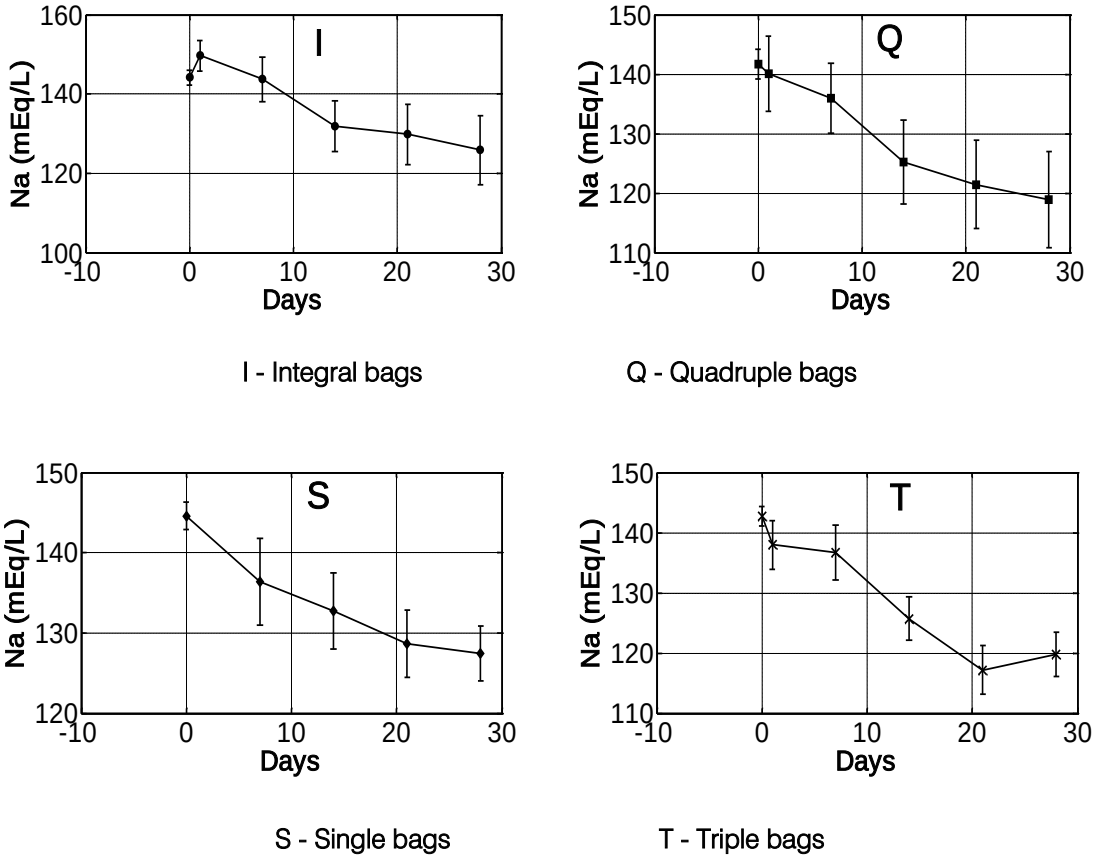



*Figure – 1:* Plasma levels of Sodium (in m $EqL^{-1}$) in various bags on different days of storage

*Table – 2:* Plasma levels of Potassium (in mmol $L^{-1}$) in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=17) | | | | | | | |
| 5.04 ± 1.09 | | 12.52 ± 6.29 | 16.78 ± 6.42 | 20.84 ± 7.5 | 26.11 ± 1.88 | 14.45 | <.001 |
| INTEGRAL (n=14) | | | | | | | |
| 5.54 ± 2.14 | 5.95 ± 3.27 | 9.58 ± 4.33 | 16.95 ± 5.37 | 21.69 ± 7.33 | 27.19 ± 11.7 | 20.04 | <.001 |
| TRIPLE (n=52) | | | | | | | |
| 6.68 ± 5.64 | 13.59 ±11.85 | 18.80 ± 8.79 | 23.34 ± 9.72 | 30.86 ± 9.72 | 31.16 ± 8.47 | 59.50 | <.001 |
| QUADRUPLE (n=15) | | | | | | | |
| 7.57 ± 5.58 | 9.93 ± 7.37 | 15.05 ± 6.31 | 21.24 ± 9.61 | 26.48±11.06 | 30.88±13.07 | 15.12 | <.001 |

*Note: Values are represented as mean ± SD*

*Effect of processing and storage on Plasma Potassium levels*

For all types of bags, there was a statistically significant ($p<.001$) difference in Mean Potassium values on different days of storage.(Table 2) There was a progressive increase in mean potassium values with increasing days of storage as shown in Figure 2.



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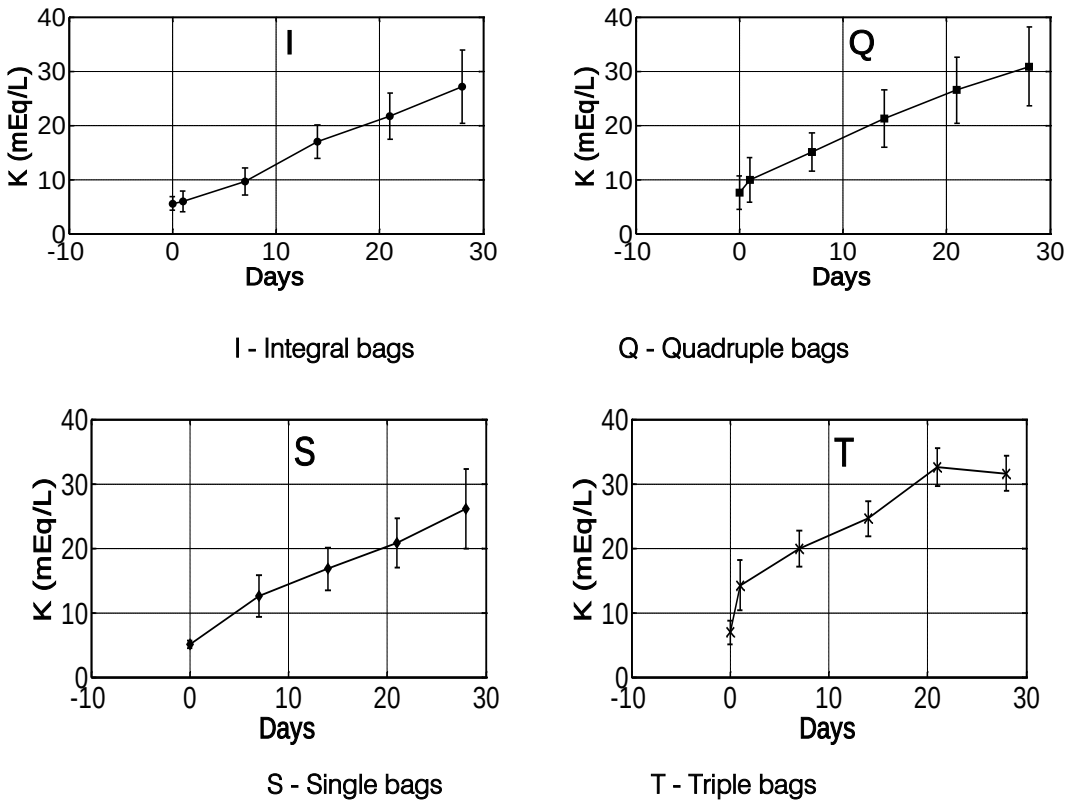



*Figure – 2:* Plasma levels of Potassium (in mmol $L^{-1}$) in various bags on different days of storage

*Table – 3:* Plasma levels of Chloride (in mmol $L^{-1}$) in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=17) | | | | | | | |
| 104.88 ± 2.96 | | 95.29 ± 9.45 | 92.59 ± 8.57 | 88.29 ± 4.77 | 87.29 ± 5.91 | 18.46 | <.001 |
| INTEGRAL (n=14) | | | | | | | |
| 104.14 ± 2.71 | 108.36±5.27 | 103.00±7.14 | 92.71± 5.09 | 87.21 ± 5.87 | 83.71± 6.27 | 45.70 | <.001 |
| TRIPLE (n=54) | | | | | | | |
| 97.81 ± 11.46 | 94.59±15.45 | 95.3 ± 10.34 | 86.27±11.61 | 75.98±11.34 | 77.91±14.74 | 29.92 | <.001 |
| QUADRUPLE (n=17) | | | | | | | |
| 99.94 ± 8.22 | 99.82 ± 9.81 | 93.47±18.96 | 85.65±15.18 | 82.12±15.57 | 82.53±15.05 | 5.71 | <.001 |

*Note: Values are represented as mean ± SD.*

*Effect of processing and storage on Chloride levels*

For all types of bags, there was a statistically significant ($p<.001$) difference in Mean chloride values on different days of storage.(Table 3).There was a progressive decrease in mean chloride values with increasing days of storage as shown in Figure 3.



