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Research Article

Association of Tuberculin Skin Test with Plasma Inflammatory Cytokines in Healthy Nigerian Healthcare Workers

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ABSTRACT

Tuberculin skin test (TST) is among the screening criterion for tuberculosis and inflammation is known to be associated with *Mycobacterium tuberculosis* infection. Therefore, there is need to investigate the relationship between TST and inflammatory cytokines [tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ)] and oxidative stress biomarkers [3-Nitrotyrosine (3-NT), inducible nitric oxide synthase (iNOS)] in apparently healthy Nigerian healthcare workers.

Four millilitres of blood samples were collected from 34 participants for determination of TNF-α, IFN-γ, 3-NT and iNOS using ELISA. The TST was performed using 0.1 mL of 5 TU purified protein derivative and the diameter of induration was evaluated at 72 hours. Higher levels of TNF-α (10.10±4.31; 11.84±5.81; 12.95±6.96; 14.59±4.71 pg/mL) and IFN-γ (1.30±1.10; 1.44±1.25; 1.49±1.10; 2.10±1.56 pg/mL) were found at indurations of <5 mm, 5-9 mm, 10-14 mm, ≥15 mm, respectively. However, highest levels of 3-NT and iNOS, were found at indurations of 10-14 mm. Tuberculin skin test correlated positively with TNF-α, IFN-γ, iNOS ($p>0.05$) and age ($p<0.05$), while 3-NT was inversely correlated with TST ($p>0.05$). Almost half of the healthcare workers had indurations of ≥10 mm, suggestive of exposure to environmental *Mycobacterium*. This group of Nigerians should be made to undergo further diagnostic procedures for tuberculosis at regular intervals to establish that the positive TST is due to development of antibodies to *Mycobacterium* species but not as a result of actual infection.

Keywords: *Active tuberculosis, Tuberculin skin test, Healthcare workers, Inflammatory cytokines, Oxidative stress biomarkers*

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INTRODUCTION

Tuberculin skin test (TST), also known as Mantoux test or Purified Protein Derivative (PPD) test, is one of the oldest screening tests carried out on the skin for diagnosis of tuberculosis (TB) using PPD. This test is a delayed-type hypersensitivity (Type IV) reaction, which is based on the immune response of the body to intradermal injection of PPD. It is a heterogeneous mixture of more than 200 mycobacterial antigens that are common to *Mycobacterium tuberculosis* (MTB), *Mycobacterium bovis bacillus of Calmette and Guerin* (BCG), and non-tuberculous mycobacteria (NTM) (Andersen *et al*., 2000; Asuquo *et al*., 2009; Gualano *et al*., 2019). The TST has been in existence for more than a century and it is still widely used in the diagnosis of latent TB in high endemic places (Nayak and Acharjya, 2012; World Health Organization, WHO, 2018).

Infection with *MTB* results in development of skin sensitivity to tuberculin (Reichman, 1979). Generally, this infection occurs when an individual inhales a droplet aerosol containing the *Mycobacterium* over a long period of time. In almost all infected individuals, the tuberculin test elicits a measurable reaction (Reichman, 1979). The Centres for Disease Control and Prevention (CDC) identified 3 cut-off levels for positive TST reactions and defined them as follow: (1) An induration of ≥5 mm is considered positive in HIV-infected person, recent contacts of TB case patients, fibrotic changes on chest radiograph consistent with prior TB, patients with organ transplants and other immunosuppressed patients receiving the equivalent of >15 mg/d of prednisone for 1 month or more. (2) An induration of ≥10 mm is considered positive in recent immigrants (i.e., within the last 5yr) from high prevalence countries, injection drug users, residents and employees of high-risk congregate settings, Mycobacteriology laboratory personnel, persons with clinical conditions that place them at high risk, children < 4 years of age, infants, children, and

adolescents exposed to adults in high-risk categories. (3) An induration of ≥15 mm is considered positive in persons with no risk factors for TB (CDC, 2000). However, because of the low specificity and sensitivity of TST, TB may need to be confirm with other TB screening/diagnostic tests such as chest radiograph, diagnostic microbiology (sputum smear/culture), TB blood test, which measures the patient's immune system reaction to *MTB* and Nucleic Acid Amplification Tests (NAAT) also known as Genexpert test (CDC, 2005; 2009).

Tuberculosis associated inflammation is reported to be central to progression from latent TB to active TB. The *MTB* uniquely modulates fundamental inflammatory processes including recruitment of immune cells to the infected lung and production of inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukins (ILs) -2, -6, -12, -15, -17, -18, -23 and -27, interferon-gamma (IFN-γ) and T-helper-1 (Th1) cells (Tomioka *et al.*, 2011; Kaufmann and Dorhoi, 2013). Among these cytokines, TNF-α and IFN-γ have been identified to be the most important agents of the anti-mycobacterial cytokine cascade. This is as a result of the formation and maintenance of the granuloma that is mediated by TNF-α acting in synergy with IFN-γ in the activation of macrophages to produce effector/oxidative stress molecules such as inducible nitric oxide synthase (iNOS) and nitric oxide ($^{\cdot}NO$) (Chan *et al*., 2001; Pereira-Suárez *et al*., 2006; Cooper and Khader, 2008; Kulkarni and Madrasi, 2008; Lin and Flynn, 2010). The $^{\cdot}NO$ produced by iNOS in the activated macrophages reacts with superoxide radicals ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), which is an unstable metabolite. These $ONOO^-$ react with protein tyrosine to produce 3-nitrotyrosine (3-NT) that are more stable end-products (Ischiropoulos, 1998).

