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SMOKING HABIT AFFECTS THE QUALITIES OF STORED LEUKODEPLETED RED BLOOD CELLS

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ABSTRACT

Background: *Pack Red Cell (PRC) is one of the most widely used blood products. Indonesia has a high number of smokers who are potential donors. Smoking is a habit that can cause radicals, triggers oxidative stress so that transfusion becomes ineffective.*
Objective: *This study aimed to determine the relationship between smoking habits and methemoglobin, glutathione, and glucose 6 phosphate dehydrogenase (G6PD) in leukodepleted PRC (PRC-LD) blood bags during storage.*
Methods: *PRC-LD from the Jakarta Red Cross Transfusion Unit were grouped into non-smoker donors (NP), light smoker donors (PR), and moderate smoking donors (PS). Analysis for methemoglobin, glutathione, and G6PD was performed on days 0, 14, 21, and 35h. The analysis was done spectrophotometrically.*
Results: *Statistical analysis indicates that methemoglobin was increased on days 21 and 35, glutathione levels decreased progressively on days 0, 14, 21, and 35. The G6PD activities decreased markedly on day 35 in all groups. A significant relationship was found between methemoglobin and glutathione, as well as G6PD and glutathione.*
Conclusion: *Smoking habits make the storage blood condition worse.*

Keywords*: Methemoglobin, Glutathione, G6PD, smoking, storage blood*

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INTRODUCTION

One of the blood components that is often used is Packed Red Cell (PRC).[1] The American Academy of Blood Bank (AABB) defines PRC-LD as a blood component of PRC that has a leukocyte count < $5x10^6$ per unit of blood bag. PRC-LD is safer to use because of minimal transfusion reactions and lower risk of transmission of cytomegalovirus (CMV) infection.[1–3] During storage of PRC, alterations that will affect its viability and utility in transporting oxygen from the lungs to the tissues are known as Storage Lesion, in decreased pH, glucose, ATP, lactic acid acumulation and loss of other red cell functions. During the collection of donor blood, about 1-5% of red blood cells will be damaged.[4–6] Red blood cell stored in the blood bank undergoes a progressive damage due to metabolic impairment which eventually reduce the erytrocyte antioxidative capacities.[7] It is not clear.[8]

Cigarette smoke contains 4000-7000 types of oxidants One puff of cigarette smoke contains 10^{17} free radicals in the tar phase and 10^{15} in the gas phase.[9,10] Red blood cells are continuously exposed to endogenous and exogenous sources of reactive oxygen species (ROS) which can damage red blood cells. To overcome this effect there are antioxidant systems in cells, enzymatic as well as non enzymatic. Enzymatic antioxidants consist of glutathione peroxidase (G-Px), superoxide dismutase (SOD), catalase (CAT), and peroxiredoxin-2 (PRDX-2), whereas non-enzymatic antioxidants consist of vitamin C, vitamin E and glutathione.[11–13] Oxidative lesion produced when the oxidant attack overewhelm the antioxidant capacities.[14]

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Glutathione is the main antioxidant in cells. Reduced glutathioe (GSH) reduces any peroxide into water or hydroxyl containin compound and at the same time oxide themself into aoxidized form of glutathione or GSSG. As this reaction is very important to cope oxidative stress, the availability of GSH should be assured and this is carried out by enzyme glutathione reductase, which absolutely dependent to reduced NADP (NADPH). NADPH supply depend on activities of glucose 6 phosphate dehydrogenase (G6PDH), a crucial enzyme in hexose monophosphate shunt pathway. Methemoglobin (metHb), a species of hemoglobin in which the heme Fe^{2+} is oxidized to Fe^{3+}, can not bind O_2 anymore. MetHb is formed by any oxidation process, whether by autooxidation (a normal process) or by any absorbed external oxidant, as in smoking habit.

Until now, there are no regulations regarding smoking in donor recruitment, and there is no limit on the safe storage of PRC-LD blood related to methemoglobin, glutathione and G6PD activity levels during blood storage.[2,15]

METHOD

This study used a cross-sectional design to analyze the relationship between smoking habits and levels of methemoglobin, glutathione and G6PD activity in PRC-LD blood bags during storage. This research protocol received ethical approval from the FK-UIRSCM health research ethics committee with the number KET.1085 /UN2.F1/ETIK/PPM.00.02/2020. The samples were obtained from 36 donors who came to Blood Transfusion Unit of Indonesian Red Cross in Jakarta from September 2020 to November 2020. All samples were grouped into 3, 12 from non-smoker donor, 12 from light smoker donor, and 12 from moderate smoker donor. Blood from each donor was collected into 350 mL double bag containing the anticoagulant CPDA-1. PRC was prepared by separating blood components through rapid centrifugation at 5000 rpm at 4^{o}C. PRC is processed into leukodepleted PRC by filtration according the methodsdescribed by Haemonetics, the producer. Briefly, the PRC passes through a filter which retains the leukocytes and lets flow the erythrocytes which are collected into another bag.The collecting bags were labelled according to groups and dates such D0, D14, D21 1nd D35. Hemolysates from 25% blood suspension were prepared from each sample and stores in refrigerator -20^{o}C for further analysis.

