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Efficacy of L Carnitine for Prevention of Gamma Irradiation Induced Oxidative Damage in Red Blood Cells

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Abstract:-

- ***Introduction***

Storage of red cells is associated with some significant biochemical and metabolic changes. Red cells when exposed to oxidative stress during storage as it was reflected by increasing levels of lipid peroxidation, Hb oxidation and osmotic lysis. These deleterious changes were more pronounced after gamma irradiation at 25 Gy. L carnitine, a naturally occurring antioxidant, known for its role in facilitating mitochondrial beta oxidation of long chain fatty acids and membrane repair.

- ***Materials and Methods***

A total of 30 red cell units were studied. The blood bags were divided in two aliquots. L carnitine was added to one aliquot and other half without L carnitine served as a control. Both the halves were irradiated at 25 Gy and stored for 28 days. Markers of oxidative injury and membrane damage were investigated to assess role of L carnitine as antioxidant.

- ***Results***

The mean levels of plasma K+ and supernatant Hb and MDA were significantly lower in L carnitine fortified irradiated red cells compared to gamma irradiated red blood cells without carnitine. (mean plasma Hb 0.363 gm/dl vs 0.281 gm/dl, mean K+ 51.2±4.4 mmol/L v/s 46.3±3.3* mmol/L and mean MDA 5.01±0.99 mmol/L v/s 4.04*±1.13 mmol/L). L carnitine provided beneficial effect against such radiation induced red cell damage.

- ***Conclusion***

Lower levels of markers of red cell membrane damage and oxidative injury were observed in L carnitine fortified irradiated red blood cells compared to non-irradiated. Since L carnitine is also a naturally occurring compound, it can be added to the blood bags for protection of red blood cells against oxidative damage.

Keywords:- *Oxidative Injury, Gamma Irradiation, L Carnitine, Red Cell Storage Lesion.*

I. INTRODUCTION

Red blood cells are exposed to oxidative stress during storage at 4°C which results in damage to membrane and oxidative injury contributing to storage lesions considerably. The red cell membrane damage and oxidative injury makes the erythrocytes more prone to oxidative stress as shown by increase in various markers of oxidative injury such as leakage of hemoglobin and intracellular potassium ions. As the membrane integrity is compromised there is increase in osmotic fragility of red cells and loss of lactate dehydrogenase (LDH), an intracellular enzyme. The enzymes and hemoglobin are released in supernatant plasma. [1]

As a measure of prevention of transfusion associated graft versus host disease, the cellular blood components such as red cells and platelets are irradiated before transfusion. Irradiation leads to formation of reactive oxygen species (ROS) e.g. hydroxyl radical. These ROS are free radicals which are potent oxidants. When these oxidants interact with red blood cells and other blood components which are formed by proteins and lipids, they cause oxidation of membrane by lipid peroxidation. [2, 3]. Similarly, gamma irradiation of blood components prevents TA-GVHD but during the process of irradiation there is damage of red cell membrane which reduces their survival [4]. The damage caused to the red cell membrane after irradiation makes it porous as membrane integrity is compromised. This leads to leakage of intra corpuscular components such as intracellular potassium, free Hb and makes the membrane to lose lipid as lipid vesicles [5,6,7]. There is a gradual increase in Thiobarbituric acid reactive substances (TBARS) / malondialdehyde (MDA) levels are observed in red cells which are exposed to irradiation which suggest oxidative process occurs due to irradiation [8,9]. Loss of membrane integrity due to oxidation of membrane lipids and protein results in cell death [10].

Oxidative damage occurring during storage of red blood cells and after irradiation can be prevented either by incorporation of antioxidant in the bags or by supplementing the donor with a course of antioxidants. Various antioxidants have been studied to evaluate the

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protective affect against oxidative injury occurring to red cell during gamma irradiation [11, 12].

L-Carnitine, facilitates mitochondrial beta oxidation of long chain fatty acids which has been used for prevention of storage lesions during red blood cell storage [13]. Red cells have substantial amounts of L-carnitine and its esters and also contain carnitine palmitoyl transferase (CPT), an enzyme. This enzyme helps in transference acyl moiety from coenzyme A to L carnitine [14]. L-Carnitine has shown its role in stability of red cell membrane under different stressful conditions. It has also been reported that L-carnitine may be involved in repair mechanism of red cell membrane phospholipids after oxidative challenge by replacing oxidized fatty with acyl carnitines in membrane phospholipids [15].

