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Reduction of Oxidative Stress and Storage Lesions (RCSL) in Red Blood Cells: Analysis of Ascorbic Acid (AA), N-Acetylcysteine amide (AD4), and Serotonin (5-HT)

By Shanmuka Gadiraju

Abstract

Oxidative stress is a common occurrence in red blood cell (RBC) storage in blood banks throughout the world. Typically RBC units stored under routine standard protocol (stored in SAGM-CPD additive solution) can only be kept up to forty-two days for transfusion usage before being discarded. I am studying the effects of Ascorbic Acid (AA), N-acetylcysteine amide (AD4), and Serotonin or 5-hydroxytryptamine (5-HT) as additives in blood bank storage to find out if these additives can reduce storage-induced oxidative stress on red blood cells (RBCs), as well as to understand how potential blood storage additives can affect the shelf life of blood and post-transfusion recovery in patients. I conducted a literature review by studying various journal articles that examined metabolism to proteomics and the synergy of the different additives. These various additives significantly alleviated a range of signs of oxidative stress on RBCs including but not limited to replenishing glutathione (GSH), decreasing percent hemolysis, inhibiting the phospholipid rearrangement, and encouraging ATP production. By reducing these symptoms of oxidative stress, RBCs are able to last longer without any significant changes biochemically, and decrease the chances of post-transfusion complications such as Graft vs Host Disease (GVHD). The new additive solution could potentially increase the patient's post-transfusion recovery rates as well as increase the shelf life of RBC storage units past the standard forty-two days.

Introduction

RBC stored units used for transfusions can last up to forty-two days before they must be discarded due to extensive physiological and biochemical changes during storage (D'Alessandro et al., 98). But, prior to forty-two days, there are changes within the first twenty-four hours of storage itself. In high Human Development Index (HDI) countries such as the United States, fifteen million units are used to care for 300 million

people at a rate of 40,000 units every day (Hess, s9). The average RBC transfusion is roughly three pints, but a car accident victim could need as much as 100 pints (“Blood Facts and Statistics”). The median annual blood donation rate is approximately 15,000 units in high-income countries, suggesting a clear deficit of blood for transfusions nationwide (“Blood safety and availability”). Hess described standard blood, which was stored in the anticoagulant citrate, phosphate, and dextrose (CPD) solution with proper filtration through either the buffy coat removal system or the platelet-rich plasma method of component manufacture (s9). By 1983, there were four principal additive solutions: AS-1 (Adenine and Sodium), AS-3, SAG (Sodium, Adenine, and Glucose), and SAGM (Sodium, Adenine, Glucose, and Mannitol) (Moore, 32). The focus is mainly on SAGM (877 mg of Sodium, seventeen mg of Adenine, 0.9 g of Glucose, and 0.52 g of Mannitol), and the concurrent problem with all of the additive solutions is that they don’t protect the cells from any storage-induced oxidative stress (Moore, 32-3).

Current inadequacies in blood storage protocols jeopardize valuable blood supplies. Improved blood preservation through the introduction of additives such as AA, AD4, or 5-HT inhibits fluctuations and changes in phospholipid content of the RBC membrane, reduces RBC hemolysis, regenerates GSH, and alleviates toxic intermediates and reactive oxygen species (ROS) produced within RBCs due to storage-induced oxidative stress (D’Alessandro et al., 98). These types of additives may be promising in increasing the shelf-life of RBCs.

Background

Raval *et al.* stated that normal hemoglobin (Hb) in RBCs contains ferrous iron (Fe^{2+}) capable of transporting oxygen. However, this Hb can be converted to methemoglobin (metHb) that contains oxidized iron (Fe^{3+}) due to the increased oxygen (O_2) affinity in stored units. Superoxide radicals mutate hydrogen peroxide and generate other oxidation by-products and radical oxygen species (87). Raval *et al.* pointed out that these storage-induced oxidative changes have been linked to aggregation of structural proteins such as band 3 and band 4.1 membrane structural proteins on the RBC

membrane (D'Alessandro et al., 111).

