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The effects of acetyl l-carnitine on the prevention of platelet storage lesions

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Abstract

Platelet storage lesion is the degradation of platelets when stored in an external environment, causing a loss in platelet viability, in turn, causing their low availability. The aim of the study is to compare the effects of l-carnitine with its derivative acetyl l-carnitine with respect to platelet count, mean platelet volume, pH, and platelet shape change. Three blood bags were used and separated into three setups: l-carnitine, acetyl l-carnitine, and saline, and were stored at 20 to 24°C. Platelet count and mean platelet volume were determined by using a hema-analyzer, and pH was determined by using a pH meter. Platelet morphology was assessed by collecting stained samples and observed under a microscope, where regular and irregular platelets were recorded. One-way ANOVA analysis for mean platelet volume, pH and morphology did not show any significant difference ($p>0.05$) among the setups, indicating that there is insufficient evidence to conclude that acetyl l-carnitine can be used as a platelet preservative.

Keywords: *platelet storage lesions, platelet preservation, l-carnitine, acetyl l-carnitine, platelet storage*

Introduction. Platelet storage lesion (PSL) is a term that covers the progressive degradation of platelets in storage [1]. This degradation is seen with the lowering of pH, platelet count and volume, and changes in platelet morphology, and accordingly there is a significant societal motivation to decrease PSLs. Whereas current platelet-storage protocols are deemed adequate, there are nevertheless societal gains to be achieved by examining alternatives. These gains include cost-per-day-of-storage, collection and disposal costs, and the associated administrative costs.

PSLs cause platelet metabolism inadequacy thus reducing the efficiency of intracellular metabolism. This further causes the platelet's shelf-life to decrease to only five to seven days [2]. The true cause of PSL has not been clearly understood; however, it has been observed to be linked to lactic acid accumulation and platelet aggregation [3]. Additionally, it has been shown that heightened metabolic activity within the platelet contributes to the production of PSLs [3].

There is research on preserving platelets using preservatives, one being l-carnitine. L-carnitine is a common ergogenic acid due to its importance in the conversion of fat into energy [4]. It can change the metabolic pathway in platelet mitochondria from glycolysis to ß-oxidation, which uses fatty acids instead of glucose to make energy available at the cellular level [1]. The use of fatty acids instead of carbohydrate complexes lowers lactic acid accumulation and thus lowers the chance of PSLs occurring in the platelets. Studies have shown that l-carnitine is a significantly effective platelet preservative [1,5] and its derivative, acetyl l-carnitine (ALCAR), has its uses in the medical field. ALCAR is also able to metabolize fats to make energy available at the cellular level; however, it is mainly used to treat neurological diseases such as cerebral ischemia [6], a condition wherein there is a reduction in the supply of blood to the brain. ALCAR is vital for mitochondrial lipid transport which is important for the mitochondria's function [7]. Additionally, ALCAR has anti-aging abilities for cells [7,8] and is more effective than l-carnitine against oxidative stress [9]—the excess of production of free radicals vis-à-vis the body's capacity to neutralize them— which is a factor in producing PSLs. L-carnitine itself has been proven to be a successful platelet preservative; however, further research is needed to determine whether its derivatives exhibit the same results [5].

ALCAR is less expensive than l-carnitine; but, that alone does not signal efficacy. The motivation of researching ALCAR is to determine whether decreased per-unit application cost is dominated by shorter storage periods or increased PSLs or a combination thereof.

The study aimed to determine the effects of ALCAR as a preservative against PSLs in comparison to the tested l-carnitine. It specifically aimed to:

(i) determine platelet count, mean platelet volume, platelet pH, and platelet morphology with respect to platelet concentrates treated with 1mL each of 15mM ALCAR, saline as the negative control, and l-carnitine as the positive control at Days 0 (before treatment), 3 and 5 after application of preservatives; and

(ii) compare mean change (with respect to Day 0) of platelet count, mean platelet volume, platelet pH, and platelet morphology at Days 0, 3, and 5 among the different treatments and control.

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Methods. The study aimed to determine if ALCAR is an effective platelet preservative and to compare its efficacy with l-carnitine. Platelet count, mean platelet volume, platelet pH, and platelet morphology was observed and analyzed to determine the efficacy of l-carnitine and ALCAR. Platelet samples were obtained and separated into three blood bags to be treated with ALCAR, l-carnitine, and saline for five days. The first three parameters were measured on Days 0, 3, and 5 using a hema-analyzer and platelet morphology was observed on Day 5.