In view of the membrane lesions occurring during gamma irradiation of red cells and the potential beneficial effect of L carnitine in preventing the membrane damage, the protective effect of L carnitine in was investigated. The study was aim to assess ability of L carnitine in prevention of gamma irradiation induced oxidative and membrane injury in red blood cell components stored over a period of 28 days at 4^{o}C. The markers of membrane damage such as malondialdehyde (MDA), hemoglobin oxidation and osmotic fragility and markers of oxidative injury were studied.

II. MATERIAL & METHOD

A total of 30 packed red blood cell components were included assessing the effects of gamma irradiation and protective effect of L carnitine. Whole blood was collected in 450 ml quadruple CPDA-1 SAGM bags from whole blood donors. The blood bags were collected and processed within 6 hours of collection of units. Buffy-coat reduced packed red cells were used in the study. Leukofiltration of the blood components were not done. The red cell component were aseptically divided into two halves with the help of sterile connecting device. L carnitine, antioxidant was added to one half of the blood bag in the final concentration of 15 mmol/L. The other half which was not fortified with L carnitine served as a control. Both the aliquots were exposed to gamma irradiated at a dose of 25 Gy by a gamma irradiator (Nordion, Canada). The irradiated blood components were stored at 4^{0}C for a period of 28 days. Sampling of the components were done aseptically to prevent any contamination, on day 0, day 14 and day 28 of storage. Markers of oxidative injury ((malondialdehyde MDA levels, Hb oxidation and osmotic fragility) and membrane damage (plasma Haemoglobin (Hb), plasma potassium (K+), lactate dehydrogenase (LDH) were measured in all the samples. As a measurement of red cell indices, all samples were checked for Hb, Hct, MCV, mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW) by 3 part cell counter (ABX Diagnostica, Biomeriux, France).

- *Assessment of Red Cell Membrane Damage*

Plasma hemoglobin in red cell units was measured by commercially available portable hemoglobinometer (Plasma/Low Hb system) (HemoCue, Sweden). Potassium levels (Plasma K+) in supernatant was estimated by electrolyte analyzer (Easylyte, Medica). Quantification of lactate dehydrogenase was done using commercially available reagent kits (Bio Systems S.A. Barcelona, Spain).

- *Assessment of Oxidative Injury in Red Cells*

Oxidative injury to red blood cells monitored by measuring the levels of MDA in. a red cell suspension by the method which has been previously described by Knight et al. [11]. Briefly, a red blood cells suspension was prepared using 200 µL of red cells in 1 ml of 0.1 M NADPH. The suspension was incubated at 37^{0}C for 60 minutes after which 6% Trichloroacetic acid was added to the suspension for stopping the reaction. The mixture was kept are room temperature for 5 minutes and 2 ml of 0.6% Thiobarbituaric acid was added. The suspension was held at room temperature for another 5 minutes and then mixture was boiled at 100^{0}C for 15 minutes. Once the mixture cooled at room temperature it was centrifuged at 3000 rpm for 10 minutes. The supernatant obtained was read at 532 nm spectrophotometrically. Osmotic fragility test on red cells and quatification of methemoglobin formation was carried out by following standard protocol and technique [12, 13].

III. STATISTICAL ANALYSIS

Descriptive values such as mean and standard deviation were computed for all the parameters. For correlation of marker of red cell oxidative injury and red blood cell membrane damage was done using Pearson correlation. Paired t test was used for comparison between parameters obtained on different days of sampling. SPSS software and Microsoft Excel were used for all statistical analysis.