There are many different pathways within the RBC system such as glycolysis, GSH homeostasis, pentose phosphate pathway (PPP), and purine salvage pathway (PSP) that are affected by storage-induced oxidative stress (Pallotta et al., 380). Depleting ATP and decreasing 2,3-diphosphoglycerate (2,3-DPG) throughout the weeks of storage inhibits the full capacity of the glycolytic pathway, which in turn interferes with PPP (D'Alessandro et al., 108; Pallotta et al., 380). Inadequate ion exchange during storage such as between sodium and potassium, which is essential for the movement of waste and other important processes of life, also causes fluctuations in pH that disrupts all enzymes within pathways, as proteins can only function effectively in optimal pH (Pallotta et al., 380).

Vitamin C is a water-soluble vitamin that the human body cannot store. In its oxidized form, AA, it is one of the best natural antioxidants. Due to its necessity in the body for basic functions, and since the human body cannot actually produce it on its own; this is one of the main new additives used. Secondly, AD4, the amide form of N-Acetylcysteine, is another important additive. N-acetylcysteine is a precursor for the GSH synthesis and has been used in previous studies to reduce the oxidative stress in sickle cell anemia patients (Pallotta et al., 377). The amide form of this compound is looked at as it has higher potential as an antioxidant. The 5-HT is found in the the brain as well as in the gastrointestinal tract of the human body. For RBCs, 5-HT does not activate any receptors, but acts like a protective antioxidant instead of like a neurotransmitter (Amireault et al., 1).

Inhibition of RBC Membrane Fluctuations during Blood Storage

Although phospholipid content within the RBC membrane fluctuates and membranes change shape throughout the storage process, the use of AA, AD4, and 5-HT inhibit these changes by reducing oxidative stress through adequate supply of antioxidants to the RBC membrane.

In a study that analyzed normal and β -thalassemic patients' hemoglobin for β -hemoglobinopathies, using ^1H and ^{31}P Nuclear Magnetic Resonance (NMR)

spectroscopy to determine the relationship between phospholipid composition and oxidative stress in RBCs. Inna Freikman, *et al.* collected blood samples from both normal and β -thalassemic patients prior to transfusion and pointed out that there are several different phospholipids in the RBC membrane composition such as phosphatidylserine (PS), “lipids localized in the cytoplasmic leaflet of the membrane”, and phosphatidylcholine (PC), “lipids with the choline head” (2388).

Umakant Dumaswala’s main focus for the study was to identify the primary antioxidant defense system in banked RBCs and determine the oxidative damage to proteins and lipids. Dumaswala *et al.* collected blood samples in polyvinyl-chloride bags containing 63 mL citrate-phosphate-dextrose (CPD) anticoagulant. Dumaswala *et al.* extended Freikman *et al.*’s observation by stating that the RBC storage altered the kinetics of the “transbilayer mobility of PS, phosphatidylethanolamine (PE), PC, and the activity of aminophospholipid translocase activity” during storage of RBCs (1042).

Dumaswala *et al.* stated that malondialdehyde (MDA) formation is the primary indicator of oxidative stress in RBCs. As MDA formation increased by 27.2 after forty-two days of storage, the current additive solution is not adequate enough to protect RBCs properly against oxidative stress. A “total lipid loss of 13.4%”, can be associated to the PS externalization as stated by Freikman *et al.* , the results in a “RBC membrane vesiculation during storage” that could relate to the microparticles (MP), that are released in the supernatant or plasma (Dumaswala *et al.*, 1043-4; Freikman, *et al.*, 2391). Both PS externalization and MPs are signs that the RBCs are under oxidative stress. Freikman *et al.* proved that a combination of Vitamin C and N-acetyl-L-cysteine (NAC) curbed the effects of PS externalization and other rearrangements in the RBC membrane during storage. Freikman *et al.* experimented with *in vitro* incubation and found that RBCs shed their PS into the plasma supernatant through membrane vesicles, MPs, or PSs bound to soluble proteins due to oxidative stress. H_2O_2 and butylhydroperoxide (BHP) increase the PS composition by “three-fold”; therefore, increasing the likelihood of PS externalization, which is linked to apoptosis of the RBCs (2392). An increase in PS is potentially due to increasing oxidative stress caused by oxidants such as H_2O_2 and BHP. In turn, the PC composition decreased by “two-fold” because the total PL