Methemoglobin examination was carried out according a technique of Evelyn-Meloy as described by Arnaud.F et al.[16]For making a standard curve of methemoglobin, 0.5 mL leukodepleted PRC were mixed with 0,5 mL $K_3Fe(CN)_6$to form methemoglobin. A series of metHb concentration were made by addition of distilled water to final volume of 1 mL.The absorbance was of each concrntration was measured at a wavelength of 630 nm. For measurement of metHb, each sample of leukodepleted PRC was mixed with 0,5 mL of 20 mmol KCN and then measured its absorbance at a wavelength of 630 nm.

Glutathione were determined by Ellmann reaction.[17] A standard curvewas made by dilution a series a series of volume of glutathione PBS to a final volume of 1 mL. Into each tube was added 5% TCA (Tri Chloro acetic acid), and DTNB (5.5-dithio-bis-2-nitrobenzoic acid). The absorptions were read at 412 nm.

G6PD activities were determined using SIGMA-ALDRICH catalog number MAK015 on D0 and D35. As the enzyme produce NADPH at the glucose oxidation, the activities of G6PD in each sample was determined by production of NADP after the addition of reaction mixture of the kit. The absorbance were read at 450 nm. The activities, expressed in unit, were calculated from a standard curve formed by plotting varios concntration of NADP vs A_{450}.

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RESULTS

MetHb. In the study, it was found that there was a significant difference in methemoglobin levels between non-smoker donors, light smokers and moderate smokers.

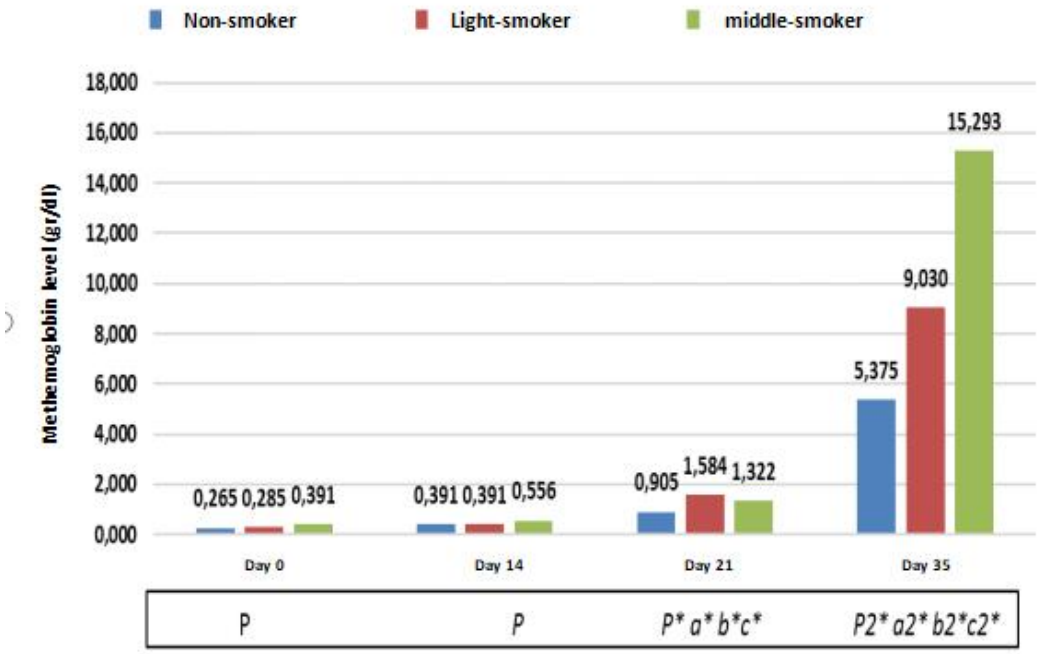

Figure 1: Methemoglobin levels between non-smokers, light smokers and moderate smokers on storage D0, D14, D21 and D35

The lowest methemoglobin level was in non-smoker D0 which was 0.265, and the highest methemoglobin level was found in the medium-smoking donor D35 which was 15.293. The increase in methemoglobin levels started at D21 in light smokers and moderate smokers p=0,000(Fig.1)