IV. RESULTS

There was a gradual and statistically significant increase in markers assessing oxidative injury of red blood cells such as hemolysis by osmotic fragility, levels of methemoglobin , and MDA and markers estimating damage to red cell membrane such as plasma Hemoglobin (Hb() , potassium (K+) and lactate dehydrogenase (LDH). The increase of all these markers were observed in both the studied group during the storage of red cells at 4^{o}C for 28 days. (Table 1)

However, a statistically significant ($p < 0.005$) protective effect of L carnitine was not observed on all parameters studied. The protective effect of L carnitine against irradiation-induced changes was seen in plasma Hb (mean plasma Hb 0.363 gm/dl in aliquot without L carnitine vs 0.281 gm/dl in L carnitine containing aliquot on day 28). (Table 1, Figure 1)

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Similarly, protective effect of L carnitine was seen by significantly lower levels of K+ levels (51.2±4.4 mmol/L v/s 46.3±3.3* mmol/L) and MDA (5.01±0.99 mmol/L v/s 4.04*±1.13 mmol/L) were observed on Day 28 in L carnitine containing aliquots. (Table 1, Figure 1)

Although, lower levels of LDH, Hb oxidation and hemolysis at 0.65 % NaCl were also observed in aliquots containing L carnitine on day 28 but these were not statistically significant on comparing with aliquots without L carnitine.

V. DISCUSSION

Red blood cell transfusion is associated with number of adverse events. Transfusion associated graft versus host disease (TA-GVHD) is a potentially fatal complication occurring due to transfusion of allogenic donor leukocytes with red cell components. There is clonal expansion and engraftment of these transfused allogenic donor leukocytes. [16]. It cannot be prevented by leukoreduction of components as even very low levels of leukocytes can cause TA GvHD. Gamma irradiation of red cell as well as other components is used to prevent TA-GVHD but it may result in damage to red cells which are exposed to irradiation and reduces their survival [4]. These effect on the red cells are evident by assessing the markers for damage of red cell membrane. The parameters which can be used to monitor damage to the red cell membrane are free hemoglobin and leakage of intracellular potassium ions, and formation of membrane lipid vesicles which represent lipid peroxidation [5,6]. The red cells are exposed to free radicals generated by gamma irradiation. Loss of membrane integrity due to oxidation of membrane lipids and protein results in cell death [10]. Thus, antioxidants if present in storage bags may provide protection red cells against irradiation induced oxidative damage. Various studies have tried tirilazadmesylate (TM) and normal plasma which contains powerful antioxidants such as vitamin E and vitamin C as an antioxidant which can scavenge ROS generated from irradiation [12].

In the present study, the changes in various markers of red cell membrane damage (plasma K+, plasma Hb and LDH) and oxidative injury to red cells (MDA levels, Hb oxidation and osmotic fragility) were estimated. These changes were compared in gamma irradiated red cells both in presence and absence of an antioxidant when stored at 4^{o}C for a period of 28 days. We have used an antioxidant, L carnitine to study its protective effect on irradiation induced red cell injury.

L-Carnitine, is a naturally occurring antioxidant which is known for its role in facilitating mitochondrial beta oxidation of long chain fatty acids have been used for prevention of storage lesions during red blood cell storage [13]. It has been demonstrated that L carnitine and its esters have a positive effect on the stability of red cell membrane under various adverse conditions. It has also been reported that it may be involved in repair mechanism of red cell membrane phospholipids after oxidative challenge [15].

In view of the membrane lesions found with storage of red cells and the potential beneficial effect of L carnitine in preventing the membrane damage, we evaluated the effect by addition of L carnitine to the blood bag containing red cells. This was assessed in red cells exposed to gamma irradiation and stored for a period of 28 days at 4^{o}C.

In the present study, L carnitine provided a protective effect against red cell membrane damage after gamma irradiation, which was reflected by statistically significant ($p < 0.005$) lower levels of plasma Hb in carnitine fortified red cells. The mean levels of supernatant Hb and supernatant K+ were significantly lower in L carnitine fortified red blood cells units compared to red blood cells without L carnitine (Table 1).

We have seen in the present study that gamma irradiation results in oxidative damage to red cells. A gradual and statistically significant increase in MDA levels, Hb oxidation and osmotic lysis was observed in gamma irradiated red cells during storage for 28 days. We investigated whether L carnitine can decrease or prevent this deleterious effect of gamma irradiation. L carnitine did provide protection against radiation induced oxidative damage, as MDA levels were significantly lower in L carnitine containing aliquots. The mean levels of other markers of oxidative injury (Hemoglobin oxidation and osmotic fragility) were lower in L carnitine fortified irradiated red cells compared to non-irradiated red cells, though the difference was not statistically significant ($p > 0.005$, Table 1, Figure 1).