composition generally remains the same. RBCs treated with antioxidants decreased PS by “2.8-fold” via NAC and by “2.6-fold” via Vitamin C. suggest that the antioxidants reverse some of the rearrangement of phospholipids caused by oxidative stress as the antioxidants decrease PS in the RBC membranes which decreases the chance for PS externalization. However, just decreasing the amount of PS in a membrane doesn’t mean that there is less oxidative stress. Using N-ethylmaleimide (NEM), “a specific inhibitor of aminophospholipid translocase, an enzyme that translocates PS from the outer membrane leaflet back to the inner leaflet...” the PS amount in the membrane decreased, but NEM caused more PS externalization, which resulted in more PS shedding (Freikman *et al.*, 2392). This suggests that even though the PS composition decreases, the PS composition could increase in the plasma supernatant via MPs or in other ways.

In addition to the changes in PS and PC previously stated by Freikman *et al.*, Dumaswala *et al.* claimed that there were other changes to the RBC membranes such as the fact that the densitometric scanning of the gels showed that the carbonylation of the band 4.1 protein increased significantly from 0.4 mmol in fresh RBC ghosts to 3.4 mmol after forty-two days. Dumaswala *et al.* noticed that by day eighty-four, total RBC membrane protein aggregates increased by forty percent. Angelo D’Alessandro also looked at other changes in the RBC membranes by determining that “echinocyte and stomatocyte shapes...are considered potentially reversible transformations” whereas “spherocytosis, sphero-stomatocyte, spherocyte, ovalocyte or degenerated shapes are irreversibly changed cells” (108). Some “irreversibly-altered membranes” of RBCs such as the spherocytosis increases the chance for an RBC to lyse due to the decreased surface/volume ratio compared to the typical concave structure of the RBC membrane (D’Alessandro *et al.*, 110). As the percentage of irreversible modifications increases on day seven from “below ten%” of the total RBCs to thirty-one percent on day 42, the oxidative stress on the RBCs also increased (D’Alessandro *et al.*, 110). During the second week, biochemical imbalances started shifting and more oxidative intermediates in the metabolic processes cause fluctuations in pH and the protein arrangement in the membranes. This causes some of the alterations that are both reversible and irreversible. Any alteration to membranes change permeability, as well as increase the likelihood

for lysing. These alterations suggest that the current additive solution SAGM does not sufficiently protect RBCs during storage for more than fourteen days. It is essential to look for other additives that curb oxidative stress and inhibit lysing of RBCs to extend the duration of time that RBCs can be stored.

Likewise, Freikman *et al.* looked at other changes in the RBC membrane such as the “lactate/pyruvate ratios in thalassemic RBCs,” which were used as the baselines for determining the effects of oxidative stress on RBCs (2389). BHP and H₂O₂, oxidants, had completely opposite effects as they increased the ratio by “9-[fold]” due to BHP and by “5-fold” due to H₂O₂ (Freikman *et al.*, 2389). By increasing the ratio of lactate/pyruvate, the oxidants increased the oxidative stress exerted on the RBCs. On the other hand, antioxidants such as Vitamin C and NAC caused beneficial effects on the RBCs by decreasing the ratio by “4-fold” (Freikman *et al.*, 2389). This shows that Vitamin C and NAC decreased oxidative stress in RBCs as they decrease the lactate/pyruvate ratio, which is linked to oxidative stress. Although Freikman *et al.* only ran this experiment in a “incuba[tion] for 30 min at room temperature”, and these are by no means optimal conditions as emulated in RBC storage units--four degrees Centigrade and for ideally more than thirty minutes to maybe a day or more-- this short experiment shows the advantageous effects of Vitamin C and NAC as novel additives (2389).