Materials. Platelet concentrates (PCs) was isolated from whole blood using a large, specialized centrifuge at a soft spin of 110 rpm then a hard spin of 1000 rpm both for 15 minutes at 20-24 °C . The platelets were separated into three bags and stored at 20-24 °C. Citrate Phosphate Dextrose Adenine (CPDA-1) was already introduced inside the blood bag upon being purchased as it is the standard chemical which lines the bag specifically to work as a preservative. L-carnitine and ALCAR, obtained from in powdered form from Now Foods and iHerb respectively, and were each dissolved in sterile normal saline to produce 1 mL of 15 mM of l-carnitine and ALCAR solutions. After PC extraction, the preservatives were thoroughly mixed with the PCs by gently shaking the blood bag. The local blood bank that assisted with the study by supplying healthy blood samples, equipment, and guidance requested not to be named in the research paper for confidentiality purposes. Models and other specific settings of equipment used are not mentioned under the blood bank's request.

Sampling. Blood samples were acquired from a local blood bank agency at Day 0. From each of the three donors, 450 mL of blood was extracted by a phlebotomist in a triple bag. CPDA-1 contained in the bag was used as an anticoagulant after blood extraction. 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.

Extraction of Platelet Concentrate. The platelets were extracted by a medical technologist via the platelet-rich plasma method to obtain the PCs.

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. Both spins were conducted in a blood centrifuge at 20-24 °C. Separation of PC from platelet-poor plasma was done using a plasma separator by transferring the platelet-poor plasma to the primary bag. PCs with a volume of 60-70 mL was obtained from the procedure, to be used for one replicate each. The obtained PCs were tested by medical technicians to see if the platelets were infected with any disease (i.e. malaria, AIDS, hepatitis, etc.). The healthy PCs were stored at 20-24 °C with constant gentle agitation using a platelet agitator.

Separation of Setups. Each PC was used for one replicate of each setup. Blood extraction was done on a quadruple bag to enable the use of three setups: ALCAR, l-carnitine, and saline.

Before storage in the agitator, the PC was separated into three parts, each approximately at a volume of 60mL. The PC in the first satellite bag was transferred and equally divided to all three satellite bags. 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 the positive control and the third for the ALCAR 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 with 1mL saline solution. The positive control setup consisted of the PC and 1mL of 15mM l-carnitine.

Storage and Preservation. CPDA-1 has been added during blood extraction at around 63 mL per blood unit in order to prevent coagulation. The samples were 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 ALCAR.

The chosen concentration for the preservatives used in the study was 15 mM based on a pilot study of Deyhim et al. [1] in determining the best concentration and volume of l-carnitine in preserving PCs.

L-carnitine solution was prepared by dissolving the l-carnitine powder in sterile, normal saline at a concentration of 15 mM. A volume of 1 mL l-carnitine solution was added to the positive control setup [1]. ALCAR was prepared and added to the variable setup the same way as l-carnitine. The preservatives were added to the PCs one day after blood extraction.

One mL of l-carnitine and ALCAR each were introduced into the platelet bag via aseptic infusion one day after extraction. Insulin syringes were used after filtering the preservative solution through a 0.22 μm filter. Sterile normal saline was used in order to dissolve the l-carnitine and ALCAR powders. As a control, an equal volume of 1 mL of saline was also added to the third setup. The site of puncture was sealed and a stripper was used to mix the preservative with the blood bag contents. A biosafety cabinet level II located in a nearby hospital from the site of storage was used. Transportation of samples was done using an approved Styrofoam box and was returned after application of preservatives.

Data Gathering. After extraction, platelet count and mean platelet volume were measured by running a sample of 1mL through the hema-analyzer three times and taking the average. The pH was tested using a pH meter by sampling a 1mL volume of the PCs and washing the bulb of the pH meter after every measurement. All successive measurements were taken on Days 3 and 5 of storage.

Platelet morphology was analyzed by photographing microscope smears of the platelets in each 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. Discoid and irregular shapes were noted among the platelets. Six photographs were taken from each slide and were gridded and printed into paper for manual counting. A four-by-four area was used to count the platelets. The number of irregularly-shaped platelets was divided by the total number of platelets to obtain the percent change of platelet morphology.

Statistical Analysis. One-way ANOVA and paired t-test (α=0.05) was used to compare the mean changes of Day 0 and Day 3, and Day 3 and Day 5 between all setups. Post-hoc tests were conducted to see if there were any significant differences. The results were verified by a statistics teacher of Philippine Science High School – Western Visayas Campus.