Nigeria has been listed by WHO as an endemic TB country (WHO, 2019), and a high percentage of her people live in conditions of social deprivation, with no access to adequate medical care (Asuquo *et al*., 2009). Therefore, there is need to determine the occurrence of latent TB in Nigerian populations, especially in people working in the hospitals. This study aimed at investigating the relationship between TST and inflammatory cytokines [tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ)] and oxidative stress biomarkers [3-Nitrotyrosine (3-NT), inducible nitric oxide synthetase (iNOS)] in apparently healthy Nigerian healthcare workers.

MATERIALS AND METHODS

Ethics*:* The study protocol was carried out in accordance with the declaration of Helsinki and was approved by the joint University of Ibadan/University College Hospital Institutional Research Ethics Committee. All participants were briefed about the study and informed consent was obtained from each participant before they were enrolled into the study.

Research Design, Description of Participants and Blood Collection: Thirty-four male and female participants who were researchers and who had been working in the laboratories for at least two years were recruited into this cross-sectional study from the University College Hospital, Ibadan, Nigeria. The inclusion criteria are: (i) age between 18 and 65 years, (ii) non-pregnant/lactating female, (iii) apparently healthy participants without clinical symptoms of tuberculosis. Participants with malnutrition, poor living conditions, immunosuppressive infections such as human immunodeficiency virus (HIV) were excluded from the study. Similarly excluded from the study were participants with recent contact with TB patients, organ transplants, and clinical conditions that place them at high risk. From each of the participant, 4 mL of whole blood samples were collected into EDTA tubes immediately after reading the tuberculin diameter at 72 hours. All blood samples were centrifuged at 4000 rpm for 10 minutes to extract plasma for the determination of TNF-α, IFN-γ, 3-NT and iNOS. The plasma samples were stored at –20ºC until analysis.

Tuberculin Skin Test (TST): This test was performed using 5 tuberculin units (TU) PPD (BB-NCIPD Ltd, Sophia, Bulgaria). About 0.1 mL of the antigen was injected intradermally into the dorsal surface of the forearm of each participant using a disposable tuberculin syringe. The results were evaluated 72 hours later and the diameters of the indurated areas were recorded in millimetres. A transverse induration of <5 mm was considered to be negative (non-reactive to tuberculin antigen), between 5 and 9 mm was considered positive for those who had contact with TB patients within the last five years, between 10-14 mm was considered positive for healthcare workers and ≥15 mm was considered positive for persons with high risk for TB (CDC, 2000). All the participants had been vaccinated with BCG during childhood as part of the mandatory Nigerian National Vaccination Program.

Determination of plasma TNF-α and IFN-γ: The plasma samples were prepared according to the manufacturer's protocol (Abcam Inc. USA). The principle involved the use of a pre-coated wells of the microtiter strips with a monoclonal antibody specific for either TNF-α or IFN-γ. Samples, including standards of known TNF-α or IFN-γ concentrations, control specimens or unknowns were pipetted into these wells. The standards or samples and a biotinylated monoclonal antibody specific for TNF-α or IFN-γ were simultaneously incubated for 3 hours at room temperature and washed. After washing, the enzyme Streptavidin-Horseradish Peroxidase (Streptavidin-HRP), that binds the biotinylated antibody was added, incubated for 30 minutes at room temperature and then washed. Tetramethylbenzidine (TMB) substrate solution was added, which act on the bound enzyme to induce coloured reaction products. This was read on spectrophotometer using 450 nm as the primary wavelength and 620 nm as the reference wavelength (ELISA reader: Spectra Max Plus 384 Molecular Devices LLC, USA). The intensity of the coloured products is directly proportional to the concentrations of TNF-α or IFN-γ present in the samples.

Determination of plasma iNOS and 3-NT: The iNOS and 3-NT were determined using ELISA kit from Elabscience Biotechnology Inc., China and samples were prepared according to the manufacturer's protocol. The technique was based on the principle of competitive-ELISA. In this principle, iNOS or 3-NT had been pre-coated on the microtiter plate

provided. During the reaction, iNOS or 3-NT in the samples or standards compete with fixed amount of iNOS or 3-NT on the solid phase supporter for sites on the biotinylated detection antibody specific for iNOS or 3-NT. Excess conjugate and unbound samples or standards were washed from the plate and Avidin conjugated to HRP were added to each microplate well and incubated. Then a TMB substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of stop solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm with an ELISA reader (Spectra Max Plus 384 Molecular Devices LLC, USA). The concentrations of iNOS or 3-NT in the samples were then determined by comparing the optical density (OD) of the samples to the standard curves.

