

## Nitroblue Tetrazolium Reduction Test, Malondialdehyde And Ascorbic Acid Levels In Sepsis And Non-Haematological Malignancies

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**Abstract:** Malignancies and infections are associated with oxidant – antioxidant imbalance. The polymorphonuclear leukocytes generate huge amount of reactive oxygen species. The end product Malondialdehyde generated is responsible for the damaging effects on DNA and cell membrane. This study was undertaken to assess the oxidative stress using nitroblue tetrazolium scores and malondialdehyde levels, and to assess antioxidation by vitamin C levels in patients with sepsis and non-hematological malignancies. Nitroblue Tetrazoleum(NBT) reduction test, serum Malondialdehyde(MDA) and vitamin C levels were estimated among 30 healthy persons,30 septic cases,30 malignant cases and 30 with both sepsis and malignant cases. All three parameters of all four groups were compared. In our study, NBT & MDA both of which are markers of oxidative stress were increased in patients with sepsis and malignancy when compared with control groups and the  $p$ -value < 0.001 was statistically significant. In contrast these groups showed decrease in Vitamin C levels which acts as an antioxidant. Increased NBT and MDA levels serve as diagnostic and prognostic markers for increased oxidative stress in patients with sepsis and malignancy and antioxidant therapy may be initiated for the well being of these patients.

**Keywords:** NBT, Malondialdehyde, Vitamin C, Sepsis, Oxidative- stress, Antioxidants

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### I. Introduction

In aerobic life, oxidative stress arises from both endogenous and exogenous sources. Despite antioxidant defence mechanisms, cell damage from oxygen free radicals is ubiquitous<sup>1</sup>. White blood cells have a fundamental role in defence against invading micro organisms and the recognition and destruction of neoplastic cells as well as their role in acute inflammatory reactions. On neutrophil activation, a significant increase in oxygen consumption, termed respiratory burst occurs that leads to the production of free radicals responsible for microbial killing<sup>2</sup>. Immune cells use reactive oxygen species [ROS] in order to support their functions and need adequate levels of antioxidant defences to avoid harmful effects of an excessive ROS production.<sup>3,5</sup>

The polymorphonuclear leukocytes generate huge amounts of ROS that induces lipid peroxidation, which affects unsaturated fatty acids localized in cell membranes. The end product Malondialdehyde (MDA) generated is responsible for the damaging effects of free radicals on DNA and cell membranes<sup>6</sup>. Lipid peroxidation products diffuse from the site of inflammation and can be measured in the blood<sup>7</sup>. Both cancer and infection are associated with oxidant-antioxidant imbalance<sup>8</sup>. The damage produced by reactive oxygen species may be prevented by the antioxidants like Catalase, superoxide dismutase, vitamin C, E<sup>5</sup>. The present study was undertaken in patients with sepsis and malignancy to assess oxidative stress using histochemical Nitroblue tetrazolium test (NBT) and measuring Malondialdehyde (MDA) levels. The antioxidant status was studied by measuring vitamin C levels.

### II. Objectives Of The Study

- To assess the oxidative stress and antioxidant levels in patients with sepsis and non haematological malignancies.
- Role of Nitroblue tetrazolium (NBT) in patients with sepsis and non-hematological malignancies.
- To assess serum malondialdehyde (MDA) levels as a marker of lipid peroxidation in patients with sepsis and non-hematological malignancies.
- Vitamin C as an indicator for antioxidant levels in sepsis and non-hematological malignancies.
- To compare NBT scores, MDA levels and Vitamin C levels in sepsis and non-hematological malignancies.

### III. Materials And Methods

The present study comprises of 120 patients, from Dr. Pinnamaneni Siddhartha Institute of Medical Sciences and Research Foundation, Vijayawada. The study was undertaken during June 2012 to September 2014.

The study constitutes of 4 groups with 30 subjects in each group:

1. Healthy volunteers as control group,
2. Patients with sepsis,
3. Patients without infection with non haematological malignancy,
4. Patients having infection and non haematological malignancy.