Valeria Pallotta collected blood samples from the Italian National Blood Centre from ten healthy male donors and stored the units at four degrees Centigrade for up to forty-two days in CDP-SAGM or CPD-SAGM with AA and NAC. Pallotta *et al.* calculated the untargeted metabolomics analyses with an “Ultimate 3000 Rapid Resolution High Performance Liquid Chromatography (HPLC) system and an electrospray hybrid MicroTOF-Q mass spectrometer equipped with an ESI-ion source” (378). Pallotta *et al.* already established in previous experiments, that there were better results when both Vitamin C and NAC were used together instead of only using one of the antioxidants (377), supporting D’Alessandro’s *et al.* claim that Vitamin C alone won’t have enough antioxidant powers to curb storage-induced oxidative stress in RBCs. The “decreased levels of glucose” showed signs of the absorption of ascorbate instead of D-glucose suggesting that the cells converted the ascorbate into its reduced

form and transported into the cell via glucose (GLUT) transporters as stated by J. May. The combination of both ascorbate and NAC works well because the NAC targets the GSH pathway, which is ideal in decreasing the oxidative stress in RBCs. However, a combination of AD4 and ascorbate would work even better as stated by Amer, Atlas, and Fibach (“AD4 attenuates oxidative stress in β -thalassemia blood cells”) since AD4 was significant in lowering percentage of RBCs that lyse as well as doubling the amount of GSH regenerated compared to NAC (251-2). Ascorbate and AD4 would become a stronger antioxidant force to increase the life of RBC units in storage.

Reduction of Hemolysis during Blood Storage

Hemolysis is a common occurrence during normal blood storage, but with AA, AD4, and 5-HT, the percent hemolysis significantly decreases in RBCs. This holds the RBCs intact longer in storage.

Jay Raval collected blood samples that were whole blood derived (WBD), group A⁺, pre-storage leukoreduced, stored in AS-5 solution and procured from a US FDA licensed collection facility in Pittsburgh, Pennsylvania. Raval *et al.* tested for several biochemical processes such as “blood gas measurements for pO₂, pCO₂, pH, and metHb levels in supernatants and RBC lysates” (88). Raval *et al.* calculated hemolysis using the percent hemolysis equation “ $((100 - \text{Hct of sample}) \times \text{fHb}_{\text{control}}) / (\text{Hb}_{\text{aliquot}})$ ” where the “fHb_{control} is the mean free Hb concentration in the supernatant of the unrocked control sample and Hb_{aliquot} is the mean total Hb concentration of the RBC aliquot at a Hct of 40%” (89). Raval *et al.* demonstrated that AA, 5.86 mmol L⁻¹ didn’t reach statistical significance until day fifty-six, where there was a difference of twenty-two percent between the AA units compared to the saline controls; however, this was outside of the time frame of storage (89). Although there was a trend of lower levels of hemolysis in AA treated RBCs compared to the saline controls, there was not a significant difference until day fifty-six, which is past the standard storage of the forty-two days. This proves that AA will not be sufficient enough on its own to provide enough strength to curb oxidative stress in terms of hemolysis. Hemolysis is an important aspect and if the possible benefits occur outside of the intended timeframe, a higher concentration of AA

should be used or a combination of other additives should be used to determine if the lower hemolysis could be statistically significant before forty-two days of storage.

