The effects of acetylcarnitine on the prevention of platelet storage lesions

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Abstract

Platelet storage lesion is the degradation that platelets experience when stored in an external environment, causing a loss in platelet viability and leading to a shelf life in platelets. This study aims to prevent platelet storage lesion by using preservatives to slow down its effects. The main aim of the study is to compare the effects of l-carnitine, an alternative preservative that lengthens platelet shelf-life from seven (7) to ten (10) days, with its derivative acetylcarnitine in respect to pH, platelet count, platelet volume, and platelet shape change. Three blood bags were used and separated into three setups, with either addition of l-carnitine, acetyl carnitine or saline (control) and stored at 22°C. Platelet count and mean platelet volume were determined by the use of an ABX Micros ES 60 hema-analyzer, and pH was determined by using a pH meter. Platelet morphology was observed by collecting stained samples and observed under a microscope, where regular and irregular platelets were recorded. Due to an anomaly, platelet count was not included in the analysis. One-way analysis of variance for mean platelet volume, pH and morphology did not show significant difference ($p>0.05$) among the setups, showing that further studies should be done to determine if acetylcarnitine might be a possible preservative for platelets.

Keywords: *Platelet storage lesion, L-carnitine, Acetylcarnitine, Preservatives, Platelet viability*

Introduction. Platelets are the blood cells that are responsible for blood clotting and the sealing of injuries. They are fragments that are created from the division of megakaryocytes [1]. They are indispensable to patients that require large amounts of platelets, such as in surgeries, accidents, and illness. But due to the short shelf-life of platelets, constant donations must be done. Platelets are highly fragile and cannot be stored using the same methods as other parts of blood such as being frozen [2]. Platelets are stored via a platelet bag after extraction, where preservatives are added. They are then placed in an agitator to induce constant motion needed for them not to aggregate, as well as to provide a constant circulation of air through the semi-permeable blood bags [3].

Platelet storage lesion (PSL) is an umbrella term that refers to the progressive changes that occur in platelets during storage, leading to platelet degradation. PSL have been observed to be caused by platelet activation during the preparation and storage of the platelet concentrates (PC). It has also been shown that lactate accumulation plays a major role in the formation of PSL [4]. Platelet storage lesion significantly affects platelets' functional integrity, along with their ability to recover and survive. PSL covers changes in shape, functionality, pH, release of granule content, and decrease in agonist-induced aggregation. It is also responsible for shortening the shelf-life of platelets to 5-7 days [5].

L-carnitine is a potentially good prospect. Multiple researches back its potential as a good blood preservative [5,6,7] to be added along with CPDA-1. When platelets undergo glycolysis, the byproduct pyruvate increases the acidity of the medium, contributing to platelet storage lesion [8]. L-carnitine changes the metabolic pathway of the platelets from glycolysis to ß-oxidation, a pathway that uses fatty acids instead of glucose, which slows down the acidification of the medium [5,9]. This allows it to extend the shelf life of platelets to a longer extent when added to the composition of CPDA-1. The research opted to follow the procedures of the closest related study [4].

A derivative of l-carnitine, acetylcarnitine (ALCAR, or Acetyl l-carnitine) is most commonly used to treat neurological diseases [6] and has been observed to give anti-aging abilities to cells [7]. Acetylcarnitine is the acetylated version of l-carnitine, and by structure can be said to be quite similar in function. It has been shown to have great benefits on metabolism concerning fat transfer [7]. Acetylcarnitine's preservation qualities upon blood, specifically platelets, have not been thoroughly tested. The proposed study aims to determine whether acetylcarnitine can achieve the same quality and length of shelf-life as l-carnitine. As its preservative capabilities have not yet been fully tested [6], the research tests to see if it also has similar preservative capabilities as l-carnitine.

The study aims to determine the effects of acetylcarnitine as preservative against platelet storage lesions in comparison to l-carnitine. It specifically aims to:

(i) Determine the following parameters with respect to platelet concentrates treated with 1mL of 15mM acetylcarnitine, saline as the negative control, and l-carnitine as the positive control at days 0 (before treatment), 3 and 5 after application of preservatives.

(ii) Compare mean change (with respect to Day 0) of each of the following parameters at days 3 and 5 among the different treatments and control.

Methods. The study is an experimental procedure that would require three set ups: platelets treated with acetylcarnitine, l-carnitine (positive control) and saline (negative control) with three replicates for each setup. All blood platelet samples were applied with the standard CPDA-1 preservative. Other variables were held constant and the only manipulated variable is the type of additive to be used (l-carnitine, acetylcarnitine, and saline). The pH, platelet count and volume of the different samples and sets were then compared to see which one is significantly more effective after a storage time of five days.

