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RESEARCH ARTICLE

METHEMOGLOBINEMIA AND RAISED SERUM THIOBARBITURIC ACID REACTIVE SUBSTANCE LEVELS IN MALARIA

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Abstract

Background: Methemoglobinemia is found in patients with vivax and falciparum malaria. We study the relation between Methemoglobinemia and lipid peroxidation which is a marker of reactive oxygen species and its role as a biomarker in prognosis of patients with falciparum malaria.

Material and Methods: 133 patients of falciparum malaria and 25 control subjects were enrolled for the study. Blood was collected for estimation of Methemoglobin, thiobarbituric acid reactive substances (TBARS) calorimetrically. TBARS reflected amount of lipid peroxidation that indirectly measures reactive oxygen substances (ROS). The correlation between Methemoglobinemia, TBARS, parasites, and outcome of falciparum malaria was analyzed.

Results: The present study enrolled 133 patients of falciparum malaria of which 48 were with uncomplicated (UM) and 85 patients with severe falciparum malaria (SM) and 25 normal persons as controls. All were followed up to 7 days. Out of 85 patients 2, 3, 4, and 5 organ dysfunctions constituted 19 (22.3%), 38 (44.5%), 19 (22.1%), and 9 (12.0%) patients, respectively. The concentration of Met-Hb in UM was 4.1 ± 1.2 % ($p < 0.001$) and increased with SM to 11.1 ± 7.2 % (Table-1). After 7 days of treatment it tends to return to normal ($p < 0.001$). The level of TABRS was high in UM compared to controls and higher in SM than UM ($p < 0.001$). There is a significant correlation between Met-Hb and TABRS and with parasitemia. A positive correlation exists between parasite count and Met-Hb level ($r = 0.930$, $p < 0.0001$), between parasite count and serum TBARS level ($r = 0.948$, $p < 0.0001$) and between Met-Hb and serum TBARS level ($r = 0.917$, $p < 0.0001$). In total 15 cases (17.6%) died during the study and the patients who died had a high Met-Hb (14.5 ± 3.6 %) compared to survivors 11.4 ± 4.6 % ($p < 0.001$) and high TABRS (6.5 ± 2.6 nmol/ml) compared to the survivors 5.9 ± 3.6 nmol/ml ($p < 0.001$).

Conclusion: In the present study we found Methemoglobinemia is high among patients with UM and SM. Met-Hb also high among the patients

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who died. Methemoglobinemia is directly correlated to parasitic count, TABRS, and mortality. It can be used as a prognostic marker of SM.

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Introduction:-

The disease malaria is caused by protozoan parasite belonging to the genus Plasmodia which is an intraerythrocytic parasite. Consequent to invasion of RBC by the parasite a lot of metabolic and pathological alterations occur in the RBC that ultimately influences the hosts tissues and organs leading to complications¹.

Hemoglobin (Hb) is one of the most affected molecules during malaria infection because parasites degrade about 60-80% of Hb to use it as a source of amino acids required for the development of parasite during intraerythrocytic schizogony².

In this process, heme is oxidized to ferric form of Hb known as methaemoglobin (Met-Hb) which is incapable of oxygen transport. Elevated level of Met-Hb has been detected in malaria and has been associated with adverse outcome³. Hb degradation generate free heme and reactive oxygen species (ROS) which are deleterious to parasite and host. In the presence of ROS free cell free Hb dimers oxidized to Met-Hb, releasing heme prosthetic group. Free heme is highly toxic and along with Met-Hb has a pathogenetic role in various forms of severe malaria including cerebral malaria⁴.

Further, there are reports of life threatening methemoglobinemia with history of ingestion of chloroquine and exposure to aniline dye with antimalarial^{5,6}.

ROS damage all the major biomolecules e.g. proteins, carbohydrates, nucleic acids, and lipids⁷. The oxidation of lipids is known as lipid peroxidation and the extent of lipid peroxidation is also a measure of ROS. The oxidation of lipids results in a rise of Malonaldehyde and it is estimated by thio-barbituric acid (TBA) method which has been used as an index of oxidative damage because of its sensitivity and simplicity⁸. Enhanced lipid peroxidation as evidenced by raised thio-barbituric acid reactive substances (TBARS) has been detected among patients with falciparum and vivax malaria and correlated with severity⁹. Therefore, we have undertaken this study to estimate Met-Hb in adult falciparum malaria and correlate it with parasitemia and TABRS that reflect oxidative stress and its usefulness in the prognosis of patients with SM.

