



# Use of Nitroblue Tetrazolium Test: Revisited in Context of COVID-19

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Coronavirus disease 2019 (COVID-19) is a pandemic caused by  $\beta$  coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-COV2) in which the virus binds to host cells via angiotensin-converting enzyme receptor, and the primary T cell immune response leads to recovery in asymptomatic/mild infections.<sup>1,2</sup> However, in severe and critical type of illness, SARS-COV2 elicits an aberrant immune response involving interplay of events such as oxidative stress, lymphocytic cytolysis, release of cytokines, chemotaxis, neutrophilia, and formation of neutrophil extracellular traps (NETs).<sup>3</sup> Increased levels of circulating NETs indicate neutrophil activity. The viral pathogenicity and altered immune response together led to deleterious series of events culminating in acute respiratory distress syndrome (ARDS) and coagulopathy.

Oxidative stress lies at the heart of many diseases and is defined as an imbalance between production of toxic reactive oxygen species like hydroxyl ion, superoxide anion, hydrogen peroxide ( $\text{OH}^-$ ,  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$ ), and antioxidant mechanisms (ascorbic acid, glutathione, and vitamin E and A) leading to oxidative damage.<sup>4–6</sup> This oxidative damage can be lipid peroxidation and DNA (deoxyribonucleic acid) oxidation. These can lead to cell and nuclear membrane damage and nuclear decondensation, and oozing out of nuclear material forming NETs. Oxidative stress can be measured by techniques like detection of reactive oxygen species by using carboxy dihydrodichlorofluorescein diacetate (carboxy H2DCFDA)/flow cytometry based dichlorofluorescein (DCF) dye test and an age-old technique of nitroblue tetrazolium test (NBT) that detects the oxidative burst activity. NBT was introduced by Park et al<sup>7</sup> in 1968 by which neutrophils can be divided into NBT-positive and NBT-negative depending upon whether they reduce the dye to blue black

formazan compound or not.<sup>7</sup> NBT with stimulating agents like phorbol myristate acetate (PMA) is a conventional test for screening for chronic granulomatous disease and its carriers.<sup>8</sup> In healthy subjects, we usually do not expect to see much NBT positivity in unstimulated smears and we see significant NBT positivity with PMA substrate stimulation. Also, PMA has been used to develop NETs in in-vitro settings.<sup>9</sup>

Here, we would like to present our brief experience of NBT test positivity in unstimulated (NBT-US) samples of COVID-19 patients from intensive care unit (ICU), when we tried to study the metabolic activity of neutrophils. All the patients were either on noninvasive or invasive mechanical ventilation and hence were categorized as severe ARDS. These patients' age ranged 23 to 67 years with a mean age of 42 years, and there were 8 males and 3 females. The ICU/hospital stay ranged from 3 to 31 days with a mean of 14.09 days. The absolute neutrophil count ranged from 7,740 to 24,990/ $\mu\text{L}$ . The absolute lymphocyte count ranged from 410 to 2,010/ $\mu\text{L}$ . The neutrophil to lymphocyte ratios ranged from 3.85 to 28.24 with a mean of 13.28. Also, an age- and gender-matched small control of non-ICU non-COVID-19 patients was taken.

An NBT stock solution without stimulant was prepared by taking 20 mg of NBT powder (nitroblue tetrazolium powder by Sigma, catalogue No. 1.24823, Sigma-Aldrich Chemicals Pvt. Ltd., Bommasandra Jigani Link Road, Anekal Taluk, Bangalore) and dissolving in 20 mL normal saline. This stock solution is kept under refrigeration. Steps for the NBT test assay performed on peripheral blood were as follows: a tube each for test and control was taken. A 100  $\mu\text{L}$  of the unstimulated stock solution was put in each of the tube. After that 100  $\mu\text{L}$  of ethylenediaminetetraacetic acid sample of patient