Sampling. The blood samples were acquired from the blood bank at the start of day 0. The blood bank ensured that no contaminants or pathogens that can affect the results were present in the samples by analysis of Transfusion Transmitted Infections (TTIs). In order to ensure confidentiality, no name, age, sex, or any other personal data of the donor was given to the researchers.

Preparation of Preservatives. L-carnitine solution was prepared by dissolving the powder form of l-carnitine in sterile, normal saline at a concentration of 15 mM. A volume of 1 mL l-carnitine solution was added to each setup [5]. Acetylcarnitine was prepared and added to the variable setup the same way as l-carnitine. The preservatives were added to the PC one day after blood extraction. 15 mM was the chosen concentration based on a pilot study of Deyhim et al. [5] in determining the best concentration and volume of l-carnitine in preserving PC.

Separation of setups. Each platelet concentrate was used for one replicate of each setup. Blood extraction was done on a quadruple bag to enable the use of three setups:

Before storage in the agitator, the PC was separated into three parts. The PC in the first satellite bag was transferred and equally divided to all the satellite bags and checked with a weighing scale. Approximately 60 mL PC each was transferred to both the second and the third satellite bags. The first bag became the negative control setup, the second bag the positive control and the third for the acetylcarnitine variable setup. The primary bag was used to contain unwanted red blood cells and platelet-poor plasma, which was returned to the blood bank.

The negative control setup consisted of the PC and added 1 mL saline solution. The positive control setup consisted of the PC and 1 mL of 15 mM l-carnitine.

Extraction. Each donor had one 450 mL of blood extracted for platelets by a phlebotomist in a triple bag. Citrate Phosphate Dextrose Adenine version 1 (CPDA-1), already contained in the blood bag upon purchase (standard for platelet bags to contain preservative), was used as an anticoagulant after blood extraction. The platelets were then extracted by a medical technologist via the platelet-rich plasma method of extraction to obtain the platelet concentrate.

After extraction, the blood unit was subjected to a soft spin of 110 rpm for 15 minutes using a large, specialized centrifuge machine. Afterwards, the platelet-rich plasma was collected and transferred to the first satellite bag then subjected to a hard spin of 1000 rpm for 15 minutes [25]. Both spins were conducted in a blood centrifuge at 20-24oC. Separation of platelet concentrate from platelet-poor plasma was done using a plasma separator by transferring the platelet-poor plasma to the primary bag. 60-70 mL of PC was obtained from the procedure, which was then used for one replicate. The obtained PCs were then further tested by medical technicians to see whether the platelets are not infected with any disease (i.e. malaria, AIDS, hepatitis, etc.). The healthy PC were then stored at 20-24°C with constant gentle agitation using a platelet agitator.

Storage and preservation. CPDA-1 would have already been added during blood extraction (contained in the blood bag) at around 63 mL per blood unit in order to preserve the platelets and prevent coagulation. The samples were then stored at a volume of 20 mL at 20-24°C with constant gentle agitation in their respective satellite bags. Three replicates were prepared and each replicate consisted of the three setups: l-carnitine (positive control), saline (negative control), and acetylcarnitine.

Application of preservatives. Preservatives and saline were introduced after one day of storage. Aseptic infusion is necessary so addition of preservatives and control was done under class II laminar flow using an insulin syringe through the blood bag tube. The site of puncture was then applied a stripper to mix the preservative with the blood bag contents and heat sealed.

Biosafety cabinet level II is located in another nearby hospital from the site of storage, so transportation of samples was done using an approved Styrofoam box and transported back after application of preservatives.

Quantitative Analysis of samples. After extraction, the initial platelet count and platelet volume was recorded using a hema-analyzer (ABX Micros ES 60). Platelet concentrate pH were then analyzed using a pH meter. Subsequent recordings of platelet count, platelet volume, and platelet pH were done on the third and fifth days. Recording was done three times per analysis in order to see a more accurate data.

Qualitative Analysis of samples. Platelet morphology was analyzed by photographing microscope smears of the setup and manually counting the ratio of activated platelets to the total number of platelets. For a qualitative comparison, microscopic analysis of the samples involving the shape and concentration of platelet change was also done to show their shape and configurations. The platelets would be viewed manually and it would be decided if the platelets were irregular or discoid based on the parameters of a functioning discoid platelet. Discoid and irregular shapes were noted among the platelets. Regular and irregular platelets were counted manually on different parts of the slide. The irregular shaped platelets were then divided over the total number of platelets to get their percentage.