Pallotta *et al.* extends the observation that AA was not sufficient enough, where AA, with a concentration of 0.23 mM, had an even higher percent hemolysis of ninety-five percent compared to the saline control's percent hemolysis of ninety-two percent (377). Instead, Raval *et al.* used an AA of at least 8.78 mmol L⁻¹ and had percent hemolysis levels that were statistically significant within the time frame at day forty-two of storage with a difference of thirty-nine percent between the AA storage units and the saline controls (90). 2.93 mmol L⁻¹ and 5.86 mmol L⁻¹ are nearly not strong enough to make enough of an effect on RBCs to help reduce the oxidative stress caused by storage, but RBC stored in 8.78 had enough statistically significant data starting from day twenty-one, which is well before the maximum number of days of storage suggesting that AA with the proper concentration can curb oxidative stress and reduce percent hemolysis. Pallotta *et al.* may have only supported Raval *et al.*'s data that AA wasn't enough on its own because it didn't use even close to enough concentration of AA of only 0.23 mM compared to Raval's minimum of 2.38 mmol/L. Pallotta *et al.* demonstrated that a combination of both Vitamin C, at 0.23 mM, and N-acetylcysteine (NAC), at 0.5 mM, had a greater effect on percent hemolysis compared to Vitamin C alone in both Pallotta *et al.*'s 3% increase and Raval *et al.*'s 22%. Pallotta *et al.* had a 41% decrease in percent hemolysis with the combination of both AA and NAC compared to the saline control (377).

Although a combination of both AA and NAC proved to be a decent combination stated by Pallotta *et al.*, Amer, Atlas, & Fibach showed that the amide form of NAC, AD4, had more beneficial effects on percent hemolysis compared to NAC. Johnny Amer experimented with four-month-old mice that were bred to have the same conditions as the patients with B-thalassemia conditions; these mice were injected with AD4 and blood samples were collected prior and two hours after the AD4 treatment (250). Amer, Atlas, & Fibach calculated hemolysis by comparing the percentage of lysed RBCs to RBC input. Amer, Atlas, & Fibach tested the protection of RBCs from lysis and phagocytosis by macrophages through AD4 treatment by incubating thalassemic

RBCs in PBS overnight with different concentrations of AD4 and NAC; NAC and AD4 inhibited lysis, up to seventy percent for NAC and ninety-seven percent for AD4 at two mM, *in vitro* for intravascular hemolysis (252). Likewise, Amer, Atlas, & Fibach demonstrated that AD4 reduced phagocytosis by macrophages, as well as extravascular hemolysis by fifty percent whereas NAC reduced phagocytosis by macrophages by twenty-seven percent (253). The control had a full 100% lysis whereas at two mM the NAC treatment “inhibited lysis...[by] 70%” and AD4 treatment “inhibited lysis...[by] 97%” (Amer, Atlas, & Fibach, 252). Both NAC and AD4 reduce the percentage of RBC lysis significantly; however, even starting with 0.2 mM, there’s already a big difference between NAC and AD4 treatments. By 0.4 mM, AD4 reduced RBC lysis by sixty percent whereas NAC treatment reduced RBC lysis by eighty-five percent. This distinction between NAC and AD4 demonstrates that AD4 is a better treatment method because inhibiting more RBC lysis leads to RBC longevity.

Though both Pallotta *et al.* and Raval *et al.* used AA and AD4, Amireault *et al.* extends this observation by adding that 5-HT also adds to inhibiting percent hemolysis in stored RBCs. Pascal Amireault collected blood, purified using Histopaque 1083, used an electronic hematology particle counter, and stored in four degrees Centigrade. Amireault *et al.* incubated the RBCs in four degrees Centigrade and observed hemolysis in the RBC with TPH^{-/-} and wide-type (WT) genotypes for fourteen days. Amireault *et al.* calculated that the half-life of the RBC survival for both genotypes. Amireault *et al.* showed that 5-HT decreased percent hemolysis by around twenty-five percent on day forty-two of storage while none of the 5-HT precursors or the saline controls could mitigate the oxidative stress that caused 100% hemolysis by day forty-two of storage (4). In addition, Amireault *et al.* also showed that 5-HT, 100 mM, emulated other existing anti-oxidants such as Trolox, 100 mM, a derivative of Vitamin E, suggesting that 5-HT has an extrinsic protective effect on RBCs and acts as an antioxidant instead of a neurotransmitter (4). “The dose-dependent protective effect of 5-HT on mouse RBC” is important to consider because there are several side effects to having too much 5-HT in the body (Amireault *et al.*, 2013, 2). The forty-eight percent increase in half-life with the addition of ten mM of 5-HT shows that 5-HT increases the life of RBCs up to as much as 304% with the