Material and Methods:-

This study was undertaken at the Department of General Medicine, Veer Surendra Sai Institute of Medical Sciences and Research, Burla, Sambalpur.

After permission from Ethical Committee we enrolled patients of uncomplicated falciparum malaria (UM), severe falciparum malaria (SM), and healthy controls from Medicine outdoor and indoor for determination of Met-Hb from January 2017 to December 2019.

The diagnosis of malaria was made with detection of asexual form of malaria parasite from Giemsa stained peripheral blood smear or with rapid diagnostic test and Quantitative Buffy Coat. SM was diagnosed according to the criteria of World Health Organization¹. Patients of SM were treated with Artesunate as per guidelines of WHO¹⁰.

Patients with history of ingestion of chloroquine, primaquine, pamaquine within 15 days had been excluded from the study. Patients with pregnancy, G-6PD deficiency, exposure to dye are also excluded.

On admission, blood was collected for biochemical investigations complete blood count, blood glucose, renal function test, and liver function test in all patients.

For determination of Met-Hb 5 ml of EDTA blood was collected and within 2 hours RBCs were separated from plasma by centrifugation with the buffer solution Tris HCL. The washed RBCs were lysed and hemolysate was used for the determination of Met-Hb concentration by the method of Evelyn and Mallory calorimetrically^{11,12}.

TBARS estimation was done calorimetrically by the method of Satoh. For this, 0.5 ml of serum was taken and to it 2.5 ml of trichloroacetic acid was added and centrifuged at 3500 rpm for 10 minutes. The precipitate was washed with sulfuric acid and next 3.0 ml of TBA was added, heated for 30 minutes, 4.0 ml of n-butanol was added, and chromogen was extracted by centrifugation at 3000 rpm for 10 minutes. The absorbance was measured at 532 nm in spectrophotometer⁷.

Statistical analysis was performed by student's t test and Pearson's rank correlation with SPSS version 11.

Results:-

The present study enrolled 133 patients of falciparum malaria and 25 control subjects. Out of 133 patients, 48 were with uncomplicated (UM) and 85 patients with severe falciparum malaria (SM) and all were followed up to 7 days. There was a male preponderance in the study (4:1), and they were in the 20-35 years of age group; females were in 40 to 50 years of age. Out of 85 patients with SM hepatic failure was the most common organ system failure (n=58; 68.2%), followed by neurological (n=50;58.8%), renal (n=40;47.8%), hematological (n=30; 35.2%), and, respiratory failure (n=15; 17.6%). Two, 3, 4, and 5 organ dysfunctions constituted 19 (22.3%), 38 (44.5%), 19 (22.1%), and 9 (12.0%) patients, respectively. Cerebral malaria, hepatic, and renal involvement was the most common combination (n=38,44.5%) of organ dysfunctions found in this study.

The concentration of Met-Hb in UM was 4.1 ± 1.2 % ($p < 0.001$) and increased with SM to 11.1 ± 7.2 % (Table-1). After 7 days of treatment it tends to return to normal ($p < 0.001$). The level of TBARS was high in UM (2.01 ± 0.61 nmol/ml)

compared to controls (1.05 ± 0.29 nmol/ml) and higher in SM (4.2 ± 1.03 nmol/ml) than UM ($p < 0.001$). The mean parasitic count was 1157.0 ± 516.8 /cmm among UM and 4383.8 ± 1274.6 /cmm. There is a significant correlation between Met-Hb and TBARS and with parasitemia. A positive correlation exists between parasite count (/μL blood) and Met-Hb (%) level on first day of admission ($r = 0.930$, $p < 0.0001$). Positive correlation also exists between parasite count (/μL blood) and serum TBARS level in nmol/ml ($r = 0.948$, $p < 0.0001$) and between Met-Hb (%) level and serum TBARS level in nmol/ml ($r = 0.917$, $p < 0.0001$). Scatter diagram showed a positive relation between Met-Hb and TABRS (Fig-1), parasitic count with TBARS and Met-Hb (Fig-2,3).