Data analysis. Since there were three setups to be analyzed, the one-way ANOVA statistical tool was used. All four parameters of the three setups were analyzed via one-way ANOVA. If there was variance among the four setups, post-hoc tests would have been conducted to see which of the setups have differences.

Safety procedure. During the conduct of the research, personal protective equipment was worn at all times to prevent contamination of the samples. After the experiments, blood plasma and instruments in contact with them were treated as a biohazard and disposed off accordingly.

Results and Discussion. The results showed that all three setups have almost the same parameters, with p-values above the critical range of 0.05 on platelet count, mean platelet volume, and pH, and morphology. Overall, the present results cannot confirm acetylcarnitine to be a viable candidate as a platelet additive.

Platelet count initially had a low count on the first analysis, but more than doubled on the second test three days after. This data showed up as a consistent anomaly, appearing as well during the preliminary studies. It is unknown exactly why this occurs in the experiment. So far, no related research has been found to explain this phenomenon, therefore the data cannot be analyzed. It is possible that this may be due to the measurement on the first day, where platelet count was always low even during preliminary experiments.

Mean platelet volume measurements were taken at the same time platelet count was being measured. The results showed a trend in which the volume of the platelets gradually increased during subsequent analyses. Similar to platelet count, statistical analysis showed that the results are statistically insignificant, with p-values of 0.12, 0.24, and 0.30 on the first, second, and third measurements respectively.

Platelet pH did not change much during the duration, only dropping 0.02 at most at any given sample. They do, however, slightly decreased throughout the duration. Statistical analyses showed insignificant results, with p-values of 0.12, 0.22, and 0.70 on the first, second, and third measurements.

In the research that was undertaken, the factors that were focused on included the acidity of the platelet medium and the metabolic activity of the platelets. With the current technology used for platelet storage, much is being looked into the metabolic activity of the platelets. In the body, platelets derive up to 80% of their energy through beta-oxidation [5]. The energy from beta-oxidation comes from fatty acids, reducing the reliance on glucose. Glucose is important in platelet metabolism, as prevention of glucose intake into the platelets decreased platelet production and platelet activation in mice. In vitro, however, requires different mechanisms. Prevention of platelet activation is necessary for long term storage and increased platelet viability. Platelet metabolism shifts to glucose during storage, increasing lactate concentration, which is a byproduct of the metabolic pathway. This accumulation leads to the decrease of plasma pH, inducing platelet activation.

The researches of Deyhim et al. [5] and Sweeney et al. [7] used l-carnitine to prolong platelet shelf-life. By switching the metabolism of platelets from depending on glucose to fatty acids, the metabolites that are produced during glucose metabolism, primarily lactate, is eliminated. This change can help prevent the lowering of the pH inside the blood bag, minimizing platelet storage lesion. The study that was conducted followed these observations and tested to see if another chemical such as acetylcarnitine could be capable of obtaining similar results. The final data showed that although there is insignificant difference in comparison to L-carnitine, acetylcarnitine was shown to be safe in being used in platelets, as shown in the parameters. A factor that is relevant in this research is time, as shown that the results of platelet count and volume of Deyhim et al. [5] were significant only on the last day of measurement, and pH showing insignificant results. More significant results could have been obtained should the duration of the study have been increased.

Mean platelet volume was measured similarly to the platelet count by running a sample of 1µl through the hemoanalyzer three times and taking the average. Successive measurements were then taken on the third and fifth days of storage. One-way ANOVA was used to determine if there is significant difference among the samples.

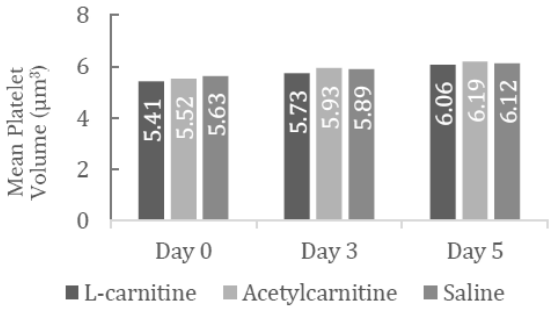

Figure 1. Mean platelet volume at different days of storage in L-carnitine (blue), Acetylcarnitine (red), and Saline (yellow/control) treatments.