highest dose used in Amireault *et al.*'s experiment with 5-HT (2). Future studies need to be conducted to determine which amount of 5-HT will cause these potential side effects and use as much 5-HT in RBCs without causing too many negative biochemical changes while alleviating the oxidative stress by exerting “an extrinsic protective effect on the RBC[s]” (Amireault *et al.*, 2). The RBCs stored in AA demonstrated a decrease in the accumulation of RBC micro-particles by sixty-nine percent compared to standard storage (CPDA-1 alone; Fig. 1B).

Adequate Replenishment of GSH throughout Storage

GSH is the main antioxidant system within RBCs. Any additive that replenishes GSH throughout storage such as AA, AD4, and 5-HT help alleviate the oxidative stress due to sufficient GSH in the RBC during storage.

Dumaswala *et al.* provided evidence that “GSH and the dependent enzyme, glutathione-peroxidase (GSH-PX), is the key antioxidant defense system in stored erythrocytes” (1042). Catalase and GSH-PX form an important defense system to combat H_2O_2 produced by the superoxide dismutase (SOD) reaction (Dumaswala *et al.*, 1045). Dumaswala *et al.* calculated using Beutler's equation for GSH and GSH-PX and found a decreased by 0.6 mmol/mL in GSH from day zero at 2.3 mmol/mL to 1.7 mmol/mL on day forty-two (1046). In addition, Dumaswala *et al.* found increasing amounts of GSH, homocysteine, and cysteine throughout storage; extracellular GSH increased from zero mM on day zero of storage to one mM on day forty-two of storage to 1.9 mM on day eighty-four of storage, which is indicative of increasing oxidative stress due to the movement of GSH to the RBC membranes to counteract the increasing free radicals (1046). D'Alessandro *et al.* collected blood from donors and separated the RBCs from the other components of blood and suspended the RBCs in SAGM additive solution. D'Alessandro *et al.* extracted membrane and cytosol proteins on days zero, fourteen, and thirty-five (108). D'Alessandro *et al.* supported Dumaswala *et al.*'s claim that GSH decreased during storage of RBCs. D'Alessandro *et al.* showed a parallel increase in GSSH while there was a decrease in GSH (111). In addition, D'Alessandro

et al. established that other proteins part of the GSH pathway relocated to the membrane throughout storage such as “such as glutathione S-transferase, glutathione peroxidase and thioredoxin-like protein 1 (hsa00480 – KEGG pathway annotation)” by day fourteen (111). This relocation of the components of the GSH pathway emphasizes the effects on oxidative stress on the RBC’s metabolic pathways. As the components move away from their optimal location, the level of protection diminishes significantly.

Contrary to previous statements of D’Alessandro *et al.* and Dumaswala *et al.*, Pallotta *et al.* showed that the use of AA and NAC counteract the current routine effects on GSH by increasing GSH within the RBC units throughout storage. Pallotta *et al.* demonstrated that with AA, 0.23 mM, and NAC, 0.5 mM, GSH and any other proteins and intermediates involved in the GSH pathway on RBCs were up-regulated to produce more GSH throughout storage. Pallotta *et al.* pointed out that rate limiting precursors to the GSH synthesis pathway such as cysteine and other thiol metabolism were as up-regulated, which works in conjunction with the increase in over GSH in storage (383). Pallotta *et al.* demonstrated that cysteine increased from one mM on day zero to six mM on day forty-two, about times times more, in the supplemental solution compared to a decrease from one mM on day zero to 0.5 mM on day forty-two in the saline control (383). Pallotta *et al.* demonstrated that methionine increased from zero mM on day zero to 115 mM on day forty-two in the supplemental solution compared to an increase from zero mM on day zero to twenty mM on day forty-two in the saline controls (383). Without any stored additives, the GSH would be transported outside of the RBC, as there was an increase in extracellular GSH as it was pumped outside. The decrease in GSH results in oxidative stress in the RBCs because the GSH is the main primary antioxidant system of the RBCs (Pallotta *et al.*, 377). The decrease of the major antioxidant system demonstrates that the standard additives used to store RBCs is not sufficient enough to prevent oxidative stress compared other additives such as AD4, which specifically targets the GSH regeneration process (Amer, Atlas, & Fibach, 254).