Healthy blood donors were taken as the control group. Thorough clinical history was taken and haematological tests done were within normal limits. Criteria for sepsis was adapted from the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference and, the diagnostic criteria advanced by the 2001 International Sepsis Definition Conference<sup>1</sup>. Two or more of the following are required:

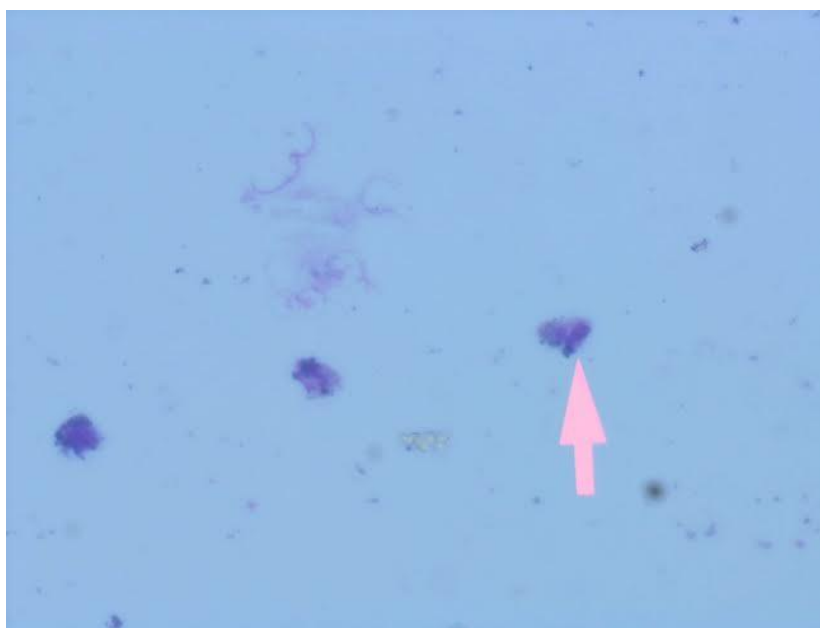
- 1) Body temperature  $>38^{\circ}\text{C}$  or  $<36^{\circ}\text{C}$
- 2) Heart rate  $>90$  beats per minute
- 3) Respiratory rate  $>20$  breaths per minute or arterial  $\text{CO}_2$  tension less than 32 mm Hg or a need for mechanical ventilation
- 4) White blood count greater than  $12,000/\text{mm}^3$  or  $<4000/\text{mm}^3$  or  $>10\%$  immature forms.

The cases were later confirmed by microbiological culture.

Non haematological malignant cases were diagnosed by FNAC, endoscopic biopsies and tumor markers. The cases were later confirmed by histopathological examination.

Informed consent was taken from all the cases and the study was approved by the ethical and research committee of Dr. PSIMS & RF. 10 ml of blood was collected from all cases, of which 0.1ml of blood was used for histochemical Nitroblue tetrazolium test, 1ml of blood was used for estimation of Malondialdehyde (Thiobarbituric acid method) and another 4 ml of plasma was used for estimating vitamin C levels (2,6-dichlorophenolindophenol titration method). Statistical analysis was carried using Epi-info version 7 software.

**III.1 Histochemical nitroblue tetrazolium test<sup>2,3</sup>:** 0.1 ml of EDTA anticoagulated blood is taken into a polyethylene test tube, mixed with equal volume of 0.2% nitroblue tetrazolium in pH 7.2 phosphate buffered saline. The mixture is incubated in a moist  $37^{\circ}\text{C}$  chamber for 30 minutes. Smears are made from the incubated blood sample and counterstained with Giemsa for 20 minutes. The slides are washed with 7.6 pH buffer and dried. A total of 100 consecutive polymorphonuclear neutrophils are identified and the percentage containing large blue black formazan crystals are recorded as NBT score. Only polymorphonuclear neutrophils which are identified with certainty are counted.



**Fig . 1-** NBT positive neutrophils under oil immersion field

**III.2 Estimation of Malondialdehyde (MDA) in whole blood<sup>4</sup>:** Malondialdehyde as end product of fatty acid peroxidation reacts with thiobarbituric acid to form a pink coloured complex having an absorption maximum at 535nm. MDA values were calculated from absorbance co-efficient of MDA – TBA complex at 535nm.