Many naturally occurring antioxidant such as human plasma and a powerful synthetic compound, tirilazadmesylate for prevention of oxidative injury after irradiation successfully have been studied and protective have been reported [12]. Since L carnitine is also a naturally occurring compound, the presence of this compound in the blood storage bags may render protection to red cells against such damage.

It has been suggested that the presence of L carnitine in the preservative medium during red cell storage may spare the use of ATP from the ATP pool during re-acylation of phospholipids for membrane repair [17]. This metabolic process associated with the possible biophysical action which may explain the reduced levels of markers of membrane damage in L carnitine stored red cells in the present study.

Our results indicate that L carnitine does provide beneficial protective effect against oxidative injury to red cells during storage which is expected as previously reported that L carnitine is involved in repair mechanism of red cell membrane phospholipids after oxidative challenge [17]. Other workers have explored the effect of various antioxidants on the oxidative injury to red cells during storage [11,12,18,19]. Epps et al, have reported decreased levels of lipid peroxidation and osmotic lysis in tirilazadmesylate treated red cells [20]. Knight et al, found increasing levels of MDA during storage after addition of

metal chelator such as desferoxaminemesylate effectively reduced MDA accumulation [11].

Similarly, protective benefits of trolox and mannitol against progressive RBC hemolysis has been reported. It was observed that trolox was able to inhibit reduction GSH levels , LDH leakage and lipid peroxidation whereas mannitol was not able to show similar effects.[21].

Supplementation only without proper concentration of storage solution doesn't ensure adequate protection to RBC protection during storage against damage to membrane and oxidative injury. The protective benefits of vitamin C on red cells may be concentration dependent. The protective effect of antioxidants such as vitamin C is dependent on many things. The products such as inflammatory cytokines, proteases, elastases, active lipids and Hb degradation products which are released when red blood cells are stored for longer durations can have a detrimental effect. The antioxidant property may work as pro-oxidant and promote oxidative injury. [22]. It has been postulated that vitamin C alone may not be able effectively reduce oxidative stress. Therefore, further studies on vitamin C along with other antioxidants in storage solutions may give more profound results [23].

Presently, American Association of Blood Bank (AABB) recommend 28 days of storage and BCSH guidelines recommend 14 days storage of red cells post irradiation. These limits have been there because radiation induced red cell damage progressively increases with the storage of irradiated red cells. As we have observed a beneficial effect of L carnitine against gamma irradiation induced oxidative damage to red cell membrane, it would be of significance to assess whether L carnitine fortification of red blood cells extends the storage period beyond 28 days post irradiation.

Gamma irradiation damages red cells and reduces their survival as evidenced by red cell membrane damage and loss of membrane lipid vesicles. Irradiation process leads to formation of free radicals which are reactive oxygen species (ROS). When these oxidants interact with red blood cells and other cellular blood components which are formed by proteins and lipids, they cause oxidation of membrane by lipid peroxidation. L carnitine provided beneficial effect against such radiation induced red cell damage. Lower levels of markers of red blood cell membrane damage and oxidative injury to red cells were observed in L carnitine fortified gamma irradiated red cells compared to non-irradiated. Since L carnitine is also a naturally occurring compound, it can be added to the blood storage bags to protect red cells against such damage.

We have used only single dose of irradiation 25 Gy and stored for 28 days only. It would be more informative to study the dose effect by irradiating red cells at 50 Gy. Larger studies are needed on this subject with prolonged storage period (42 days). Though it also advocates for studying different concentration of L carnitine and in combination with other antioxidants such as vitamin C and E.

Conflict of interest: The authors had no conflicts of interest to declare.

Ethical Approval: The study was conducted with ethical approval of the institutional ethical committee.

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Irradiated Red Cells						
	Without L carnitine			With L carnitine		
Parameters	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Hemoglobin (gm/dl)	17.9±1.1	18.0±1.1	18.1±1.2	18.1±1.1	18.1±1.1	18.0±1.2
Plasma Hb (gm/dl)	0.022±0.01	0.119±0.03	0.363±0.07	0.021±0.01	0.081#±0.06	0.281*±0.06
K+ levels (mmol/l)	7.0±1.1	26.9±3.4	51.2±4.4	6.0±1.1	25.0±3.2	46.3±3.3*
LDH levels (IU/L)	72.6±20.6	287.9±65.1	587.4±30.2	68.2±13.2	278.6±49.8	563.1±24.3
MDA levels (mmol/L)	2.16±0.23	3.28±0.27	5.01±0.99	2.61±0.18	3.16±0.45	4.04*±1.13
Hb oxidation (%)	1.24±0.50	2.11±0.42	3.5±0.85	1.25±0.46	2.09±0.53	3.03±0.86
% hemolysis at 0.65 NaCl	1.03±0.41	10.6±2.88*	18.5±2.96	1.01±0.38	10.4±2.78	16.5±2.59