In total 15 cases (17.6%) died during the study. Out of them 5 (33.3%) belonged to 3 whereas rest 10 (66.6%) had 4 or more organ dysfunctions. Analysis of death out of patients with number of organ dysfunction showed that 1 (5.2%), 4 (10.5%), 5 (26.3%), and 5 (55.5%) belonged to 2, 3, 4, and 5 organ dysfunctions, respectively (Fig.4). The patients who died had a high Met-Hb (14.5 ± 3.6 %) compared to survivors 11.4 ± 4.6 % ($p < 0.001$) and high TBARS (6.5 ± 2.6 nmol/ml) compared to the survivors 5.9 ± 3.6 nmol/ml ($p < 0.001$).

Discussion:-

Methemoglobin (Met-Hb) is generated by oxidation of the heme iron moieties to the ferric state. Due to high oxygen affinity, Met-Hb does not deliver oxygen at tissue level causing hypoxia^{2,4}. Therefore, the oxidation of Hb to Met-Hb is prevented by the presence of an efficient Met-HB reductase system in the RBC to keep Met-Hb concentration less than 1% in blood. When Met-Hb in blood is more than 2%, it is known as methemoglobinemia¹³. The causes may be congenital or acquired. Congenital Met HB arises from globin mutation that stabilize iron in the ferric state (Hb M Iwata, or mutation of enzyme that reduce Met-Hb to Hb (Methemoglobin reductase, NADP diaphorase). Acquired Met-Hb is caused by toxins that oxidize heme iron including drugs like dapson, primaquine, pamaquine, sulfonamides¹³.

Malaria is an infective cause of methemoglobinemia. In the erythrocytic stage, Hb that is taken up by the food vacuole of the parasite is oxidized to Fe^3 form and formed super-oxides^{2,3}.

The present study showed that Met-Hb is a frequent finding among patients with falciparum malaria and it is directly related to parasitemia and degree of lipid peroxidation as evidenced from rise of TBARS. In uncomplicated malaria it is about 4% whereas it increased about 3fold (up to 11%) with severe malaria. The Met-HB increased with different complications and was high among patients with death. High Met-Hb was found not only in adult malaria but also in childhood malaria in Tanzania¹⁴. It is also found in all species of malaria and among patients with falciparum malaria with Sickle cell disease (HbSS) and trait (HbAS)¹².

The mechanism of Met-Hb was due to oxidative stress during the development of the parasites inside RBC. The present study revealed that there is a positive correlation between TBARS concentration and parasitic count. It suggests that increased activity of Met-Hb is due to oxidative injury which is due to parasitemia. Further it is coupled with decreased antioxidant enzyme. Experimentally, it has been found that erythrocytic methemoglobin reductase activity is decreased in *P.yoelli* infected mice causing increased Met-Hb. Raised oxidative stress compounded by compromised activity of erythrocyte redox enzymes exacerbate the tendency towards spontaneous oxidation of Hb molecule in pRBC¹⁵.

With treatment after 7 days the Met-Hb concentration decreased. It supports the reports that showed decreasing Met-Hb concentration with time after administration of antimalarial suggests decreasing level of ROS due to the killing of parasites¹⁶. The highest mean level of Met-Hb observed in this study is $14.5 \pm 3.6\%$ which is found among the patients who died. Clinical findings in patients with excessive Met-Hb correlate to blood levels and Met-Hb $< 20\%$ provokes no signs and symptoms. When it exceeds 20% it causes dyspnea with a characteristic bluish-brown muddy color resembling cyanosis and levels $> 50-60\%$ is fatal¹⁶.

In severe malaria in the presence of parasite sequestration impaired tissue perfusion and hypoxia occur and it has an important role in its pathogenesis. Reduction of oxygen carrying capacity for even with a modest concentration of Met-Hb is likely to exacerbate tissue hypoxia¹⁴. In the present study we found the mortality is 15.2% . The mortality increases with increasing in number of organ dysfunction and the level of Met-Hb also high among the patients who died.

In summary the present study showed that Met-Hb is high among patients with SM and correlate with increased lipid peroxidation and parasitic count. It can be used as a biomarker for prognosis of SM.

Table-1:- Met-Hb and TBARS in falciparum malaria.