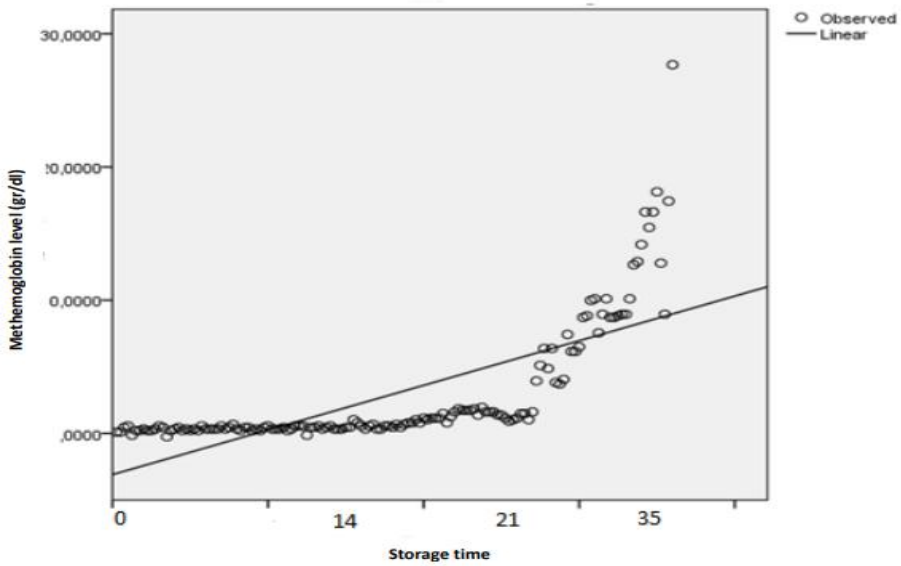

Figure 2: The relationship between storage time and changes in methemoglobin levels in non-smokers, light smokers and moderate smokers.

There is a positive correlation between methemoglobin levels and storage time. With regression equations: methemoglobin levels = 0.084 x storage time -3,089, with a value of p=0.000 and a value of R = 0.544 (Fig.2)

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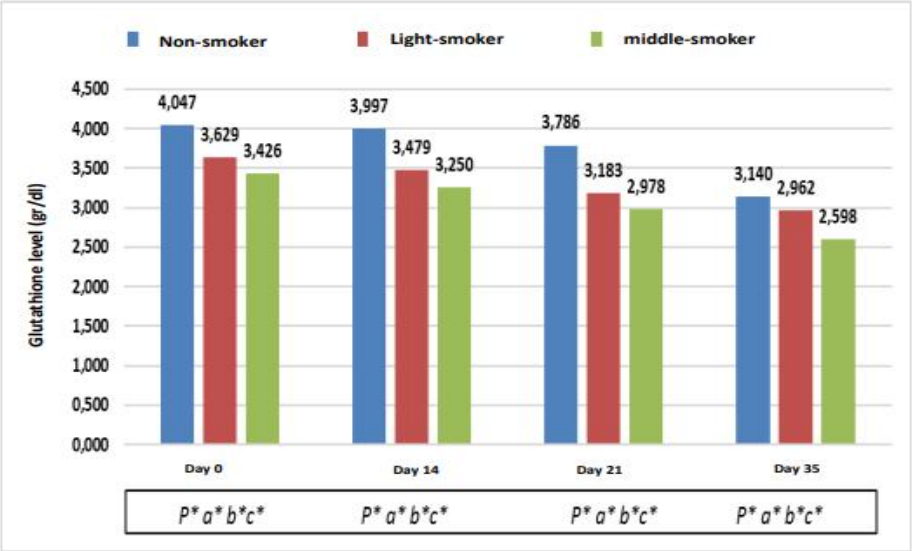

Figure 3: Glutathione levels between non-smokers, light smokers and moderate smokers in D0, D14, D21 and D35 storage

There was a decrease in GSH levels among non smoker donor groups, light smokers, and moderate smokers in H0, H14, H21 and H35. Statistical tests with the Anova test p = 0.000 results in H0, H14, H21 and H35 showed differences between groups of non smoker donors, light smokers, moderate smokers (figure 3)

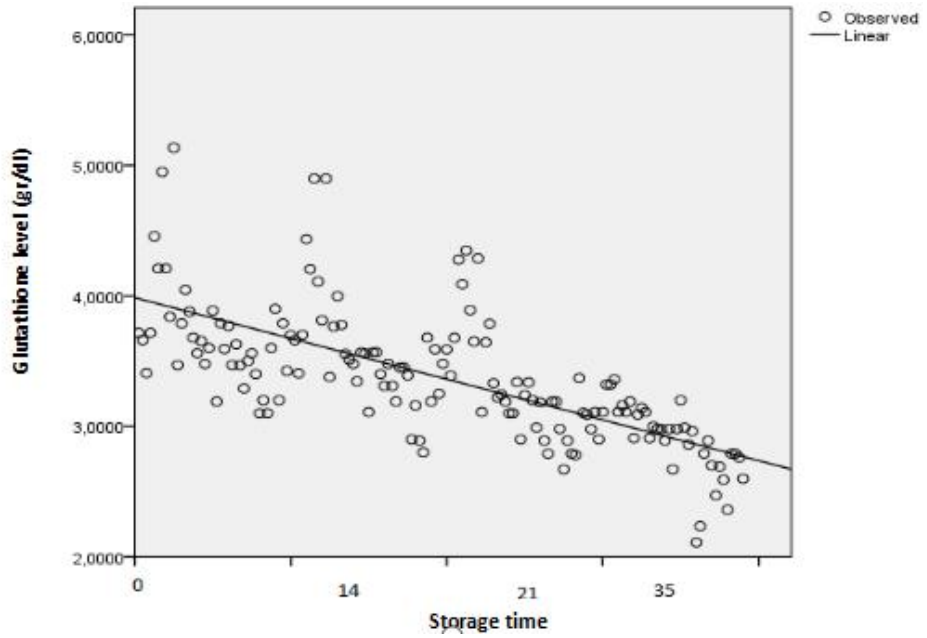

Figure 4: The relationship between storage time and glutathione levels during storage