Table 1:- Comparison of irradiated red cells with and without L carnitine stored at 4ºC over a period of 28 days. Levels of plasma Hb, K+ and MDA were significantly lower in L carnitine containing aliquots (* $p<0.005$)

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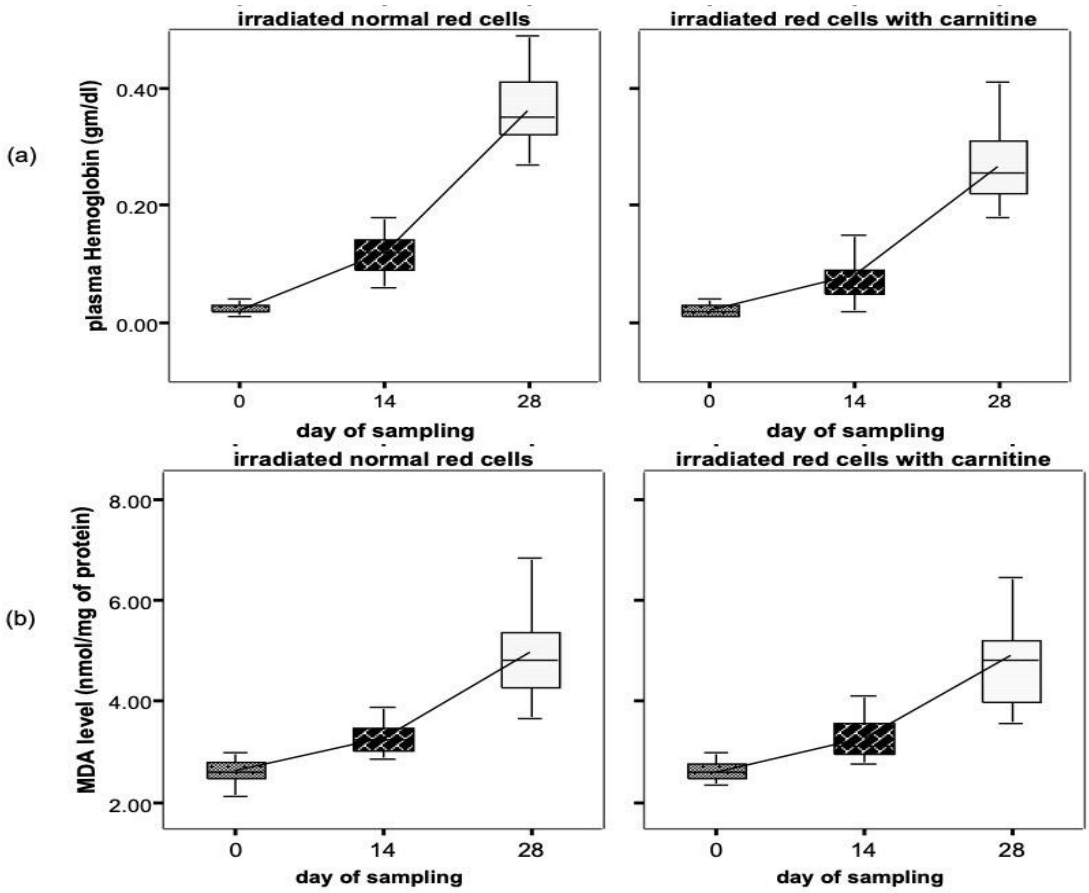

Legend to Figure:

Figure 1: (a) Comparison of plasma hemoglobin on day 0,14,28 in irradiated red cells with or with L carnitine. There was gradual increase in the levels during storage in day 0,14,28. The plasma Hb was significantly raised in irradiated red cell without L carnitine in comparison to irradiated red cells with L carnitine. (b) MDA levels increased significantly on day 28 of storage in irradiated red cells without L carnitine, showing the protective effects of L carnitine on red cells.