Categories	Met HB (%) (Day-1)	Met HB (%) (Day-7)	P value	TABRS (nmol/ml) (Day-1)	TABRS (nmol/ml) (Day-7)	P value
Control (n=25)	0.1 ± 2.1			1.05 ± 0.29		
UM (n=48)	3.79 ± 1.18	0.86 ± 0.51	<0.001	2.01 ± 0.61	1.1 ± 1.2	<0.001
SM (n=85)	11.16 ± 4.4	2.13 ± 1.07	<0.001	4.2 ± 1.03	0.9 ± 2.5	<0.001
Anemia (n=30, 35.2%)	9.2 ± 1.4	2.4 ± 2.1	<0.001	4.8 ± 0.4	1.2 ± 1.1	<0.001
Jaundice (n=58, 68.2%)	9.6 ± 1.5	3.7 ± 1.2	<0.001	4.5 ± 0.5	1.2 ± 3.2	<0.001
ARDS (n=15, 17.6%)	10.9 ± 2.3	2.5 ± 1.3	<0.001	4.6 ± 4.3	0.9 ± 1.3	<0.001
Cerebral malaria (n=50, 58.8%)	11.8 ± 3.5	2.4 ± 0.8	<0.001	3.8 ± 1.5	0.8 ± 1.8	<0.001
MODS (n=85)	10.5 ± 5.2	1.7 ± 0.8	<0.001	5.2 ± 0.2	1.4 ± 0.8	<0.001
Survivors (n=70, 82.3%)	11.4 ± 4.6	1.1 ± 2.8	<0.001	5.9 ± 3.6	0.7 ± 2.8	<0.001
Death (n=15, 17.6%)	14.5 ± 3.6			6.5 ± 2.6		

Fig.1:- Scatter diagram between Met-Hb and TBARS.

Simple Scatter with Fit Line of Serum Methemoglobin in % on Day 1 by Serum Thiobarbituric Acid Reactive Substance in nmol/ml on Day 1

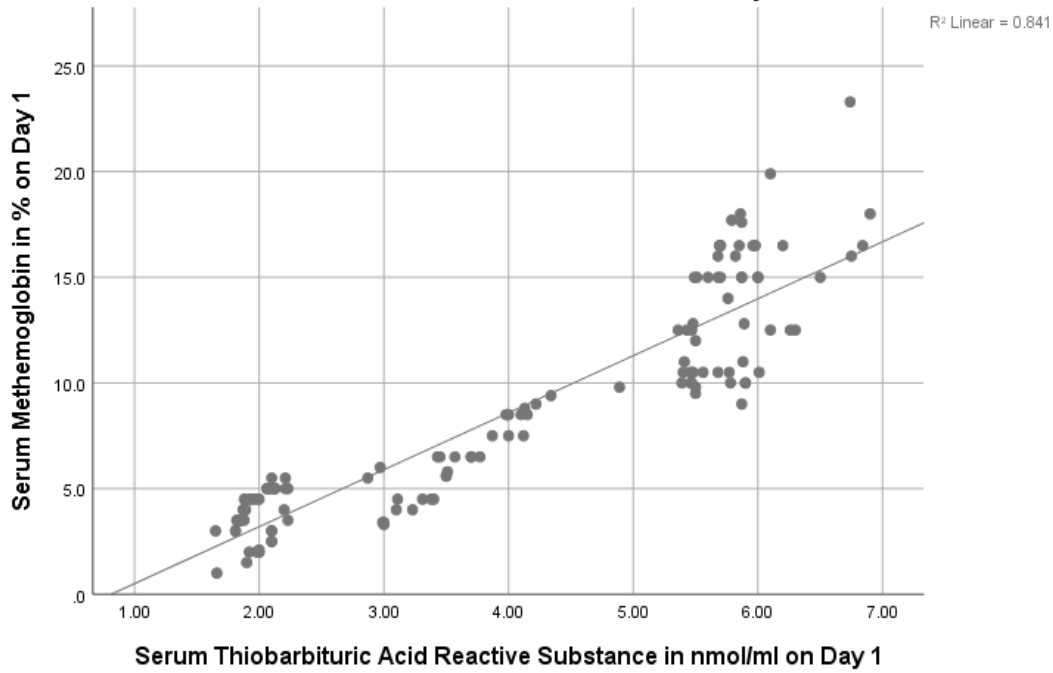


Fig.2:- Scatter Diagram between parasitic count and TBARS.