**Table 1**

	Blank(ml)	Test(ml)
Whole blood	-	0.75
Distilled water	0.75	-
Thiobarbituric acid reagent	3	3

Calculation:

Malondialdehyde (nanomoles/ml)

$$= \frac{\text{Absorbance of test} \times \text{total volume}}{\text{Nanomolar extension coefficient} \times \text{sample volume} \times 100}$$

$$= \frac{\text{Absorbance of test} \times 3205.1}{100} \text{ nmol/l}$$

**III.3 Determination of plasma ascorbate by 2,6- Dichlorophenolindophenol titration<sup>5</sup>:** Plasma is separated immediately after collection of blood. 4 ml of plasma is taken into a clean dry test tube and mixed with 4 ml of trichloroacetic acid solution and the mixture is centrifuged for 2 minutes. 200µl of the dye is pipetted into another clean test tube and the dye is titrated using the supernatant obtained from the former solution until the reddish colour is discharged.

Calculation: As 200µl of the dye is equivalent to 8µg ascorbate,  
 Plasma ascorbate (mg/dl) = 1.6/ml titration

#### IV. Results

**IV.1 Age distribution:** In the control group, 86% of the people were in the age group of 20 -40 years. In patients with sepsis majority (46%) of the patients fell in the 20–40 years age group. 50 % of patients with malignancy were in 40-60 years age range, whereas in patients with malignancy and infection 57% of the patients were in this range and 36% were > 60 years of age. None of the patients with malignancy and malignancy with infection were <20 years of age.

**IV.2 Gender distribution:** 93% of the controls were males and only 7% were females. In patients with sepsis 73% were males and 27% were females. In patients with non haematological malignancies 56% were males and 44% were females. The male to female ratio in patients with non haematological malignancy with sepsis was 1:1.

**IV.3 WBC counts and morphology:** The haematological parameters in sepsis had higher levels of total WBC count greater than 11000cells/µl with increased neutrophils in the differential counts.

**IV.4 Nitroblue tetrazolium values:** In smears, neutrophils containing blue black precipitate in the cytoplasm were counted and the score was expressed as a percentage. The values obtained in controls had a range from 3% to 12%. Values above 10% were seen in only 3 of the controls. The values in patients with sepsis ranged from 08% to 30% with 53% of the patients in the range of 20 -30%. In cases with malignancies only one patient showed normal NBT level of less than 10% whereas majority fell in the range of 11-20% accounting for 53% of the malignant patients. The distribution was in a higher range among patients with both sepsis and malignancy with 33% of patients having levels higher than 30% positivity of NBT values and majority showed levels from 21 -30% NBT positivity being 60% of these cases.

The mean value of histochemical nitroblue tetrazolium (NBT) test in controls was 6.06±2.34%. The value in sepsis was 20.96±4.16%, in patients with non haematological malignancies was 20.2±4.54% and in patients with non haematological malignancies with sepsis was 28.1±4.58%. The levels of NBT was significantly increased (p<0.01) in all the three categories of cases compared to the controls.

**IV.5 Malondialdehyde values:** The mean value of MDA in controls was 0.39±0.12 nmol/ml. The value in sepsis was 1.55±0.37 nmol/ml, in cases with non haematological malignancies was 1.34±0.36 nmol/ml and in cases with non haematological malignancies with sepsis was 2.11±0.31 nmol/ml. The mean value was high in

the cases and showed significant increase ( $p < 0.01$ ) and particularly very high in cases with both non haematological malignancy and sepsis compared to other groups.

**IV.6 Vitamin C values:** The normal values of Vitamin C levels are 0.5 to 1.5 mg/dl<sup>6</sup>. The range of vitamin C obtained in controls examined was from 0.58 to 1.46 mg/dl and the mean value of Vitamin C in controls was  $0.96 \pm 0.22$  mg/dl. In patients with sepsis, range was from 0.28 to 0.76mg/dl. 11 patients had normal levels of vitamin C. The mean value in cases with sepsis was  $0.45 \pm 0.13$ mg/dl. The levels of vitamin C in patients with malignancy were in the range of 0.24 to 0.66 mg/dl and the mean calculated was  $0.45 \pm 0.11$ mg/dl and 12 patients had normal levels of Vitamin C. In patients with both non haematological malignancies and with sepsis and only 8 patients had levels  $> 0.5$  mg/dl and the mean value was  $0.39 \pm 0.11$ mg/dl.

**TABLE 2 : Comparison of parameters between control and case groups:**

The t test, ANOVA test, Tukey HSD test, Mann-Whitney test performed on the values for MDA and Vitamin C showed to be statistically highly significant ( $p$  value  $< 0.01$ ) in all the case groups when compared with controls.