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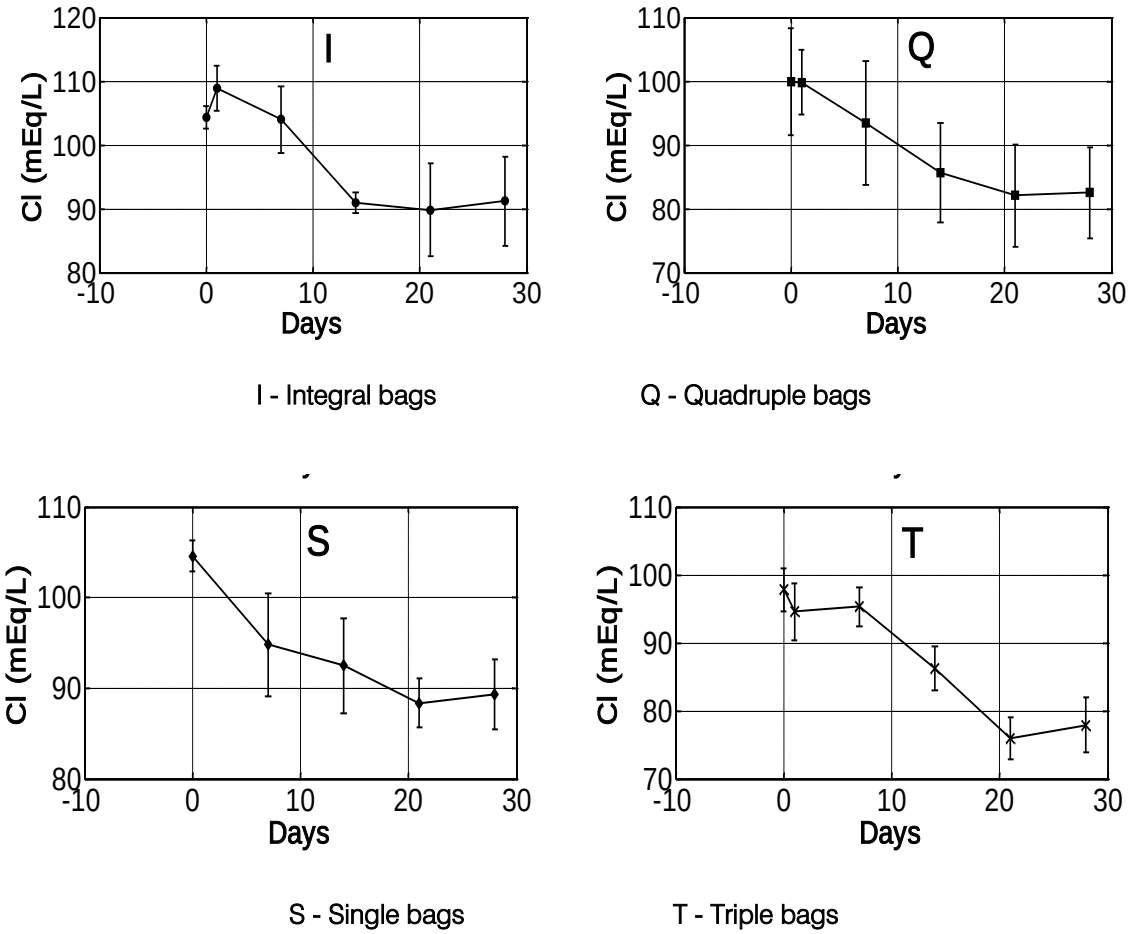



*Figure – 3:* Plasma levels of Chloride (in mmol $L^{-1}$) in various bags on different days of storage

*Table – 4:* Levels of pH in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=15) | | | | | | | |
| 7.19 ± 0.6 | | 6.91 ± 0.41 | 6.99 ± 0.61 | 6.93 ± 0.65 | 6.88 ± 0.54 | 0.75 | >.05 |
| INTEGRAL (n=13) | | | | | | | |
| 7.02 ± 0.56 | 6.98 ± 0.57 | 7.08 ± 0.49 | 6.91 ± 0.57 | 6.79 ± 0.51 | 6.73 ± 0.54 | 0.86 | >.05 |
| TRIPLE (n=52) | | | | | | | |
| 7.08 ± 0.5 | 7.05 ± 0.56 | 6.98 ± 0.57 | 6.97 ± 0.47 | 6.93 ± 0.55 | 6.81 ± 0.48 | 1.67 | >.05 |
| QUADRUPLE (n=17) | | | | | | | |
| 7.25 ± 0.48 | 7.12 ± 0.6 | 7.02 ± 0.48 | 7.14 ± 0.58 | 6.89 ± 0.43 | 6.76 ± 0.39 | 2.16 | >.05 |

*Note: Values are represented as mean ± SD.*

*Effect of processing and storage on pH levels*

For all types of bags, there was a gradual decrease in the pH, but there was no statistically significant (p>.05) difference in Mean pH values on different days of storage. (Table 4, Figure 4)



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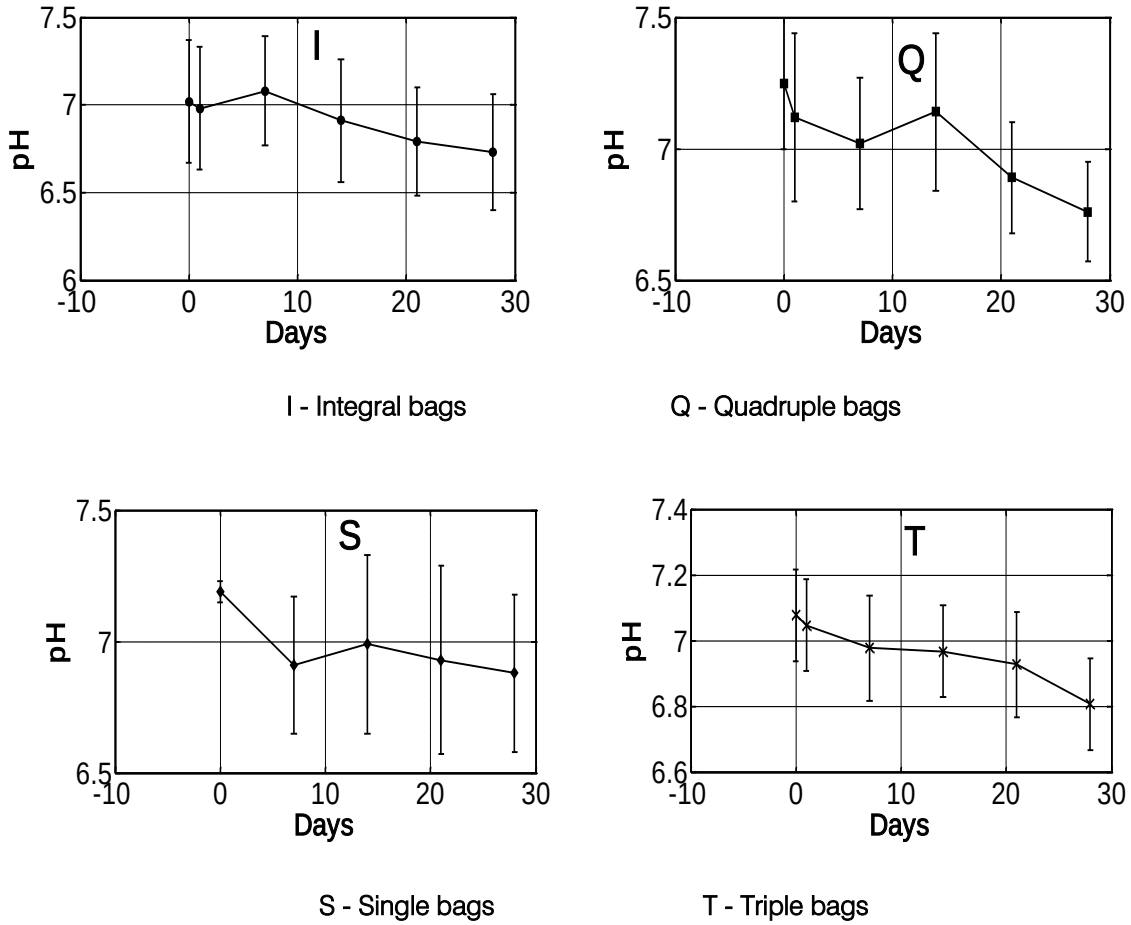