Simple Scatter with Fit Line of Serum Thiobarbituric Acid Reactive Substance in nmol/ml on Day 1 by No of Parasites / microliter of blood

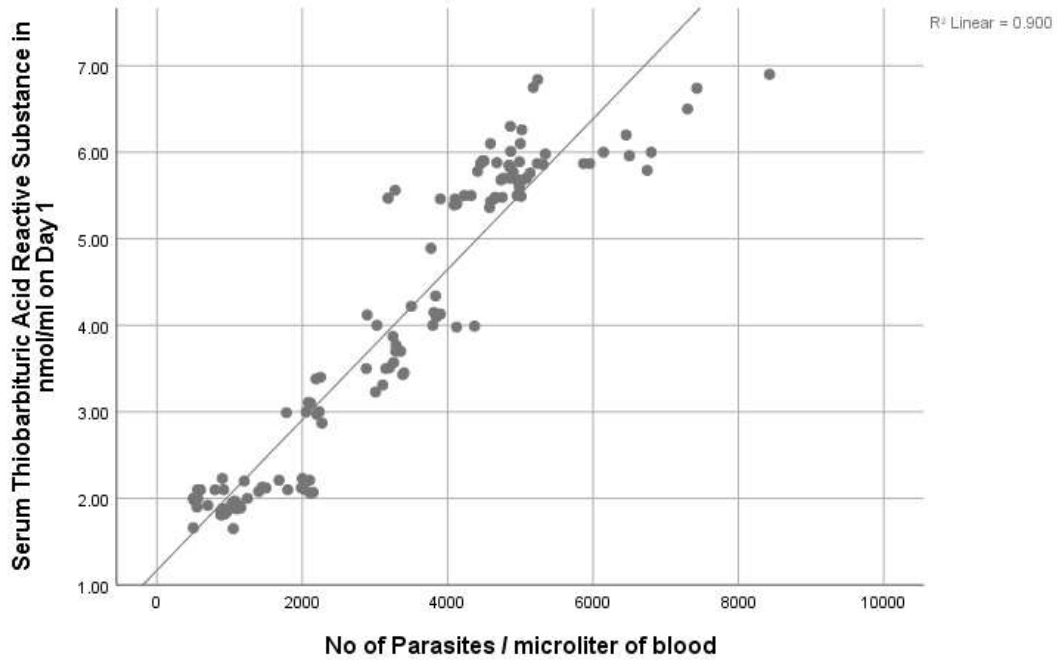


Fig.3:- Scatter Diagram between parasitic count and Met-Hb.

Simple Scatter with Fit Line of Serum Methemoglobin in % on Day 1 by No of Parasites / microliter of blood