There is a negative correlation between GSH levels and storage time. With regression equations: GSH levels = -3,986 - 0,008 x storage time, with a value of p=0.000 and a value of R = 0.483 (Fig.4) *G6PD*. As mentioned above, due to the limitation of reagents, the activities of this enzyme were determined only on D0 and D35.

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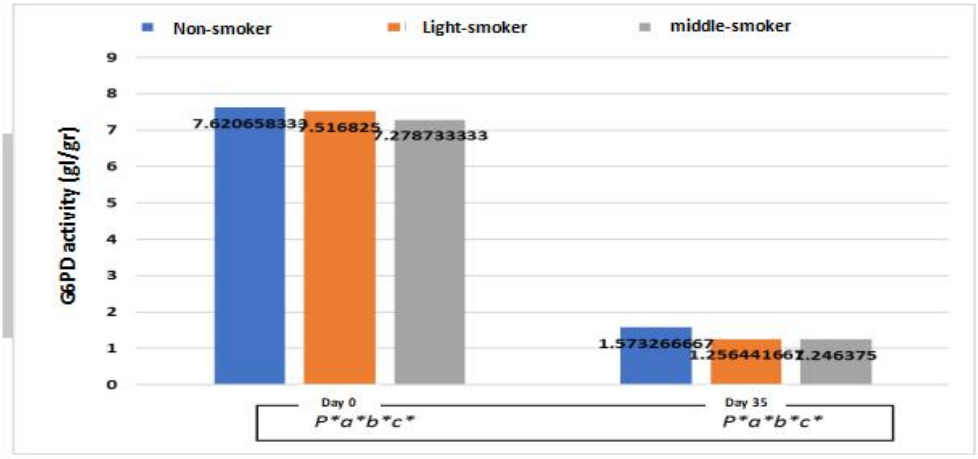

Figure 5: G6PD activity between non-smoker, light smoker and moderate smoker donors in D0 and D35 storage

It was found that, there were significant differences between G6PD activities in non-smokers, light smokers, and moderate smokers blood. G6PD activities were higher in non-smoker than in the smokers' blood. The highest G6PD activities were found in D0 non-smokers' blood. The lowest G6PD activities were found in D35 moderate smokers. There were significant difference between D0 and D35 in the three groups. (Fig 5).

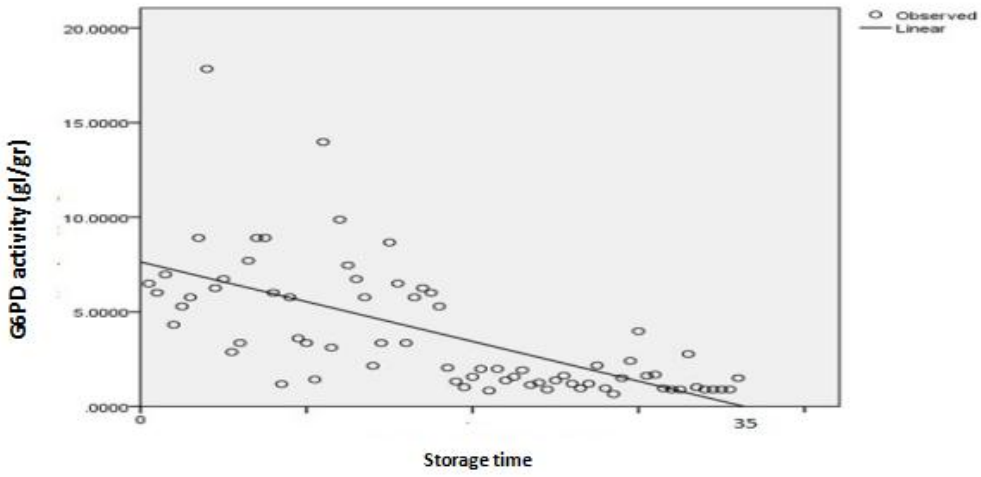

Figure 6: the relationship between G6PD activity and storage time

There is a negative correlation between G6PD activity and storage time. With regression equations: G6PD activity = 7,637 - 0,105 x storage time with a value of p=0.000 and a value of R = 0,434 (Fig.6). *Corelation between metHb and GSH levels*. Corelation analysis showed that there are significant negative relationship between metHb and GSH levels in all groups. (Figs 7,8 and 9). The slopes are greater in smoking groups, which suggested the contribution of smoke content in addition to role of duration of storage