*Figure – 4:* Levels of pH in various bags on different days of storage

*Table – 5:* Levels of LDH (IU/l) in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=16) | | | | | | | |
| 936.8±659.96 | | 1332.9±619.6 | 1553.7±689.8 | 1546.6±715.9 | 1994.00±922.1 | 4.86 | <.01 |
| INTEGRAL (n=12) | | | | | | | |
| 614.6 ± 266.1 | 602.7±309.3 | 679.8 ± 481.7 | 913.7 ± 600.5 | 971.5 ± 664.9 | 993.8 ± 844.7 | 1.59 | >.05 |
| TRIPLE (n=56) | | | | | | | |
| 878.5 ± 533.2 | 1485.02±1042.3 | 1529.3±585.7 | 2036.7±738.3 | 2289.6±708.6 | 2507.7 ± 753.9 | 42.15 | <.001 |
| QUADRUPLE (n=17) | | | | | | | |
| 735.2 ± 631.4 | 582.8 ± 319.9 | 1062.9±754.2 | 1996.8±1030.9 | 1871.6±727.6 | 1969.0 ± 857.6 | 12.73 | <.001 |

*Note: Values are represented as mean ± SD.*

*Effect of processing and storage on LDH levels*

For Single bags, Triple and Quadruple bags a statistically significant difference ($p<.001$) in LDH was found across difference days of storage. There was a gradual increase in LDH with increasing days of storage. (Figure 5) No statistically significant difference ($p>.05$) difference in mean values of LDH on different days was found in case of Integral Bags.(Table 5)



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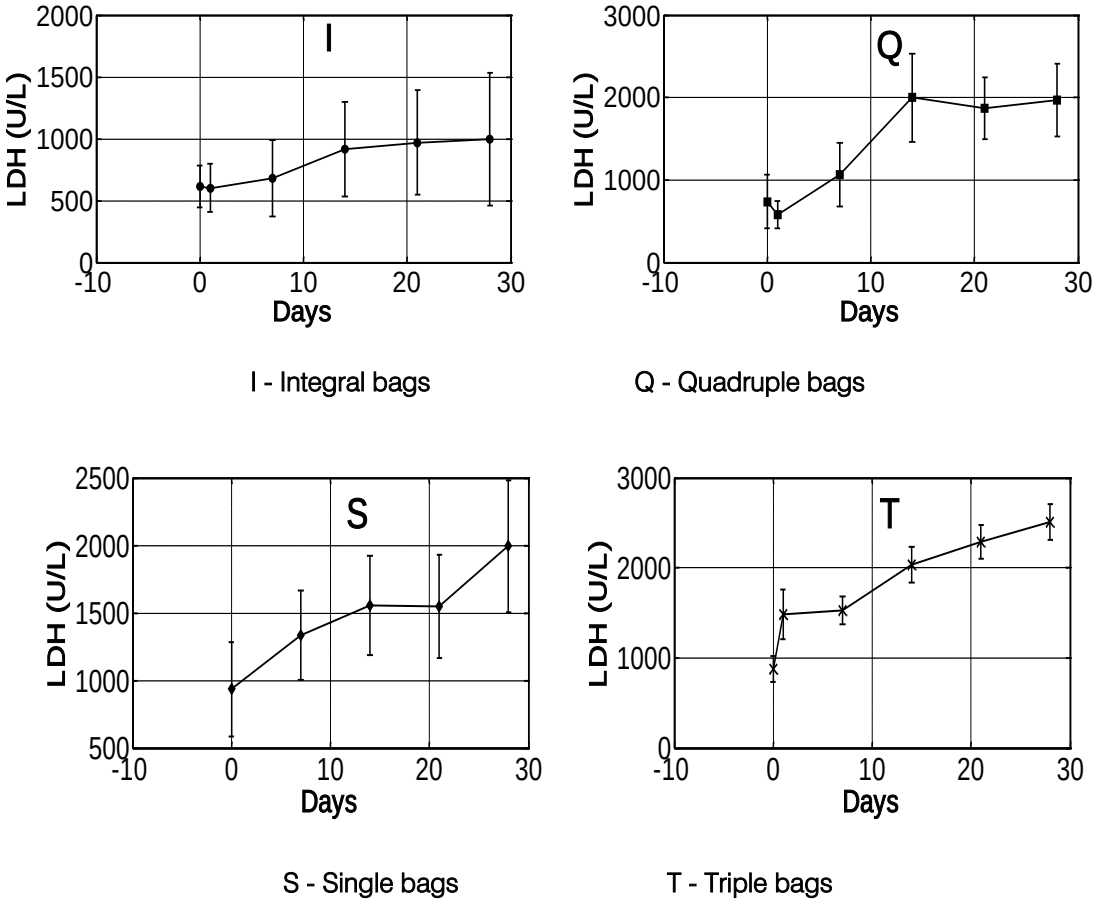



*Figure – 5:* Levels of LDH (IU/l) in various bags on different days of storage

*Table – 6:* Levels of TLC ($\times 10^9$/unit) in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=15) | | | | | | | |
| 6.32 ± 1.61 | | 5.54 ± 2.49 | 4.59 ± 1.85 | 4.13 ± 2.05 | 4.11 ± 2.14 | 3.32 | >.05 |
| INTEGRAL (n=12) | | | | | | | |
| 6.3 ± 1.3 | 0.16 ± 0.29 | 0.05 ± 0.07 | 0.04 ± 0.09 | 0.08 ± 0.12 | 0.03 ± 0.05 | 258.90 | <.001 |
| TRIPLE (n=58) | | | | | | | |
| 7.11 ± 1.91 | 9.08 ± 3.32 | 8.27 ± 3.64 | 9.08 ± 7.8 | 8.19 ± 5.42 | 8.78 ± 10.4 | 0.86 | >.05 |
| QUADRUPLE (n=18) | | | | | | | |
| 6.21 ± 1.54 | 2.51 ± 1.47 | 2.36 ± 1.76 | 2.42 ± 1.49 | 2.24 ± 1.25 | 1.86 ± 0.98 | 33.46 | <.001 |

*Note: Values are represented as mean ± SD.*

*Effect of processing and storage on Total Leukocyte count*

There was a decrease in levels with storage period as shown in Table 6 .The decrease was found to be significant for only Integral and Quadruple Bags with increasing days of storage ($p<0.001$). Figure 6 shows the decrease after processing on Day 1.



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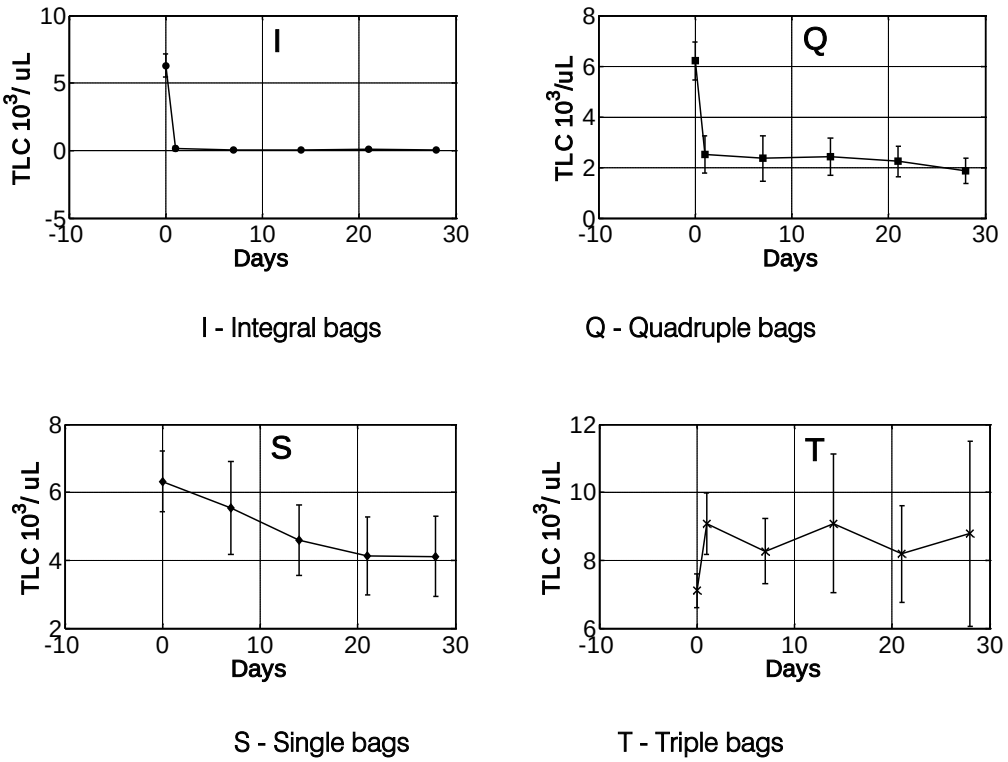