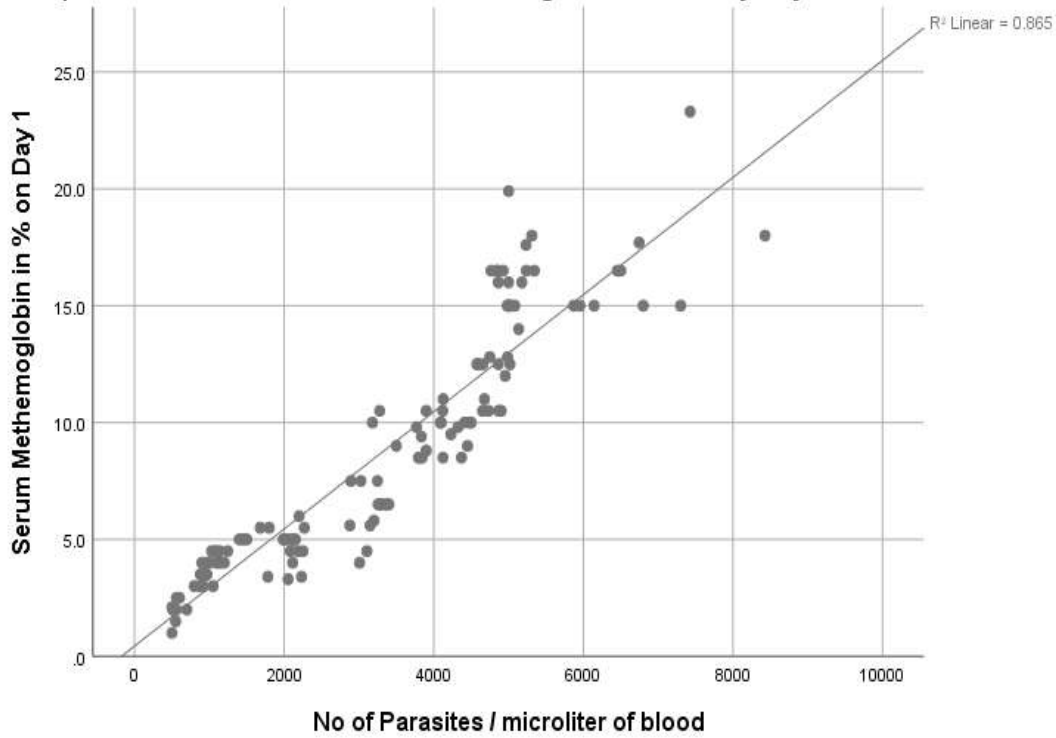
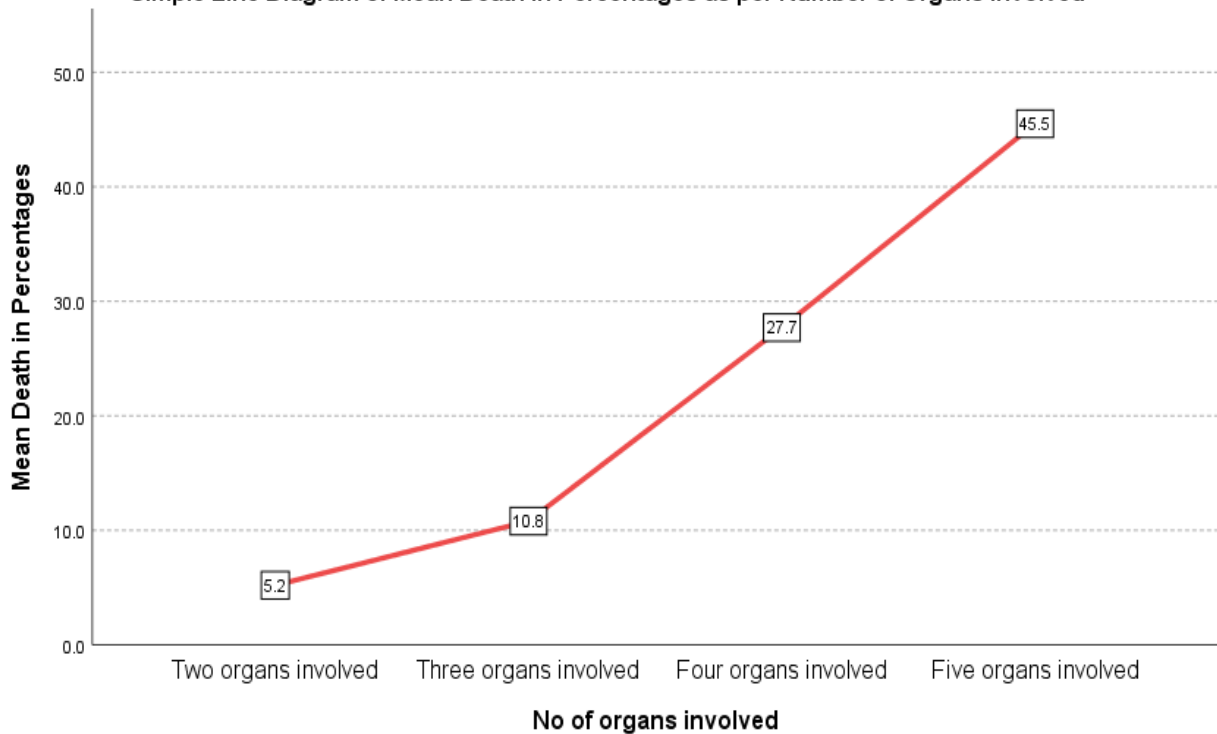


Fig.4:- Death in percentage and number of organ dysfunction.

Simple Line Diagram of Mean Death in Percentages as per Number of Organs Involved



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