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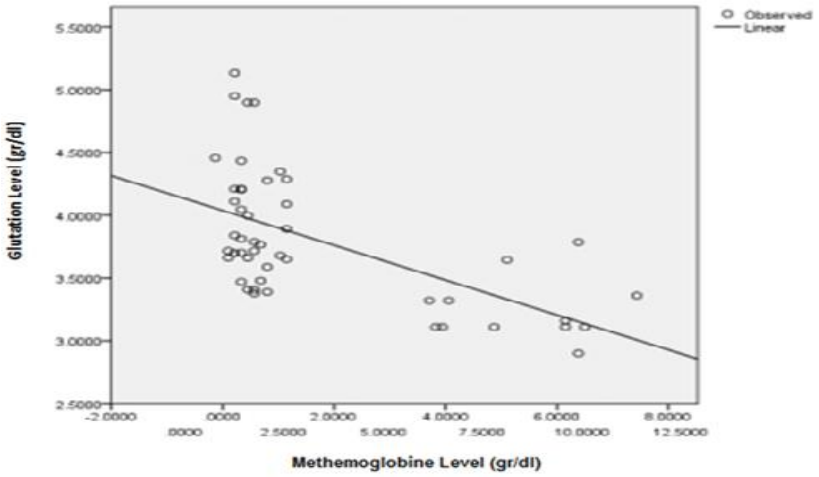

Figure 7: The relation ship between methemoglobin level and glutation level non smoker donor

There is a negative and significant correlation between methemoglobin levels to glutathione levels r = 0.341 and p = 0.000. Methemoglobin level= 4,038 – 0,139 x Gluthatione level. The higher the methemoglobin level, the lower the glutathione level.

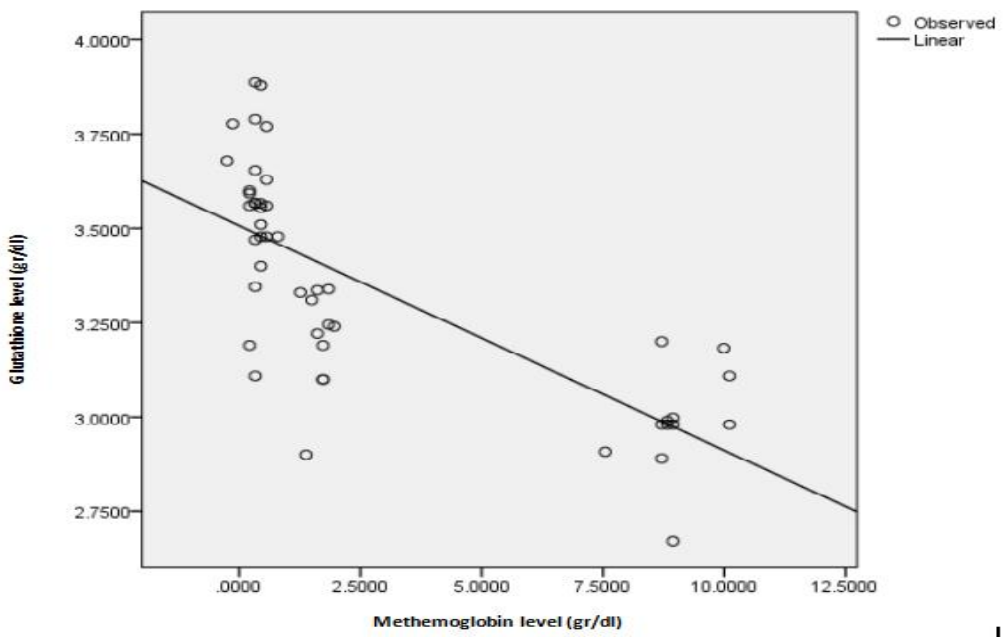

Figure 8: The relation ship between methemoglobin level and glutation level light Smoker Donor

There is a negative and significant correlation between methemoglobin levels to glutathione levels r = 0.546 and p = 0.000. Methemoglobin level= 3,507 – 0,060 x Gluthatione level. The higher the methemoglobin level, the lower the glutathione level.