*Figure – 6:* Levels of TLC ($\times 10^9$/unit) in various bags on different days of storage

*Table- 7:* Levels of Hb (g/dL) in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=17) | | | | | | | |
| 12.08 ± 1.75 | | 9.76 ± 4.07 | 8.58 ± 4.1 | 8.08 ± 4.1 | 8.32 ± 3.81 | 3.56 | <.01 |
| INTEGRAL (n=12) | | | | | | | |
| 13.43 ± 1.11 | 18.27 ± 2.12 | 18.28 ± 1.38 | 17.66 ± 1.45 | 17.23 ± 1.95 | 16.67 ± 1.59 | 14.91 | <.001 |
| TRIPLE (n=58) | | | | | | | |
| 13.79 ± 1.65 | 16.09 ± 2.79 | 14.54 ± 3.41 | 14.55 ± 3.49 | 13.56 ± 3.16 | 12.78 ± 3.94 | 7.42 | <.001 |
| QUADRUPLE (n=17) | | | | | | | |
| 13.48 ± 1.36 | 16.87 ± 3.09 | 17.54 ± 2.26 | 17.41 ± 2.04 | 17.04 ± 2.54 | 15.73 ± 2.73 | 7.46 | <.001 |

*Note: Values are represented as mean ± SD*

*Effect of processing and storage on Haemoglobin levels*

There was a gradual decrease in levels with storage period as shown in Table 7. The decrease was found to be significant for all Integral, Triple and Quadruple groups of bags during 28 days of storage. ($p<0.001$)



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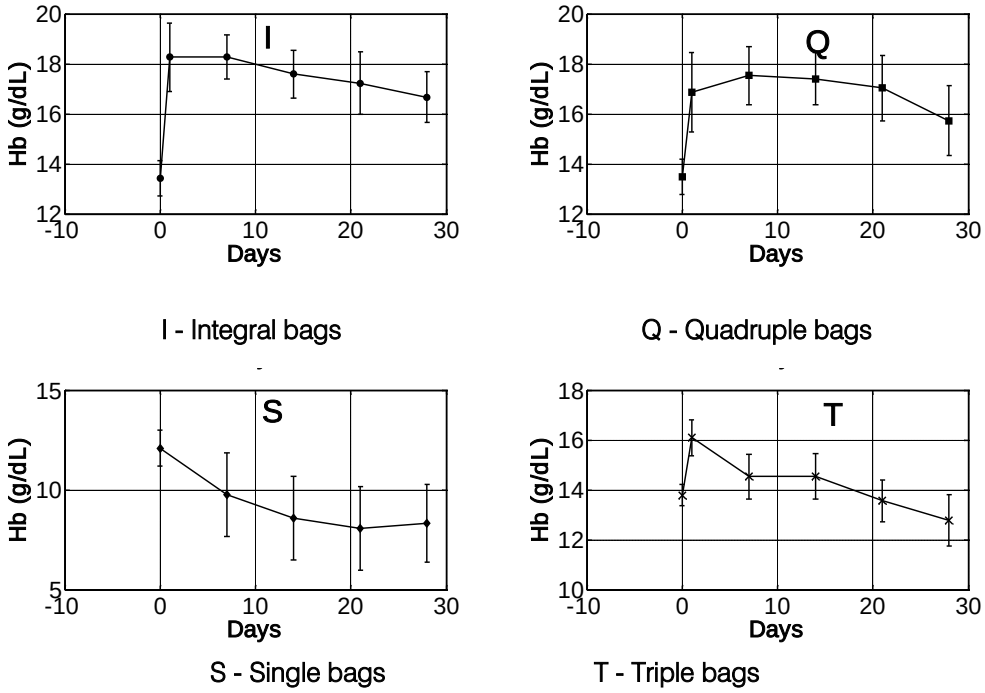



*Figure – 7:* Levels of Hb (g/dL) in various bags on different days of storage

*Table – 8:* Levels of Haematocrit in various bags on different days of storage

| Baseline Day 0 | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 | F value | ANOVA *P* |
|---|---|---|---|---|---|---|---|
| SINGLE (n=16) | | | | | | | |
| 33.28 ± 5.38 | | 29.54±11.34 | 27.4 ± 10.7 | 24.9 ± 10.8 | 25.5 ± 9.51 | 2.18 | >.05 |
| INTEGRAL (n=12) | | | | | | | |
| 37.93 ± 6.55 | 53.46 ± 12.88 | 49.90 ± 5.28 | 49.90 ± 5.25 | 52.3 ± 6.68 | 50.10 ± 7.06 | 5.41 | <.001 |
| TRIPLE (n=58) | | | | | | | |
| 37.79 ± 5.13 | 45.96 ± 8.91 | 43.12 ± 13.1 | 43.0 ± 12.64 | 41.70 ± 9.01 | 41.38±14.38 | 5.42 | <.001 |
| QUADRUPLE (n=15) | | | | | | | |
| 39.49 ± 7.73 | 52.27 ± 6.8 | 52.05 ± 5.56 | 50.70 ± 5.31 | 50.75 ± 5.27 | 44.58 ± 9.22 | 16.8 | <.001 |

*Note: Values are represented as mean ± SD.*

*Effect of processing and storage on Haematocrit levels*

There was a gradual decrease in levels with storage period as shown in Table 8. The decrease was found to be significant for all Integral, Triple and Quadruple groups of bags during 28 days of storage. ($p<0.001$)



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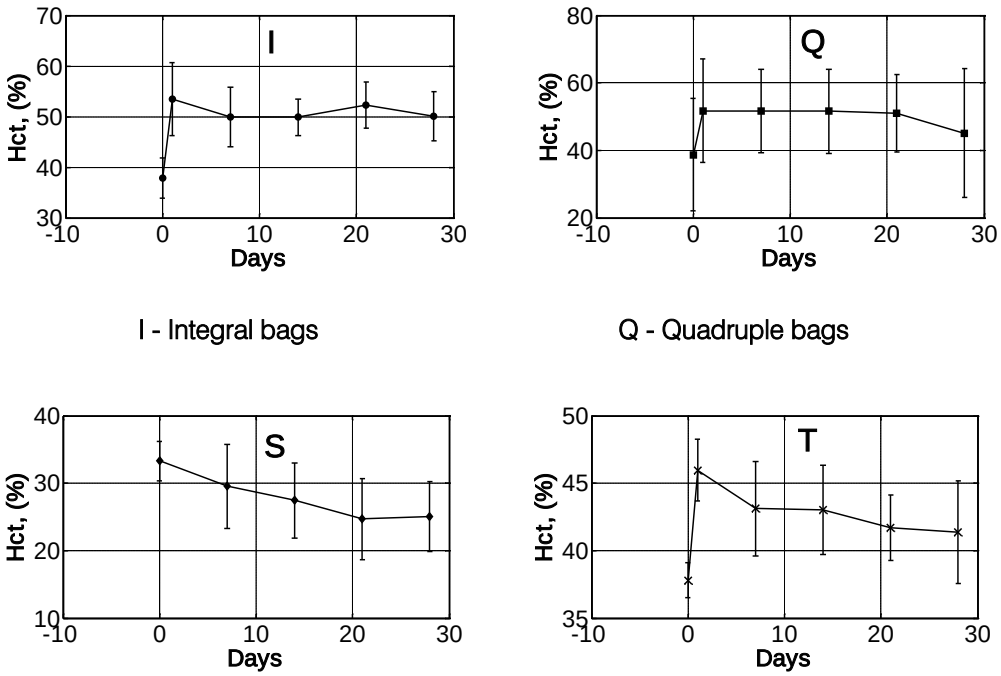



*Figure – 8:* Levels of Haematocrit in various bags on different days of storage

## VIII. Discussion

Blood banking attempts to bring the potentially life-saving benefits of transfusion to the patients who need them by making blood components available, safe, effective and cheap. Blood banks try to maximize delivering getting blood from the right donors to the right patients in a timely manner. The easiest way to assure the timely availability of blood is to have an appropriate inventory on the shelf at all times.