Safety Procedure. The researchers wore the necessary personal protective equipment such as lab gowns, surgical gloves, and lab goggles. Proper grooming (i.e. hair was short/tied up) was observed. The researchers followed the standard operating procedure of the American Association of Blood Banks, under the supervision and guidance of a hematology professional. Platelet samples, and instruments used for blood extraction and preservative application were treated and handled as biohazards and were safely disposed. Sharp objects, i.e. blood syringes, were disposed in their respective containers. The waste bags were discarded to specific medical waste contractors.

Results and Discussion. The aim of the study is to determine the effects of ALCAR on the prevention of PSLs by observing the treated platelets' platelet count, mean platelet volume, platelet pH, and change in platelet morphology. An error was made in the preparation of the l-carnitine and ALCAR solutions wherein the study used 1mL of 15 mM preservative solution then mixed with their respective PCs. This resulted in a final concentration much lower than 15 mM and thus may have contributed to the inconclusive results collected. Instead of 15 mM concentration, 1mL stock preservative concentration of 1000 mM should have been used to make the final and intended 15 mM concentration.

Despite the dubious results, there is potential value in observing the effects of small concentrations of preservatives on platelets, and hence they are presented. Other than the major flaw in the preservative preparation and application method, it is possible there are other factors that had an effect on the results.

Platelet Count. The saline setup had a smaller platelet count compared to the l-carnitine and ALCAR setups on Day 0, but each setup equalized from Day 3 of storage onwards. Days 3 and 5 showed similar results, with the biggest change on the saline setup. Day 0 showed no samples with platelet count higher than $1x10^5/mm^3$. Days 3 and 5 showed platelet counts greater than $2x10^5/mm^3$. There was an insignificant increase in platelet count between all measurements.

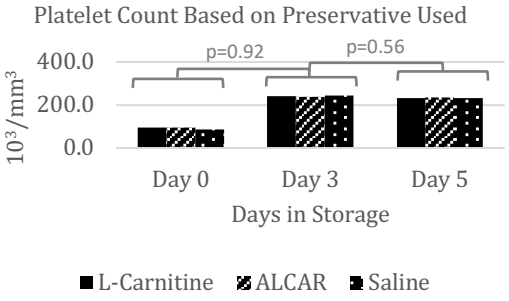

Figure 1. Platelet count means ($10^3/mm^3$) on Days 0, 3, and 5 of storage with three replicates of L-Carnitine (solid), ALCAR (lined), and Saline (dotted).

An anomaly was observed during the experiment wherein there was an increase in platelet count from Day 0 to Day 3 instead of a decrease. For this reason, the data could not be analyzed to accurately represent the effects of the preservatives on platelet count. It is unknown exactly why this occurred 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 was due to the measurement on the first day, where platelet count was low even during preliminary experiments.

Mean Platelet Volume Mean platelet volume measurements were taken at the same time as platelet count. The gathered data for all setups were 5.5 μm^3 on Day 0, 5.8 μm^3 on Day 3, and 6.1 μm^3 on Day 5 which were all below the normal range of values between 7.2 μm^3 and 11.7 μm^3 [10]. A trend whereby the volume of the platelets increased was observed during subsequent analyses.

Mean platelet volume of ALCAR setup was highest in Day 5 during preservation. L-carnitine consistently resulted in the lowest values among all measurements.

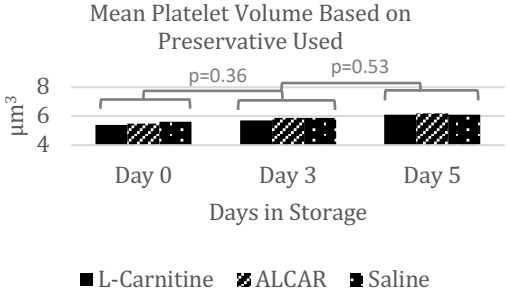

Figure 2. Mean platelet volume means (μm^3) on Days 0, 3, and 5 of storage with three replicates of L-Carnitine (solid), ALCAR (lined), and Saline (dotted) each.

Statistical analysis showed insignificant differences in mean platelet volume among all setups.