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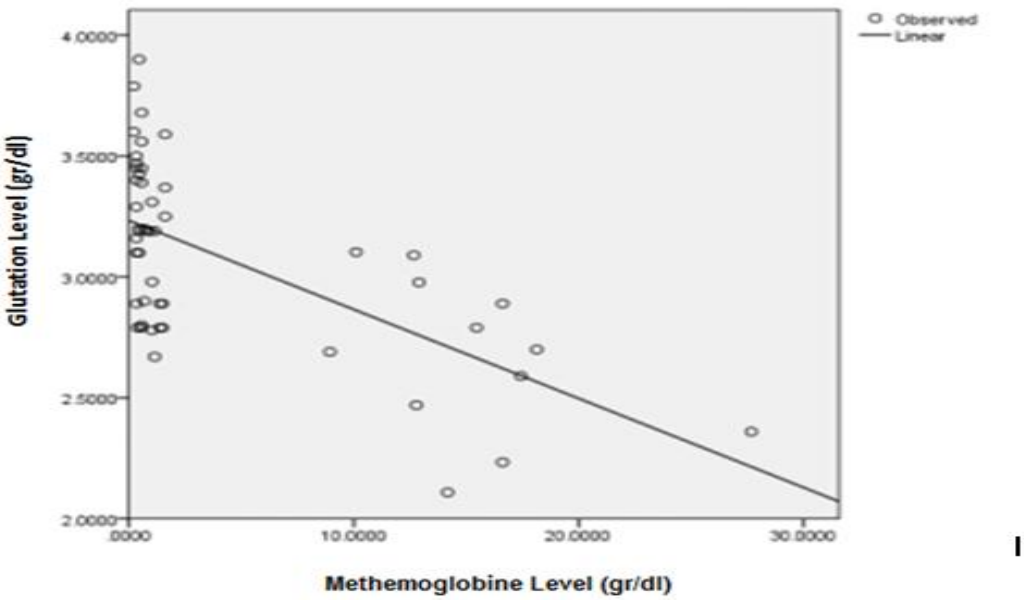

Figure 9: The relation ship between methemoglobin level and glutation level moderate Smoker Donor

There is a negative and significant correlation between methemoglobin levels to glutathione levels r = 0.394 and p = 0.000. The higher the methemoglobin level, the lower the glutathione level. The negative corelations are also found in the interaction between metHb and G6PD activities (Figs 9 and 10). The slope is greater in the smoking group, which also suggested the addition role of free radicals found in the cigarette smoke.

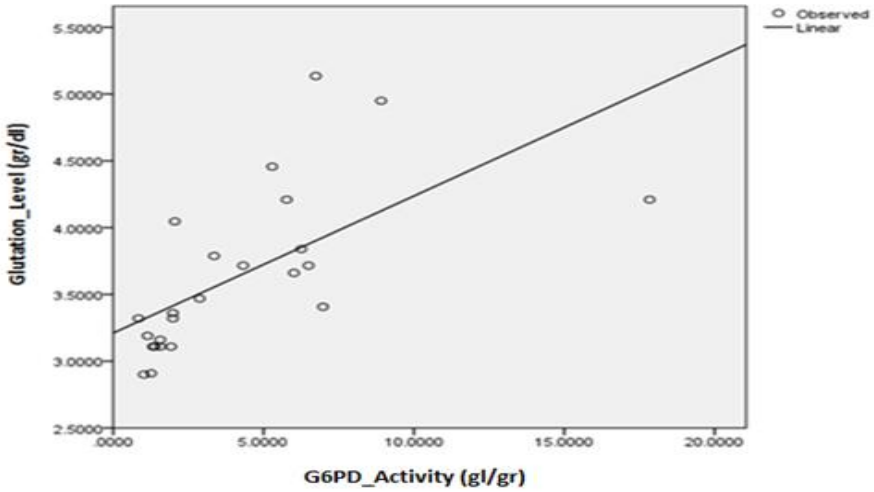

Figure 10: the relation ship between G6PD activity and gluthation level non smoker donor

There is a positive and significant correlation between G6PD activity and gluthation level r=0.408 and p=0.000. Glutathione level= 3,211 + 0,103 x G6PD activity. The higher the activity G6PD the higher the gluthatione level.

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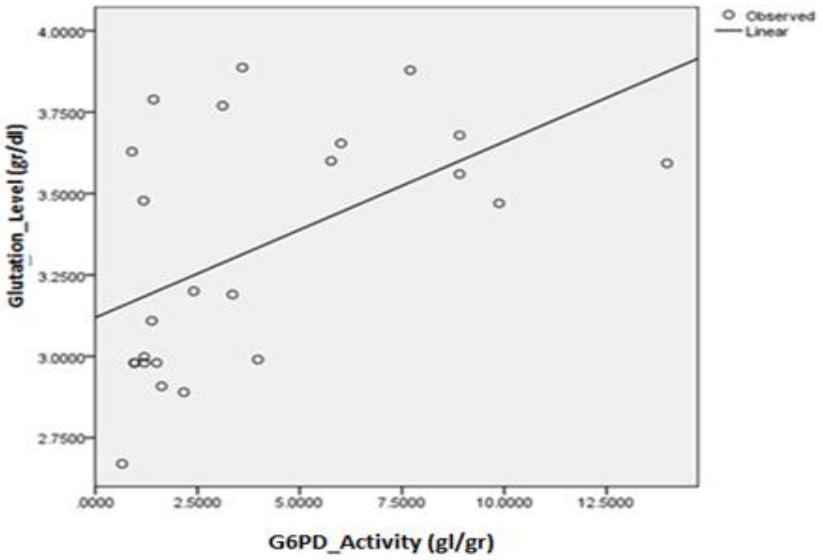

Figure 11: The relation ship between G6PD activity and gluthation level light smoker donor

There is a positive and significant correlation between gluthatione levels to G6PD activity r=0,274 and p=0.000. The higher the level of gluthatione the higher the activity of G6PD. On the contrary, there positive is corelation between the level of glutahion and G6PD activities, which suggested that the level of GSH are maintained also by activities of G6PD.(Fig.11)