Standards for blood banking have evolved in response to problems observed in the past. Donors need to be free of syphilis, hepatitis, and human immunodeficiency virus (HIV) and from a host of other diseases as well. Methods for cleaning the arms of donors should work. Blood bags should contain the appropriate solutions and be sterile. Systems for the identification of donors and patients, for the determination of antigens on their blood cells and the antibodies in their sera, and for the procedures and processes used to gather and maintain this information should be robust.

Regulatory agencies attempt to assure safety by enforcing the standards noted above. They attempt to assure effectiveness by demanding demonstrations that a reasonable fraction of red cells, platelets and plasma proteins survive after storage before licensing new blood storage systems or blood modifying products like leukocyte reduction filters. Yet the reasonable fraction for red cells is 75%, for platelets is 67%, and for plasma proteins is 80%.

Better understanding of the basic biology and better tests on which to base better standards are needed.

Red blood cells are the most commonly transfused blood component. The units are collected either as whole blood into bags containing anticoagulant citrate and nutrient phosphate and dextrose (CPD) or by apheresis into acid citrate dextrose (ACD). The whole blood is centrifuged to bring down the heavier red cells, and the red cells separated from the rest of the blood. Separation is accomplished in two different ways. One way involves draining the red cells out of a port in the bottom of the bag, leaving behind a few red cells, the buffy coat of white cells and platelets, and the plasma on top. This is called the buffy coat method, and the bags that support it are called top and bottom bags. The other method involves centrifuging the blood less hard to leave many of the platelets still suspended in the plasma. In this process, making concentrated red cells involves squeezing the platelet-rich plasma off the top to leave the red cells, the buffy coat of white cells and some platelet-rich plasma behind. This is called the platelet-rich plasma method of component manufacture. Typically, the concentrated red cells are then run through a leukocyte reduction filter, which removes most white cells and platelets, and an additive solution containing more nutrients is added to support longer storage and dilute the units so that they are less viscous and flow well during emergency administration. For the apheresis units, the collection method removes most of the white cells and platelets, and the additive solution is added directly to the collected red cell concentrate.



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The platelet-rich plasma method loses and possibly damages platelets, the buffy coat method loses some red cells, and the apheresis method is expensive.

None of these methods is particularly well optimized. The use of ACD and CPD is a legacy of the days when these were the best 3-week whole blood storage solutions available. They are acidic with a pH of 5 to 5.8 so that the dextrose does not caramelize when the solutions are autoclaved. However, mixing whole blood with these acidic anticoagulants immediately drops the pH of the resulting suspension to about 7.1 leading to rapid breakdown of red cell 2,3-diphosphoglycerate (2,3-DPG). If whole blood is drawn into neutral citrate, it would preserve 2,3-DPG better and avoid exposing many intensive care and brain injured patients to the high dextrose loads that end up in conventional blood plasma. There is enough glucose in the blood of healthy donors to support the cells until the donated blood is processed into components. In the early days of blood banking, whole blood was stored for up to five days in citrate alone.

Over subsequent weeks of storage, red cells consume dextrose through glycolysis and the hexose monophosphate shunt to produce adenosine 5'-triphosphate (ATP) and reducing substances. The production of all of these metabolic intermediates goes down over the course of storage as the glycolytic end-products, organic acids and protons, accumulate. As pH falls, the protons specifically feed back to slow the rate of glycolysis. Conventional red cell additive storage solutions support stored red cells for about 6-weeks of storage but fail rapidly thereafter. Between a pH of 7 where red cell storage typically starts and 6.5 where it ends, a unit of red cells can buffer about 7 mEq of protons. Raising the pH to 7.2 at the beginning of storage with either a less acid anticoagulant or a more basic additive solution can add another 3 mEq of buffer capacity as can the addition of physiologic amounts of bicarbonate .These changes can allow the continued production of ATP for 8–9 weeks. They result in higher concentrations of ATP at all of the points in between. Higher concentrations of ATP support the actions of the enzymes that keep negatively charged phospholipids on the inside of the membrane, exclude calcium, and limit the loss of membrane in apoptotic vesicles. These actions extend potential shelf-life by extending viability.

In the U.S. and Europe, the licensure of red blood cell storage systems has been based on measures of the viability and physical integrity of the stored cells. The viability of red cells is typically measured as the fraction of cells at the end of storage that are able to circulate. Physical integrity, a necessary but not sufficient condition for viability, is measured as fractional haemolysis.

The standard measure of viability is the 24-hour in vivo recovery. In making this measurement, about 15 mL of stored red cells are labeled with chromium 51-and reinfused into the original donor. Measurement of the total administered dose allows an estimation of the volume of dilution and timed sampling at 5, 7.5, 10, 12.5, and 15 minutes allows back extrapolation to that original concentration as well. Following the subsequent concentration allows ongoing clearance to be monitored, and recoveries greater than 75% at 24 hours are considered acceptable. Current storage systems maintaining leukocyte-reduced red cells in additive solutions provide about 84 ± 8 % viability after 6 weeks storage

A major remaining problem associated with red cell storage is that viability is very different from one donor to another. Being able to identify those donors whose cells store well is potentially useful for recipients such as children with Thalassemia or sickle cell anaemia who can become iron overloaded from repeat transfusions. From a unit with poor recovery, such a child receives all of the iron but only a fraction of the anticipated useful red cells. Giving the cells with high recovery and long survival to these children reduces the burden and cost of iron chelation therapy. We deal with this problem now by giving such children fresh cells when we can, but as the number of individuals increase, this becomes more difficult. Measures that would allow a blood banker to choose the best red cells for this situation could improve care and markedly reduce health system cost.

Sawant et al have reported significant increase in plasma potassium in red cell units over 28 days of storage for all the three types of blood bags[32]. Similar findings for plasma potassium levels in red cell units were reported by Michael et al.[32] They studied the plasma potassium concentrations, unit weights, and hematocrit of 20 units of stored whole blood. 27 units of stored packed red cells, and 20 units of packed cells prepared from stored whole blood of various ages during the 21-day storage period. They found that the plasma potassium levels increased in all the three groups with increase in the period of storage. During the 21-day storage period, total plasma potassium content per unit increased in stored packed cell units at the same rate as in stored whole blood units, because plasma potassium concentration increased in stored packed cell units at three times the rate of stored whole blood units. The mean values for total plasma potassium per unit after 21 days of storage period in mEq/unit were: stored whole blood units 5.8, stored packed cells units 4.4 and packed cells units prepared from stored whole blood units 2.5.

In literature, several factors have been implicated for abnormal hemolysis in red cell units which include inappropriate handling during processing, improper storage conditions, bacterial hemolysins, antibodies causing complement mediated lysis, and red cell membrane defects or abnormality in the blood donor. Hemolysis can also occur if the ratio of blood to



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anticoagulant is not maintained during blood collection. Excess of blood with respect to anticoagulant can cause clot formation whereas excess of anticoagulant with respect to blood may damage the red cells in the collected unit. Hemolysis during processing can occur due to various reasons like delay between collection and processing, high centrifugation speed, rapid addition of additive solution to packed red cells and variation in the quality of blood storage bags. It has been shown that mechanical stress from centrifugation or agitation can cause an increase in the hemolysis of red cell units if they are stored as packed red cells or the, units are stored for longer period (near expiry date).Mechanically stressed red cell units show progressively greater, signs of red cell damage as the storage period increases. This is because it accelerates the adverse effects of storage which can cause haemolysis. There is an increase in plasma potassium levels during storage of red cell units at 4°C because of inhibition of sodium potassium-ATPase in the red cell membrane. There is diffusion of sodium into the cells with leakage of potassium into the storage medium, until a new equilibrium is established. Red cell lysis during storage also contributes to increase in plasma potassium levels in red cell units leading to increase in plasma potassium levels with increase of storage period.