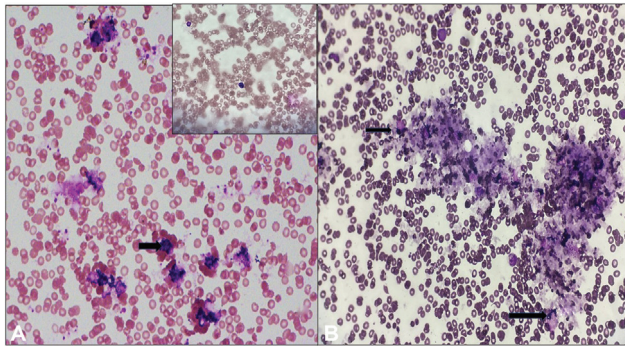
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**Fig. 1** (A) The photomicrograph showing blue black formazan (arrow) precipitates in the cytoplasm of neutrophils. Inset indicates one neutrophil showing blue-black cytoplasmic formazan precipitates in unstimulated preparation and one neutrophil without precipitates (nitroblue tetrazolium test-unstimulated [NBT-US];  $\times 200$ ). (B) Another case showing conglomerate of neutrophils with nuclear material showing less condensation and streaking resembling neutrophilic extracellular traps. The cytoplasm shows formazan compound (arrows) and at many places cytoplasmic boundaries cannot be appreciated. (NBT-US;  $\times 100$ ).

and control was put in respective tubes. After covering the tubes with aluminum foil, they were kept in an incubator for 15 minutes at 37 degrees. After incubation smears were made and air dried. Counterstain with Leishman stain was done. NBT-positive neutrophil percentage as well as absolute NBT-positive neutrophil count was calculated. NBT-positive neutrophil percentage is calculated by examining 200 consecutive neutrophils on the smear stained as mentioned above. Monocytes and eosinophils were not calculated. Absolute NBT-positive neutrophil count was calculated by multiplying the NBT percentage with absolute neutrophil count obtained from cell counter to obtain the absolute NBT-positive neutrophil count.

In the studied 11 cases of ICU COVID-19 samples, NBT-positive neutrophil percentage ranged from 6 to 53% in unstimulated smears with a mean of 13.8% ( $\rightarrow$  Fig. 1A). Absolute NBT-positive neutrophil count ranged from 585 to 4,261/ $\mu\text{L}$  with a mean of 1,509/ $\mu\text{L}$ . At places there were conglomerate of neutrophils whereby cytoplasmic boundary of neutrophils could not be much appreciated and there was nuclear decondensation and nuclear streaking. These conglomerates resembled the description of NETs ( $\rightarrow$  Fig. 1B). The average NBT percentage in the control group came out to be less than 1% and absolute NBT-positive neutrophil count ranged from 0 to 142/ $\mu\text{L}$  with a mean of 44.6/ $\mu\text{L}$ .

In the pathogenesis of COVID-19, imbalance in oxidant-antioxidant mechanism happens resulting in free radical injury along with hyperinflammation, cytokine storm causing cell damage.<sup>3-5</sup> The oxidative stress markers like increased hydrogen peroxide, structural damage to human serum albumin, increased lipid peroxidation, deficits in antioxidants like vitamin C, glutathione, thiol proteins, and trace elements (selenium) have been found in the blood of critically ill COVID-19 patients.<sup>4,10</sup> The ongoing oxidative burst of neutrophils can be studied by using NBT reduction

test.<sup>7,8</sup> What we could infer from the above finding is that the neutrophils are getting stimulated/in oxidative burst in vivo and are able to reduce NBT to formazan compound even without exogenous stimulation. To the best of our understanding, NBT test findings have not been reported in COVID-19 patients. Viral illnesses are supposed to be having low NBT percentage,<sup>11-13</sup> and the presence of higher NBT percentage may be because of intercurrent bacterial or fungal infections or more likely due to neutrophil mediated oxidative stress due to COVID-19 itself. The authors also propose that NETs can be seen with the help of this test ( $\rightarrow$  Fig. 1B). This small observation calls for larger studies evaluating NBT in comparison to standard methods of oxidative stress measurement like carboxy-H2DCFDA/flow cytometry-based DCF dye test. These techniques are the gold standard; however, they require costlier equipment's for testing like spectrofluorometer or flow cytometer and expertise in these disciplines.

To conclude, the authors would like to comment that oxidative stress lies at the heart of COVID-19 pathogenesis in severe and critically ill patients and an age-old technique of NBT can be done for evaluating oxidative stress in the scenarios where the costlier equipment requiring better techniques are not available.

#### Authors' Contributions

E.J. gave the idea of doing NBT in COVID-19 patients. She also contributed to microscopy, manuscript writing and revision. U.K. was involved in standardizing NBT test, microscopy, manuscript writing, editing, and revision. N.K. gave suggestion on manuscript and taking control population. He also contributed to proof reading and manuscript editing.

#### Conflict of Interest

None declared.

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