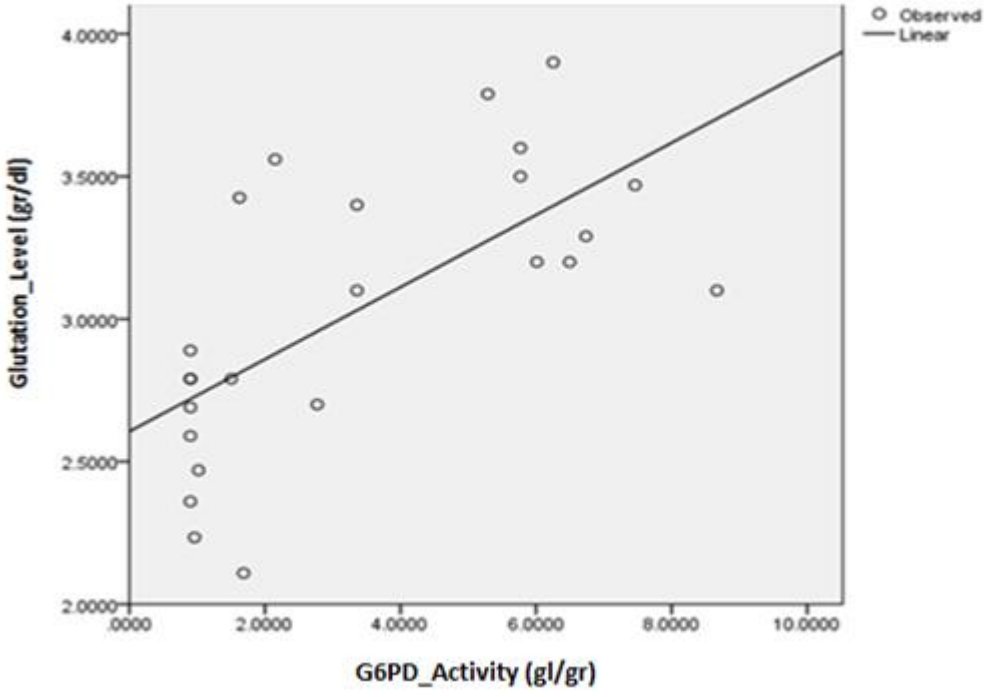

Figure 12: The relation ship between G6PD activity and gluthation level moderate smoker donor

There is a positive and significant correlation between G6PD activity and gluthation level r=0.442 and p=0.000. Gluthatione level = 2,606+0,127 x G6PD activity. The higher the G6PD activity the higher the gluthatione level.

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DISCUSSION

Cigarette is well known containing a huge number of foreign compound any many of them are oxidants or even radicals.[18] On the other hand, red blood cells main content, hemoglobin, is justly very susceptible to any oxidative attack. The iron content of hemoglobin, which in reality enable the hemoglobin to bind O_2, can easily oxidiezed even by an autooxidation process, which does not make involve any external oxidant. This fact explains, why even in normal and healthy condition, there is always oxidized hemoglobin, better known as methemeglobin. In very low concentration.[19] Hence, in the presence of a number of oxidant in alveoli, which is contamined by cigarette smoke, the methemoglobin conten increased even during 5 weeks of storage. To anticipate the increase of methemoglobin, red blood cells are equiped with reducing enzyme, methemoglobin reductase.[20–22]

Methemoglobin reductase will maintain normal methemoglobin levels below 1%.[19] Methemoglobin is formed due to biochemical processes that occur during storage. This process takes place continuously. The longer the blood is stored, the higher the methemoglobin, the faster the storage loss process.[20–22]Autooxidation of hemoglobin into methemoglobin is usually associated with the formation of hydrogen peroxide. The hydrogen peroxyde itself is reduced by catalase and glutathione peroxidase (GSH-Px).[19]

The difference in methemoglobin levels between non-smokers, light smokers and moderate smokers, depends on the amount of oxidants in the blood. It is also seen that the highest levels of methemoglobin are found in the blood of moderate smokers. The increase in methemoglobin during storage, in blood donors of non-smokers, light smokers and moderate smokers occurred starting on day 21. This happens, because there is still enough ATP to produce the coenzyme NADH which is required by methemoglobin reductase as an electron donor. And also because there are still sufficient intracellular antioxidants to convert oxidants so that not much Fe is oxidized. Red blood cells does not have mitochondria, so Adenosine Triphosphate (ATP) is obtained from the breakdown of glucose through anaerobic glycolysis, forming lactic acid. There is a decrease in pH, which will affect all enzymes activities. During storage, ATP will be reduced, the coenzyme NADH cannot be produced, methemoglobin reductase cannot work.[20,22,23]

The results of this study are supported by studies comparing the methemoglobin levels contained in the blood of leukodepleted PRC stored in anticoagulants, there are differences in methemoglobin levels between non-smokers and smokers. The difference was after 20 days of storage. They suggested that methemoglobin be measured periodically in the blood bank.24.[20,24]