For general surgical patients whose transfusions are replacement for blood lost, the extra iron helps them rebuild their own blood, but the load of effete red cells that must be cleared in the first 24 hours after the transfusion of poorly-stored blood may cause additional problems. Increased incidences of both post-operative pneumonia and metastatic cancer after transfusion are well-recognized phenomena and may in part be related to the number of non-viable red cells presented to limited clearance mechanisms. Again, a better understanding of the red cell storage lesion might improve overall blood safety.

Obvious mechanisms of the red cell storage lesion are the metabolic consequences of the increasingly acid storage environment and the oxidative injury to be associated with keeping oxygen, haeme, and iron in the same bag. Blood is collected at venous oxygen saturation, about 75%, where met-haemoglobin and superoxide generation are maximal. In healthy cells, methemoglobin reductase and superoxide dismutase are highly active, and secondary damage is limited. As stored red cells lose energy, the lifespan of these undesirable species increases with increased opportunities for secondary damage. With the exception of a few specific severe enzymatic defects that limit blood donation anyway, it is not known whose red cells are most susceptible to damage or if the addition of antioxidants such as vitamin E or n-acetyl cysteine can safely improve storage. There is a need for combined conventional storage experiments and quantitative proteomics on the red cells left in a bag after the 15 mL needed for recovery measures are removed.

*a) Red cell haemolysis*

The second standard measure in the licensure of red cell storage systems is the percent haemolysis. Haemoglobin is 98% of the non-water content of red cells, and when they rupture, haemoglobin is released into the suspending fluid. There, it can be detected as an increasingly red colour to the supernatant and measured spectrophotmetrically. In the U.S., haemolysis must be less than 1% at the end of storage and, in Europe, less than 0.8%. These numbers are arbitrary, and typical modern red cell storage systems average less than half those values.

There are three major determinants of the amount of haemolysis in any given unit of red cells. Haemolysis increases with the duration of storage, it is reduced by leukoreduction, and individual variation accounts for most of the remainder. Storage duration dependent haemolysis can be reduced by suppressing apoptosis-related microvesiculation by improving glycolytic energy flux throught the storage period. Removing most white cells early, reduces red cell damage from proteases and phospholipases released when retained white cells break down.

Haemolysis is not usually a clinical problem, but it can be for patients with ongoing intravascular haemolysis or massive transfusions. A typical unit of red cells contains about 69 gm of haemoglobin, one millimole of the 69 kD haemoglobin tetramer. Haemolysis is typically 0.4% at the end of storage, of which only half is free, outside of vesicles. Outside the red cell or the vesicles the tetramer dissociates into dimers, which in turn bind to haptoglobin dimers with 1:1 stoichiometry. Haptoglobin, normally circulates at 10–52 μM concentration in plasma, so it typically takes at least 15 units of red cells to overwhelm this system. However, patients with haemolytic anaemia may have already consumed their haptoglobin and small increases in free haemoglobin can lead to greater nitric oxide scavenging and more severe pulmonary and systemic vasoconstriction. It would be useful to be able to identify donors with very high levels of storage haemolysis.

Sawant[32] et al found that the RBC units in SAGM showed a higher level of hemolysis as compared to those in Adsol. Zimmermann[42] et al have also reported similar findings. This increased level of hemolysis in SAGM units can be attributed to the preparatory technique i.e., the PRP method in which the additive solution (SAGM) is added to the packed red cells after separation of the PRP. Sudden exposure of RBCs to additive solutions in the blood storage bags may result in either damage or lysis of the more fragile populations of RBCs due to change in osmolality. Factors relating to preparative procedures, which could have caused

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haemolysis are large variation in centrifugation speeds, rapid re-suspension of packed red cells in additive solutions and variations in blood storage bag configurations or compositions. Hogman[31] et al have reported that the type of storage containers can significantly affect RBC hemolysis during storage.

Sawant[32] in his study used the bags for storage that were made of polyvinyl chloride (PVC) with di-(2-ethylhexyl) phthalate (DEHP) as plasticizer. DEHP has been shown to decrease the rate of hemolysis during storage. Polyvinyl chloride (PVC) plasticized with DEHP is the standard material used for RBC storage bags. An extractable plasticizer like DEHP improves RBC storage by reducing hemolysis and membrane loss by microvesiculation. RBC units that are damaged during the preparative procedures and then stored in non-DEHP bags are more likely to hemolysed either during storage or further manipulation or handling. The difference in extent of hemolysis that they found in the three different blood bag types was not due to the type of plasticizer since DEHP was uniformly used in all three bag types. Zimmermann *et al*. (2003) have reported significantly lower hemolysis in RBC's in AS-1 (Adsol) compared to RBC's in SAGM. However the authors found that RBC concentrates containing SAGM from one single manufacturer had higher invitro hemolysis at the end of shelf life compared to all other manufacturers and therefore the increased hemolysis was not due to the kind of additive solution.

Improved red cell viability with Adsol causing only 0.3% hemolysis at 42 days as against 0.58% with other solutions like SAGM has been reported (Unpublished data). Studies conducted by various investigators to quantify the levels of plasma Hb in packed red cell units during storage showed significant increase in free Hb. At 26 days of storage, an unfiltered unit of packed RBC's in Adsol has plasma Hb level of 90.2 mg dl $^{-1}$ (range, 46.5 to 151.5 mg dl $^{-1)}$

Sawant reported that the RBC units stored in Adsol (TAB blood bags and BC method) had minimum haemolysis (Mean = 0.29%, Range: 0.14% to 0.5%). Maximum hemolysis (mean-0.35%, range: 0.12% to 0.7%) was associated with RBC units stored in SAGM whereas the CPDA-1 RBC units showed mean 0.31% hemolysis (range: 0.07% to 0.68%). The additive solutions SAGM-2 and Adsol have been used popularly for extended storage of RBC units. The marginal increase in hemolysis in the SAGM units could be due to the higher content of Dextrose monohydrate IP (2200 mg vs 900 mg) and Mannitol (750 mg vs 525 mg) in Adsol compared to SAGM.

The above difference in the composition of the two additive solutions might be contributory to lysis in addition to the differences in the preparatory techniques (PRP Vs BC methods). Janatpour et al have also reported higher level of hemolysis in CPDA-1 units compared to Adsol units.

During storage leukocytes break down and release a number of chemicals and enzymes such as hydrogen peroxide and proteases. These proteases released by leucocytes during storage have been reported to cause RBC lysis during storage.

There are many variables in the preparation of blood components influencing the final quality. These include the duration and force of centrifugation, the separation procedure, the configuration of blood bag sets and composition of anticoagulants and storage solutions. Therefore there is no doubt that blood components from different manufacturers differ in parameters that characterize their quality.

Although accurate evaluation of hemolysis in RBC units has relevance to transfusion recipient safety, it is also an important quality indicator of blood manufacturing processes. Hemolysis is a very important parameter for assessing the quality of stored RBC's. Hemolysis of red cell units occurs during processing for component separation and also due to repeated handling during storage, issue and transport before transfusion to the patient. The extent of hemolysis however does not exceed the permissible threshold for hemolysis up to day 28 of storage. Visual assessment of hemolysis leads to inadvertent discard of precious RBC units and therefore routine quantitative analysis for hemolysis in a blood component production setting must be done using methods like TMB or Hemocue plasma Hb analyzer. Red cell units that are likely to have excess of hemolysis for e.g., units nearing their outdate period, over-collected or under-collected units, those returned after being issued should be subjected to quantitative analysis for extent of hemolysis before a decision to discard them is taken.