Control Vs case		T	df	Z	P-value	Inference
Sepsis	MDA	-15.949	58	-6.576	.000	Highly significant
	VIT- C	11.001	58		.000	Highly significant
Malignancy	MDA	-13.475	58	-6.625	.000	Highly significant
	VIT-C	11.192	58		.000	Highly significant
Malignancy with sepsis	MDA	-28.080	58	-6.671	.000	Highly significant
	VIT-C	12.617	58		.000	Highly significant

## V. Discussion

### V.1 Nitroblue tetrazolium test:

In the present study the mean level of histochemical NBT test was significantly increased in all the three groups of patients compared to the control group .Our findings are in accordance with James R. Humbert et al<sup>2</sup>, Gordon AM et al<sup>7</sup>, and Hellum KB et al<sup>8</sup>, who conducted the study on patients with sepsis. They investigated Nitroblue tetrazolium (NBT) reduction by phagocytic leukocytes in subjects with and without infections. Acute bacterial infections were most often associated with a high percent of NBT-positive neutrophils, and showed that the NBT histochemical test to be a useful adjunct to microbiologic techniques in establishing the diagnosis in patients with suspected infection. This test proved to be useful as an adjunct in differentiating bacterial infections from non bacterial infections.

Kataeva GV et al<sup>9</sup>, observed the reaction of NBT reduction by leukocytes from patients with lung cancer and found elevated indices compared to controls. The results also showed good correlation with results from studies conducted by Philip Ashburn et al<sup>10</sup> and Jedrezejczak WW et al<sup>11</sup>, on patients with non haematological malignancies with and without infections & all of them concluded that the NBT test to be very useful for infection screening in cancer patients similarly as in normal persons.

### V.2 Malondialdehyde:

In our study there was a significant rise in the lipid peroxidation product, malondialdehyde compared to the control group ( $p < 0.01$ ) in patients with sepsis, non haematological malignancies and non haematological malignancies with sepsis.

Our study is in accordance with Irfan Altuntas et al<sup>12</sup>, Leonardo Lorente et al<sup>13</sup> and Diana Muhl et al<sup>14</sup>, in patients with sepsis, elevated MDA levels indicating increased oxidative stress.

The levels of MDA also matched with studies by Yalcin et al<sup>15</sup>, Amasyali AS et al<sup>16</sup>, in patients with carcinoma of the bladder showed increased levels of MDA indicating increased oxidative stress in these patients with high rates of progression and recurrence in their studies.

Similarly, the same MDA was used as a marker of oxidative stress by Naidu MS et al<sup>17</sup>, in cervical cancers, Richard Salzman et al<sup>18</sup>, in oropharyngeal squamous cell carcinomas, Aparna R. Bitla et al<sup>19</sup>, in carcinoma of stomach, Sanjyoti Bundebuche et al<sup>20</sup>, in ovarian cancers. They concluded with high levels of MDA in the blood of patients with these cancers indicating free radicals to play a significant role in the pathogenesis and progression of cancers.

### Vitamin C:

The mean levels of plasma Vitamin C in patients with sepsis were significantly decreased in patients with sepsis in accordance with Cowley HC et al<sup>21</sup>, Borrelli E et al<sup>22</sup>. The levels in sepsis also decreased similar to studies conducted by Yalcin O et al<sup>15</sup>, in bladder cancers, Naidu MS et al<sup>17</sup>, in cervical cancers.

Values obtained in malignancy with sepsis were found to be low similar to findings observed in the study conducted by Thomas E Ichim et al<sup>23</sup>, in which ascorbic acid levels were very low in patients with advanced cancer, and once patients develop sepsis further decrease in Vitamin C levels.

## VI. Conclusion

Nitroblue tetrazolium and Malondialdehyde, both of which are markers of oxidative stress are increased in patients with sepsis and malignancy compared to control group. In contrast these groups show decreased values of Vitamin C which acts as an antioxidant. The NBT & MDA levels are much higher in patients with both malignancy and sepsis and the p- value <0.001 which is significant statistically. The antioxidant level is also markedly reduced. Increased NBT & MDA levels serve as markers for increased oxidative stress in these diseases. In view of the raised oxidative stress, antioxidant therapy may be initiated for the better well being of these patients & requires further study in this regard.

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