Another study obtained more extreme results, namely an increase in methemoglobin on the 14th day of storage. The increasing of methemoglobin is found up to day 35 reached 96%. Increased levels of methemoglobin due to biochemical processes that occur during storage. They have recommended to use the blood for transfusions within7 days. There is a risk of storage lesions in the blood of smokers compared to nonsmokers.[20] It is recommanded abstinence from smoking for at least 16 hours before blood collection[22] The more oxidants contained in the blood, from cigarettes, the longer the blood storage, the more methemoglobin is formed. Methemoglobin levels are proportional to cotinine levels, in the blood of smokers. Oxidants in the blood can be overcome by antioxidants, both enzymatic, namely glutathione, SOD (superoxide dismutase), catalase (CAT), and non-enzymatic, namely Vitamin C and Vitamin E.[21]

The more oxidants, the more glutathione is used so that the glutathione levels will decrease. Red blood cells do not have the ability to resynthesize antioxidants. There is a relationship between glutathione produced and G6PD activity, if G6PD activity is reduced,

NADPH is reduced, and reduced glutathione production will decrease. Cigarettes can alter oxidant defenses, which is correlated with cotinine levels. Smoking is associated with increased oxidation in red blood cells, resulting in high activation of antioxidants which lead to its depletion. Cotinine-positive red blood cells show signs of oxidative stress.[15,25,26]

The mechanism of action of glutathione in tackling oxidants is to convert hydrogen peroxide into water. As a result, glutathione will be oxidized to GSSG. Glutathione will prevent hydroxyl radicals that will convert fat molecules into fat radicals (L) or lipid peroxides (LOO-). The decrease in glutathione during storage is associated with the occurrence of fat peroxidation. GSH prevents the occurrence of lipid peroxidation in red blood cell membranes. Finally preventing hemolysis and transfusion becomes effective.3 The mechanism of glutathione reduction is related to decreased G6PD activity.[21,26,27]

G6PD activity during in the blood of smokers and non- smokers decreased, the highest decrease was in the blood of moderate smokers due to the large number of oxidants in the blood. Nicotine found in cigarette can inhibit G6PD activity. Inhibition via binding to the site of the G6PD decrease in G6PD activity during storange of smokers blood. G6PD decreased significantly on average starting at 3 days of storange. G6PD is essential for protection against oxidative stress by producing NADPH.[28] NADPH is obtained from the Pentose Phosphate Pathaway. NADPH is used by glutathione to maintain intact Sulhydryl (SH) group in cell, on red blood cell membranes and to maintain iron in a functional Fe^{2+} state.[29] Decreased G6PD activity is associated with decrease ATP. Another study states that the decreased in G6PD activity was higher in unfiltered blood compared to filtered blood.[28]Ufelle et al, reported a greater decrease in G6PD activity after three weeks of storange.[26]

This study showed that there were significant differences in levels of methemoglobin, glutathione and G6PD activity between groups of non-smokers, light smokers and moderate smokers. Smoking habits exacerbate the occurrence of oxidant stress on stored red blood cells. Oxidants will be overcome by both enzymatic and non-enzymatic antioxidants. Glutathione is an enzymatic dependent antioxidant in cells. If antioxidants are not able to cope with oxidants, oxidative stress will occur. Oxidative stress threatens the survival of red blood cells. A study concluded that donor smokers with cotinine levels exceeding 10 ng/ml had higher glycolytic markers, increased oxidized glutathione, increased metabolism of the pentose phosphate pathway and increased fat.[8] Other study reported a reduction in nitrite-induced glutathione levels, up to 12% compared to baseline, along with an increase in methemoglobin levels and lipid peroxidation at the end of the study.[24]

Two molecules of reduced glutathione (GSH) will convert hydrogen peroxide to water and make reduced glutathione (GSH) of oxidized glutathione. These changes are catalyzed by the enzyme glutathione peroxidase. Meanwhile, glutathione reductase will convert GSSG into GSH again.

In this study, it was found that there was a positive correlation between G6PD activities and GSH levels. The higher the G6PD activities, the more GSH found. G6PD in this case as the caretaker on the PPP line. This is also consistent with D'Alessandro research.[7] It was stated that donors lacking G6PD had a decreased ability to recover intracellular glutathione, resulting in a tendency to oxidative stress and hemolysis in red blood cells.[30]

Another study concluded that in G6PD deficiency there was a decrease in NADPH in the PPP pathway, glutathione was reduced below normal, defense against oxidants decreased, susceptibility to oxidative stress, and hemolysis.[30] It was also recommanded to detect G6PD deficiency by measuring glutathione and GSSG levels.[31] Oxidative damage by free radicals has a negative effect on blood quality during storage.

CONCLUSION

Smoking habit impaired the function of antioxidant system in red blood cells and increased the methemoglobin content, a useless species of hemoglobin. All of these perturbations become accentuated with the course of storing time.

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