Of the various methods that are available for the assessment of haemolysis, the visual method is the simplest; however the results are often inaccurate, misleading, and result in overestimation of haemolysis in red blood cells units. In a study comparing spectrophotometric method and the photometric methods, Cardigan[28] demonstrated that both methods gave comparable results. Makroo[32] RN in his study adopted the photometric method (HemoCue plasma hemoglobin analyzer) for the assessment of hemolysis.
Adias TC and Moore Igwe B[97] in their study on Storage Related Haematological and Biochemical Changes of CPDA-1 Whole Blood have observed changes in the hematological parameters that were categorized based on whether the initial days mean values were maintained when compared with other days, (below the lowest normal value), normal (within the normal range), or high (above the highest normal value), some of the hematological parameters analyzed decreased or increased. MPV and LPCR showed drastic increase from day 14 down to day 28. This agrees with the work done by Cohl[30] et al who using Coulter Gen. S. on 40 K3 (Tripotassium ethylenediamine tetra acetate) EDTA



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anticoagulated blood specimen, found that Mean Platelet volume initially increased steadily, reaching a maximum mean percent change on day 5, and on day 6 and 7, the mean percentage change had drastically increased. The collection, processing and storage of platelets for clinical use have undergone significant changes over the last few decades and novel approaches are being investigated to develop improved platelets products on new anticoagulant and additive solutions, modified storage containers and collection sets, procedures for virus and bacterial inactivation, chemical and physical methods to maintain the shelf life in development of artificial or pseudo artificial platelet products. When the mean values of WBC on day 1 was compared to day 7, it was observed that there was rapid deterioration in granulates WBC. These changes in white blood cells are most likely due to the sum effects of the loss of individual cell characteristics specifically degeneration that is known to occur as the cell ages . Clinical implications collectively known as the RBC storage lesion, is in part related to bioreactive substances released by leucocytes in the storage medium, such as histamine, lipids, and cytokines, which may exert direct effects on recipients, but many others are related to metabolic activity with the senescence, such as membrane vesiculation, decrease in cell size, increase of cell density, alteration of cytoskeleton, enzymatic desilylation and phosphatidylserine exposure. A progressive increase in red cell distribution width was noticed in this study, after comparison of the mean cell value of day 1 and 14 and this agrees with the study of Cohl et al[30]. Other hematological parameters remained fairly stable during this study period, hence may be considered acceptable for clinical utility. In this study, potassium increased within the period of 7 days and continued subsequently. The only important electrolyte change in stored blood is that of potassium. During blood storage there is a slow but constant leakage of potassium from cells into surrounding plasma. In severe kidney disease even small amount of potassium fluctuations can be dangerous and relatively fresh or washed red cells are indicated. Potassium loss is recognized to be secondary to the changes in metabolic activity with cooling. The loss of DPG and reduced glycolytic activity are also related to decreasing pH. The leakage of potassium from cells into surrounding plasma may be responsible for the drastic progression in potassium increase in this study. Sodium on the contrary reduced, suggesting that sodium in stored whole blood may produce adverse effect after transfusion. The increase in potassium value and reduction in sodium value simply indicates the preference of component therapy to whole blood transfusion. The results reported herein have significant implications for blood transfusion managers, persons who work at the transfusion centres, public health officials, and clinicians, and suggest an urgent need for the introduction of policies for safe transfusion practices and education among blood donors and staff members at the centres. This is necessary because the demand for blood in DMCH is high as a result of excessive incidence of infections that cause anaemia, malnutrition, surgical emergencies including road traffic accidents, and obstetrical emergencies associated with blood loss. Rapid degeneration of leukocytes could lead to immunodulation related to blood transfusion. Whole blood should be leukodepleted before storage if it must be used beyond one week.

Similar effects of leucoreduction have been observed by other authors in different studies. Heaton[31] et al found significantly lower hemolysis ($p < 0.05$) and plasma potassium level in the leucoreduced group compared to the non-leucoreduced group after 42 days of storage. Leucoreduced units were found to show approximately one-third hemolysis as compared to the non-leucoreduced group. AuBuchon et al also reported that hemolysis in leucoreduced units was reduced by 5 percent as compared to the non-leucoreduced units[32]. In an another study on leucoreduction done in Canada in 2005, significant differences in hemolysis and red cell osmotic fragility were noted between leucoreduced units and non- leucoreduced units from day 7 onwards. In this study, the authors observed that by the end of 42 days of storage, the leucoreduced units showed 60 percent less hemolysis than the non- leucoreduced units. Most studies ascribe improved red cell survival of leucoreduced units, to the lack of enzymes, cytokines and free radicals derived from leucocytes in such units. Studies have shown that the presence of leucocytes thus can damage the membrane of red cells resulting in potassium leakage, acceleratel glycolysis and compromised ATP preservation. The deleterious effects of leucocytes on the quality of blood had lead to leucoreduction of red cell units. Many developed nations like Canada, France, and Britain have now adopted universal leucoreduction.

Besides the storage changes described above, there are other changes in stored red cells which are documented in literature. However, these parameters could not be included in our study due to limited resources and time constraints. These include progressive decline in the levels of 2, 3-DPG and ATP. Depletion of 2, 3-DPG level in blood results in increased affinity of hemoglobin for oxygen which decreases the oxygen delivery to tissues in transfused patients. Levels of 2, 3-DPG fall quickly during the storage of red cell units, becoming undetectable within 2 weeks. However, the clinical effect is small from this dramatic fall in 2, 3-DPG level as multiple authors have failed to find a significant effect from the transfusion of red cells depleted in 2, 3 DPG. Normalization of 2, 3-DPG level begins within hours of transfusion and is completely restored within 48 to 72 hours.89, 90 The fall in ATP level is greater when blood is stored beyond 5 weeks. ATP is

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required for many activities in red cell including maintenance of Na+-K+ ATPase pump, fed cell membrane stability, glucose transport, oxidative stress defense mechanisms, membrane phospholipid distribution, and regional vasodilation under hypoxic conditions.

Hence it is known that when red cell units are stored in the blood bank due to inherent red cell storage lesions, changes occur both in red cells and the suspending medium. Though there is a spectrum of changes which takes place in red cell units during storage, the changes of clinical significance include red cell hemolysis and increased plasma potassium levels. Red cell units with high potassium content should be avoided in situations where it can be harmful to the recipients such as cardiac patients, neonates, massively transfused patients and those with renal failure. Red cell units can be washed to reduce the plasma potassium content if fresh red cell units are not available. In addition storage, handling and processing of red cell units can have an impact on red cell storage lesions. In the literature, several factors have been implicated for abnormal hemolysis in red cell units which include inappropriate handling during processing, improper storage conditions, bacterial hemolysins, antibodies causing complement mediated lysis, and red cell membrane defects or abnormality in the blood donor. The red cell units with excessive hemolysis can be harmful to the transfusion recipient. Presently most of the blood centers use visual inspection for identification of such units. But this method is highly subjective and the supernatant colour starts appearing visibly red to unaided eyes at plasma hemoglobin concentration of 40 mg/dl, which is much below 1% hemolysis. If visual inspection is used to identify units with excessive hemolysis, it would lead to unnecessary discarding of the red cell units. Therefore when excessive hemolysis of red cell units is suspected, assessment of plasma hemoglobin may be performed using quantitative methods like TMB and Hemocue plasma method.

Benefits of leucoreduction include prevention of febrile non hemolytic transfusion reactions, transmission of leucotropic viruses, and platelet refractoriness. Hence, leucoreduction offers along with above mentioned benefits, the improved quality of stored blood. Many developed nations like Canada, France, and Britain have adopted universal leucoreduction. However it may not be practically feasible to implement universal leucoreduction in a developing country like ours; hence selective leucoreduction for specific indications can be undertaken to improve the quality of stored blood.

Red cells, platelets and plasma all have important roles in medical care, high efficacy for their primary indications, and no obvious replacements in the foreseeable future. Blood collection centres and hospital transfusion services will remain largely as they are in the immediate future. There are no alternatives.

## IX. Summary and Conclusion

Despite the advancement in red cell storage solutions, there are inherent changes that occur during ex vivo preservation of blood both within the red cells and in the storage medium, these have been collectively termed as red cell storage lesions.

a) *The most salient biochemical changes occurring in stored red cell units*

In the present study we found that the plasma potassium levels increased during the 28 day storage period in all four groups ($p < 0.001$). Since the Triple CPD + SAGM bags, Quadruple and Integral CPD + SAGM bags were subjected to processing, this finding shows the effect of processing in addition to storage on these red cell units. This observation shows that gradual hemolysis of red cells takes place with increase in the storage period of red cell units.

- Effect of leucoreduction on plasma potassium levels during storage

The effect of leucoreduction on plasma potassium levels was also studied during storage.