Platelet pH. L-carnitine dropped from 6.7 to 6.6 on Day 5 while ALCAR dropped from 6.7 to 6.6 on Day 3 of storage. Saline dropped from 6.6 on Day 3 to 6.5 on Day 5. This data is all within the normal range of values between 6.4 and 7.4 [11] and

shows insignificant difference between Days 0 and 3 and Days 3 and 5.

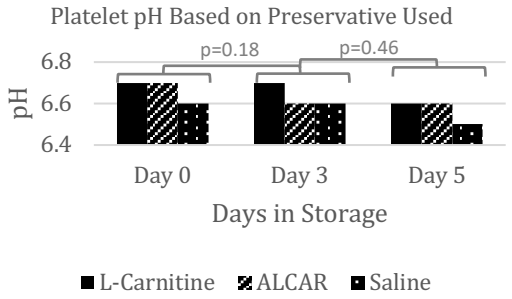

Figure 3. Platelet pH means on Days 0, 3, and 5 of storage with three replicates of L-Carnitine (solid), ALCAR (lined), and Saline (dotted).

Platelet Morphology. Statistical analysis showed that the platelet morphology results are statistically insignificant between all setups.

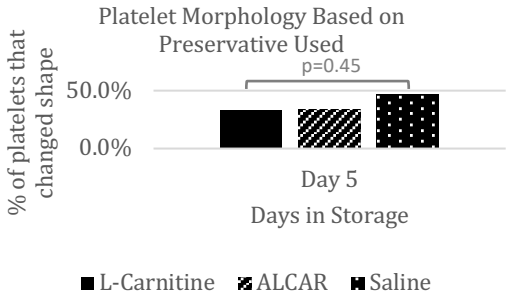

Figure 4. Platelet morphology means on Day 5 of storage with three replicates of L-Carnitine (solid), ALCAR (lined), and Saline (dotted) treatments each.

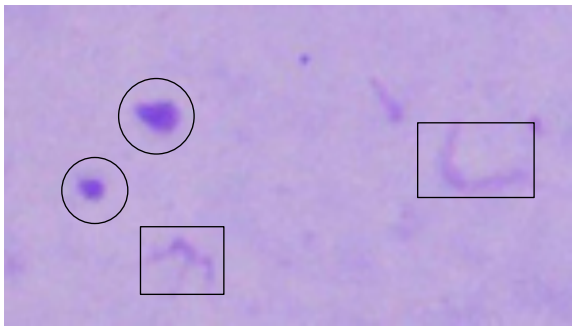

Plate 1. Regular (circled) and irregular (boxed) platelets taken from a platelet sample.

All results showed an insignificant difference in mean platelet volume, platelet pH, and platelet morphology among all setups all throughout the five-day observation period. This is because five days is the standard storage period for platelets. Previous studies showed that platelets were observed not to have significant decrease in quality during this period [12,13,14,15]. Multiple studies have since explored more options on preventing PSLs and thus increasing platelet shelf-life [14,15].

In previous literature, the focused factors included the acidity of the platelet medium and the metabolic activity of the platelets. With the current technology used for platelet storage, there is an increase in number of studies observing the metabolic activity of platelets. In the body, platelets derive up to 80% of their energy through ß-oxidation [1]. The energy from ß-oxidation comes from fatty acids, reducing the reliance on glucose. 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 [1].

The studies of Deyhim et al. [1] and Sweeney et al. [12] 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 whether another chemical such as ALCAR could be capable of obtaining similar results. The final data showed that not only is there an insignificant difference in comparison to l-carnitine, but that it cannot be conclusively said that ALCAR is a viable platelet preservative.

In the study of prolonging platelet shelf-life, platelet storage lesion is the biggest hurdle against the loss of platelet viability. It does not stem from a single variable, but instead involves multiple factors that influence platelet degradation [1,14,16]. In order to improve platelet shelf life, factors such as temperature, agitation, medium acidity, and oxygen permeability are needed to be considered. Understanding the mechanisms of platelet storage lesion involves understanding each of these factors and more in order to know their roles in maintaining platelet viability.

Limitations. The methods had the fundamental flaw of preparing the wrong concentration which may have resulted in insignificant data.

Conclusion. All four parameters showed no significant results during the entire observation period. It can be concluded that this study did not yield enough information to say that ALCAR is effective in improving platelet viability.

Recommendations. Future studies must use 1mL of stock l-carnitine solution and ALCAR solution of 1000 mM each rather than 15 mM in order to achieve to achieve more accurate results. The researchers also recommend the use of the Kruskal Wallis and Wilcoxon tests to analyze the data due to its small sample size.

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