Mean platelet volume of the acetylcarnitine setup was highest among the last two measurements during preservation. L-carnitine has consistently the lowest values among the three in all measurements. The data showed a trend where mean platelet volume gradually increased the longer the duration of the storage.

Statistical analysis of the mean showed no significant difference for the whole duration of storage ($p>0.05$). This is true for all three days of measurement. This result determines that there is no significant difference in mean platelet volume among all the samples.

The platelet pH was tested using a pH meter by sampling a 10 ml volume of the platelets and washing the bulb after every measurement. Successive measurements were then taken on the third and fifth days of storage. One-way ANOVA was used to determine if there is significant difference among the

samples.

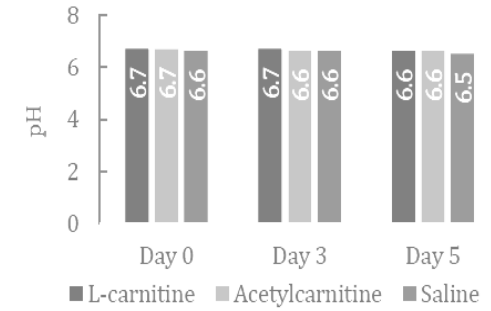

Figure 2. Platelet pH at different days of storage in L-carnitine (blue), Acetylcarnitine (red), and Saline (yellow/control) treatments.

pH values stayed almost constant throughout the entire observation period. L-carnitine dropped from 6.7 to 6.6 on the last measurement while acetylcarnitine dropped from 6.7 to 6.6 on the second day of measurement. Saline fluctuated from 6.5 to 6.6 on the second measurement and back to 6.5 again on the final measurement.

The average results of the pH showed that no significant differences are observed among the setups during the entire observation period ($p>0.05$).

Platelet morphology was analyzed by photographing microscope smears of the setup and manually counting the percentage of activated platelets to the total number of platelets. Platelets are considered to be activated if the cytoplasm seemed dispersed and granules are concentrated in the center. The morphology of the platelets was measured by eye and the activated platelets were counted with a mechanical counter. One-way ANOVA was used to determine if there is significant difference among the slides.

Saline had the highest rate of irregular platelets during the first and second measurements. Although there is an increasing trend in the l-carnitine and acetylcarnitine setups, saline decreased in irregular platelets in comparison with the other two setups during the final measurement where it reached 30.67% irregular platelets.

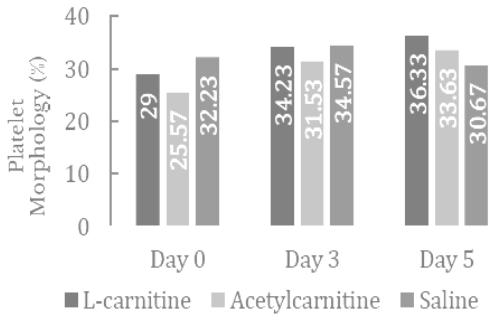

Figure 3. Mean platelet morphology at different days of storage in L-carnitine (blue), Acetylcarnitine (red), and Saline (yellow/control) treatments. Percent values acquired as a ratio of irregular cells over total number of cells.

There is no significant difference among the setup ratios during the final observation period at ($p>0.05$). This result determines that there is no significant difference in the number of irregular platelets among the samples.

Error analysis. Errors in data gathering can stem from human error in the handling of the procedures, such as the accidental swapping of samples during the analysis and inaccurate counting of platelets in the microscope. The data gathered is also limited by the capabilities of the measuring instruments. More accurate data would also require better equipment, which is beyond the financial capabilities of the researchers.

Conclusion. All three valid parameters showed no significant results during the entire observation period. It can thus be concluded that not enough information is provided to say that acetylcarnitine is more effective than l-carnitine in furthering the preservation time of platelets. The results show however that acetylcarnitine is safe and can be a possible additive for platelet preservation in the future. It is not harmful to the platelet samples and is similar to the results of l-carnitine.

Recommendations. Due to issues of time constraints and gaining access and usage of venues, the original goal of the research, which was to assess the preservative properties of acetylcarnitine in comparison to that of l-carnitine, was changed to the verification of the viability of acetylcarnitine as a preservative in the first place. This caused a change in the time span of data gathering from seven (7) days to five (5) days only, the standard preservation shelf life of platelets. It is recommended that this study be followed up so as to verify the initial goal of the capabilities of acetylcarnitine by adding a longer experimentation period. Finally, there existed a trend in the platelet count where the initial measurements were low in comparison to subsequent measurements. A potential study can also be conducted regarding that matter.

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