1. For Integral Bags, highest mean potassium was found on day 28 and the rise was maximum between day 1 to day 7 of storage. The change in potassium concentration following processing from day 0 to day 1 was not significant.
2. For Quadruple Bags CPD-SAGM bags, the highest mean potassium was found on day 28 and the rise was maximum between day 7 to day 14.
3. For Triple CPD-SAGM bags, the highest mean potassium was found on day 21, followed by day 28. However the difference between the two is not statistically significant and could be due to sampling error ($p<.05$)
4. For CPDA single bags with whole blood, the highest mean potassium was found on day 28 and the rise was maximum between day 21 to 28.($p<0.001$)

Significant mean difference ($p<.001$) was found between potassium levels of leucoreduced Integral bags and Triple Bags from days 1 to day 21 with mean for Triple Bags being greater than Integral Bags. No significant difference was found on day 28.

*The most salient haematological changes occurring in stored blood units:*

1. There is no significant difference ($p>.05$) in Haematocrit for Integral CPD+SAGM Bag with leukoreduction filter from the day of processing upto Day 28.
2. There is a significant decline ($p<.05$) in Haematocrit for Quadruple CPD+SAGM Bag on day 28 as compared to mean Haematocrit on day of processing (Day 1).



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3. There is a significant decline ($p<.05$) in Haematocrit for Triple CPD+SAGM Bags from Day 21 onwards.
4. There is no significant decline in Haematocrit for Single CPD+SAGM Bags throughout the period of storage.

*The most salient changes in total leucocyte count (TLC) occurring in stored blood units:*

1. Integral CPD+SAGM bags with Leucoreduction filter show significant decline ($p<.001$) in leucocyte count following processing after day 1.
2. Quadruple CPD+SAGM bags show significant decline ($p<.001$) in leucocyte count following processing after day 1.
3. Triple CPD+SAGM bags show no significant change in TLC over 28 days of storage.
4. Single CPDA bags show no significant changes in TLC over 28 days of storage.

*To conclude and summarize the most important findings of our study*:

1. When red cell units are stored in the blood bank, due to inherent red cell storage lesions, changes occur both in red cells and the suspending medium. These storage changes increase with the duration of storage of red cell units.
2. Prestorage leucoreduced red cell units showed significantly lower plasma potassium levels as compared to non-leucoreduced red cell units. Hence, such leucoreduced units should be used to further improve the quality of stored blood.
3. It can be concluded that the fall in haematocrit with storage is least when Blood is preserved in CPD+SAGM bags with Integral Leukoreduction filters.

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# Global Journals Guidelines Handbook 2021

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# Memberships

## Fellows/Associates of Medical Research Council

### FMRC/AMRC Memberships



## Introduction

FMRC/AMRC is the most prestigious membership of Global Journals accredited by Open Association of Research Society, U.S.A (OARS). The credentials of Fellow and Associate designations signify that the researcher has gained the knowledge of the fundamental and high-level concepts, and is a subject matter expert, proficient in an expertise course covering the professional code of conduct, and follows recognized standards of practice. The credentials are designated only to the researchers, scientists, and professionals that have been selected by a rigorous process by our Editorial Board and Management Board.

Associates of FMRC/AMRC are scientists and researchers from around the world are working on projects/researches that have huge potentials. Members support Global Journals' mission to advance technology for humanity and the profession.

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FELLOW OF MEDICAL RESEARCH COUNCIL is the most prestigious membership of Global Journals. It is an award and membership granted to individuals that the Open Association of Research Society judges to have made a 'substantial contribution to the improvement of computer science, technology, and electronics engineering.

The primary objective is to recognize the leaders in research and scientific fields of the current era with a global perspective and to create a channel between them and other researchers for better exposure and knowledge sharing. Members are most eminent scientists, engineers, and technologists from all across the world. Fellows are elected for life through a peer review process on the basis of excellence in the respective domain. There is no limit on the number of new nominations made in any year. Each year, the Open Association of Research Society elect up to 12 new Fellow Members.

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Career | Credibility | Exclusive | Reputation




## Certificate

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Career | Credibility | Reputation



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| Associate | Fellow | Research Group | Basic |
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| **Certificate,** LoR and Momento<br>**2** discounted publishing/year<br>**Gradation** of Research<br>**10** research contacts/day<br>**1 GB** Cloud Storage<br>**GJ** Community Access | **Certificate,** LoR and Momento<br>**Unlimited** discounted publishing/year<br>**Gradation** of Research<br>**Unlimited** research contacts/day<br>**5 GB** Cloud Storage<br>**Online Presense** Assistance<br>**GJ** Community Access | **Certificates,** LoRs and Momentos<br>**Unlimited** free publishing/year<br>**Gradation** of Research<br>**Unlimited** research contacts/day<br>**Unlimited** Cloud Storage<br>**Online Presense** Assistance<br>**GJ** Community Access | **GJ** Community Access |

# Preferred Author Guidelines

**We accept the manuscript submissions in any standard (generic) format.**

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3. Ensure corresponding author's email address and postal address are accurate and reachable.
4. Manuscript to be submitted must include keywords, an abstract, a paper title, co-author(s') names and details (email address, name, phone number, and institution), figures and illustrations in vector format including appropriate captions, tables, including titles and footnotes, a conclusion, results, acknowledgments and references.
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- Findings
- Writings
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- Any other original work

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3. Final approval of the version of the paper to be published.

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**Acknowledgments**

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The following is the official style and template developed for publication of a research paper. Authors are not required to follow this style during the submission of the paper. It is just for reference purposes.

***Manuscript Style Instruction (Optional)***

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swis721 Lt BT.
- Page size: 8.27" x 11'", left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

***Structure and Format of Manuscript***

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.



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***It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.***

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The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

**Author details**

The full postal address of any related author(s) must be specified.

**Abstract**

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

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A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

**Numerical Methods**

Numerical methods used should be transparent and, where appropriate, supported by references.

**Abbreviations**

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

**Formulas and equations**

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

**Tables, Figures, and Figure Legends**

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

**Figures**

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

## Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

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***1. Choosing the topic:*** In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

***2. Think like evaluators:*** If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

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***9. Produce good diagrams of your own:*** Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

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***12. Know what you know:*** Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

***13. Use good grammar:*** Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

***14. Arrangement of information:*** Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

***15. Never start at the last minute:*** Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

***16. Multitasking in research is not good:*** Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

***17. Never copy others' work:*** Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

***18. Go to seminars:*** Attend seminars if the topic is relevant to your research area. Utilize all your resources.

***19. Refresh your mind after intervals:*** Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

***20. Think technically:*** Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

***21. Adding unnecessary information:*** Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

***22. Report concluded results:*** Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

***23. Upon conclusion:*** Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

## Informal Guidelines of Research Paper Writing

***Key points to remember:***

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

**Final points:**

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

*The introduction:* This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

**The discussion section:**

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

**General style:**

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

**To make a paper clear:** Adhere to recommended page limits.



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*Mistakes to avoid:*

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

**Title page:**

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

**Abstract:** This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

*Reason for writing the article—theory, overall issue, purpose.*

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

**Approach:**

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

**Introduction:**

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

*The following approach can create a valuable beginning:*

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

**Approach:**

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

**Procedures (methods and materials):**

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

**Materials:**

*Materials may be reported in part of a section or else they may be recognized along with your measures.*

**Methods:**

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

**Approach:**

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

**What to keep away from:**

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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**Results:**

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

**Content:**

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

**What to stay away from:**

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

**Approach:**

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

**Figures and tables:**

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

**Discussion:**

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

**Approach:**

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

## The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

*Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection*.

*Segment draft and final research paper:* You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

*Written material:* You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

## Criterion for Grading a Research Paper (Compilation) by Global Journals

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| Topics | Grades | | |
|---|---|---|---|
| | **A-B** | **C-D** | **E-F** |
| ***Abstract*** | Clear and concise with appropriate content, Correct format. 200 words or below | Unclear summary and no specific data, Incorrect form Above 200 words | No specific data with ambiguous information<br>Above 250 words |
| ***Introduction*** | Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited | Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter | Out of place depth and content, hazy format |
| ***Methods and Procedures*** | Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads | Difficult to comprehend with embarrassed text, too much explanation but completed | Incorrect and unorganized structure with hazy meaning |
| ***Result*** | Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake | Complete and embarrassed text, difficult to comprehend | Irregular format with wrong facts and figures |
| ***Discussion*** | Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited | Wordy, unclear conclusion, spurious | Conclusion is not cited, unorganized, difficult to comprehend |
| ***References*** | Complete and correct format, well organized | Beside the point, Incomplete | Wrong format and structuring |

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