Amer, Atlas, & Fibach extends Pallotta *et al.*’s observations by adding that AD4 replenishes GSH better and faster compared to NAC. Amer, Atlas, & Fibach’s demonstrated the effects of NAC and AD4 on GSH levels in thalassemic cells; NAC

increased GSH slightly from 120 to 180 whereas AD4 increased GSH from 120 to 320 in one hour (251). Although Amer, Altas, & Fibach's study didn't look at the effects of AD4 and NAC on RBCs in storage post at least 24 hours of storage, the data collected within the few hours of storage can be extrapolated and follow similar trends in a longer period of storage.

Inhibition of Toxic Products and Formation of Other By-Products

Although plenty of ROS and other toxic products are produced throughout storage that promotes hemolysis and other storage lesions, the usage of AA, AD4, and 5-HT inhibit the toxic products and oxidative stress radicals resulting in less oxidative stress within the RBCs during storage.

D'Alessandro *et al.* noticed that ROS accumulated during the first twenty-one days to a total of 252.4 (109). D'Alessandro *et al.* also noticed that protein carbonylation increased until day twenty-eight to 62.1, then it reversed back to 48.2 by day forty-two. D'Alessandro *et al.* noticed that malondialdehyde increased from 3.4 mmol/mL on day zero to 7.1 mmol/mL on day forty-two (109). Amer, Altas, & Fibach determined AD4 and NAC effect on ROS by staining the cells with DCF and incubating with AD4 or NAC; the AD4 reduced ROS production more than NAC. Amer, Altas, & Fibach also performed the same test *in vivo* with thalassemic mice, where blood samples were taken before and after 2 hours of treatment; the AD4 reduced ROS and increased GSH level. Amer, Altas, and Fibach claimed that AD4 restored ninety-one percent of the endogenous thiols compared to the fifteen percent restored by NAC.

Although these free radicals peaked "at 21 days" with "252.4 units." The detrimental effects of the excess amounts of ROS in the RBCs exponentially increase through the remainder of the days of storage (D'Alessandro *et al.*, 109). The rapid accumulation of ROS prior to twenty-one days was most likely due to increase of oxidized proteins and lipids of the metabolic processes such as glycolysis and PPP. Most of the changes in the RBCs are linked to oxidative stress and ROS such as protein fragmentation, relocation of antioxidant enzymes, and vesicle formation. The SAGM solution is inadequate in inhibiting the effects of ROS on RBCs and other alternatives

should be considered in the standard protocol for RBC storage.

SODs are plasma antioxidants that interact with free superoxide (O_2^-) and convert it into O_2 or H_2O_2 . Freikman *et al.* tested the oxidant, H_2O_2 , in stored RBCs and found that the oxidant increased oxidative stress in RBCs by rearranging the phospholipids in the RBC membrane, which then causes PS externalization and MP formation (2389). The increased regulation of SODs during storage leads to the increase of H_2O_2 in the stored RBCs. Rajashekharaiiah Vani collected blood samples from male *Wistar* rats with a syringe needle. Concentrations of 10 mM, 30 mM, and 60 mM were tested and every fifth day plasma was isolated to analyze. Vani *et al.* disagreed with Freikman *et al.* and demonstrated that the addition of Vitamin C into the stored RBCs helped decrease SOD activity. Too much Vitamin C could actually cause the H_2O_2 radicals to interact with other radicals via the Fenton reaction (5). Thus, using the proper amount of Vitamin C in the storage system is paramount for reducing the overall oxidative stress in stored RBCs.

Vani *et al.* stated that TBARS is the oxidant level in the lipid peroxidation; whereas Vitamin C is a scavenging antioxidant, which combats against lipid peroxidation. Vani *et al.* initially thought that TBARS increased in the Vitamin C groups especially in 30mM and 60mM concentrations in the first five days of storage. By day ten, all the of Vitamin C groups had a significant drop in TBARS activity proving that Vitamin C counteracts TBARS and reducing the oxidative stress in RBCs (3). Vani *et al.* found out that by day twenty-five, even the stronger doses of Vitamin C, 30mM and 60mM, tended to normalize with the controls suggesting that the amount of Vitamin C was used up by around day twenty-five.

Because Pallotta *et al.* showed that NAC is “added at concentration of 0.5 mM” and “its uptake is not inhibited...until it reaches concentrations as high as 10 mM;” any additional increase in NAC would result in toxic intermediates such as homocysteine (384). Even though Amer, Altas, & Fibach (“AD4 attenuates oxidative stress in beta-thalassemia blood cells”) had the best effects using two mM AD4, Amer, Altas, & Fibach only stored their blood samples overnight whereas Pallotta *et al.* stored their blood samples for 42 days. Thus, by using up to 10 mM AD4, the toxic intermediates will not stockpile as much as using higher concentrations of AD4. Elevated levels of

homocysteine in the blood has been linked to the narrowing of blood vessels in the body which leads to early heart attacks and stroke (“Homocysteine”). These effects may or may not be present during *in vivo* trials, but its definitely a complication that should be avoided.

Pallotta *et al.* showed that ATP steadily decreased in supplemented storage compared to saline storage whereas cyclic adenosine monophosphate (cAMP) increased in saline storage while it remained constant or decreased in supplemented storage (381). As more oxidative stress causes changes in phospholipids on the RBC membrane, the more the RBC releases adenosine triphosphate (ATP) to counter those changes (Frickman, *et al.*; “Oxidative stress causes membrane phospholipid rearrangement and shedding from RBC membranes-an NMR study”; Sprague, et al.). Sprague *et al.*’s (“Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release”) hypothesis that cAMP stimulates ATP release in RBCs is supported by Pallotta *et al.*’s experiment because the ATP decreased through the storage time due to the oxidative stress negatively affecting and altering the RBC membrane in Figure 4 (381). The cAMP activity increased in normal storage conditions as more and more ATP was released to counter the mechanical changes (381). On the other hand, ATP decreased at a slower rate and cAMP remained constant through the supplemented because there was no need for ATP release as there was less oxidative stress in the RBCs stored with Vitamin C and NAC.

CONCLUSION

AA, AD4, and 5-HT as additional additives to the current preservative solution, SAGM, should combat the storage-induced oxidative stress that isn’t addressed with SAGM by preventing apoptotic-like changes in RBC phospholipid contents of the RBC membrane, decreasing the percentage of RBC hemolysis and RBC phagocytosis, maintaining GSH homeostasis, and alleviating toxic intermediates and ROS produced within RBCs during storage. This in turn will increase RBC longevity so that more blood will be able for the critical transfusions for all sorts of patients.

Further research should be conducted focusing on what concentrations of the combination of additives presented in this study alleviate the most oxidative stress.

Likewise, research should also look at other combinations as there are countless natural and synthetic antioxidants, each with its own advantages and disadvantages on how they affect RBCs. There should also be more research on the various types of additive solutions currently present such as AS-1, AS-3, AS-5, SAG, SAGM, CDP, CDP-SAGM, etc. Furthermore, the future research should also focus more on developing synthetic RBCs so that blood shortages for transfusions can be reduced.

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