Data Analysis: All data were presented as frequency and percentages or Mean ± Standard Deviation (SD). The data were subjected to statistical analysis using Statistical Package for Social Sciences version 20 (IBM SPSS, Armonk, NY, USA). Independent T-tests with equal variances not assumed and Spearman's rho correlation were used for the analysis with $p<0.05$ considered to be statistically significant.

RESULTS

The mean age of the 34 participants was 34.65±12.08 years, 19(55.9%) were females and 15(44.1%) were males. The frequency of occurrence of tuberculin diameters revealed that 12(35.3%) were non-reactive to tuberculin antigen (0-4 mm, negative); 6(17.6%) of the participants had tuberculin diameter between 5-9 mm while 7(20.6%) and 9(26.5%) had tuberculin diameters between 10-14 mm and ≥15 mm, respectively. Participants with indurations ≥15mm in diameter belong to the mean age of 39.78±8.42 years while those with mean age of 27.25±9.00 years had indurations of 0-4mm. Participants with indurations ≥15mm in diameter had highest TNF-α and IFN-γ levels compared with indurations of 0-4 mm group (Table 1). In Table 2, age significantly and positively correlated with TST.

DISCUSSION

Tuberculin skin test is one of the few methods used, since the 19th century, in detecting *MTB* infections and diagnosing TB in individuals. It is also used, as a screening tool, in epidemiological settings to measure the prevalence of TB infection in the populations (Nayak and Acharjya, 2012). Tuberculosis is considered as both community-acquired and occupational-infection disease with increasing trend in healthcare workers (Salehi *et al*., 2016). In a review of surveillance reports from the New York by Humphreys, despite a decline in TB from 3636 to 1434 in 1994 and 2002, respectively in the general populace, it increased from 2.5% to 4% in healthcare workers as shown by skin testing and other TB assessments (Humphreys, 2007). Therefore, the need to investigate the relationship between TST and inflammatory and oxidative stress biomarkers in apparently healthy healthcare workers.

All participants in this study were vaccinated during childhood with BCG, which produces intermediate TST results. It is believed that the response to this vaccination and actual vaccine given at childhood might have waned off, thus negative TST result was expected in the present study at adulthood. However, this study revealed that indurations increased with age. This result is further buttressed with the positive association observed between age and TST, suggestive of infection exposure with increase in age. In addition, Nigeria is known to be an endemic country for TB, therefore the tuberculin sizes observed may be due to exposure of these participants to the environmental *Mycobacterium* species during the course of lives. In this study, almost half of the study participants had indurations of ≥10 mm suggestive of possible tuberculosis reactivation due to environmental *Mycobacterium* species since those with malnutrition, poor living conditions, HIV, recent contact with TB patients and organ transplants were excluded. This finding is in agreement with the study of Asuquo *et al*. (2009), where a quarter of their control participants had tuberculin reactors with indurations of >10 mm. Similarly, it has been reported that 90% of people with indurations of 10 mm had *Mycobacterium tuberculosis* infection and the lifetime risk of reactivation of tuberculosis is 20% or more in such people (Brooks *et al*., 2004).

Table 1:
Inflammatory cytokines and oxidative stress biomarkers according to TST

	Tuberculin sizes						
Parameters	0-4 mm (n=12)	5-9 mm (n=6)	10-14 mm (n=7)	≥15 mm (n=9)	P1	P2	P3
Age (years)	27.25±9.00	40.67±14.33	35.57±14.21	39.78±8.42	0.074	0.197	0.004*
TNF-α (pg/mL)	10.10±4.31	11.84±5.81	12.95±6.96	14.59±4.71	0.534	0.354	0.039*
IFN-γ (pg/mL)	1.30±1.10	1.44±1.25	1.49±1.10	2.10±1.56	0.814	0.719	0.209
3-NT (ng/mL)	19.19±5.53	16.23±5.36	24.77±8.17	19.80±8.38	0.300	0.142	0.853
iNOS (pg/mL)	1181.04±946.10	880.52±103.87	1997.77±1163.45	1104.35±574.68	0.566	0.144	0.821

*Significant at $p<0.05$; TST = Tuberculin skin test; TNF-α=Tumour necrosis factor-α; IFN-γ=Interferon-γ; 3-NT=3-Nitrotyrosine; iNOS = Inducible nitric oxide synthetase; P1 = Indurations of 0-4 mm (Negative) compared with indurations of 5-9 mm; P2 = Negative compared with indurations of 10-14 mm; P3 = Negative compared with indurations of ≥15 mm.

Table 2:
Correlation of TST between inflammatory cytokines and oxidative stress biomarkers

Parameters	R - values	P - values
TST - Age	0.492*	0.003
TST - TNF-α	0.337	0.051
TST - IFN-γ	0.269	0.124
TST - 3-NT	-0.073	0.683
TST - iNOS	0.096	0.589

**Significant at p<0.05; TST = Tuberculin skin test; TNF-α=Tumour necrosis factor-α; IFN-γ=Interferon-γ; 3-NT=3-Nitrotyrosine; iNOS = Inducible nitric oxide synthetase*

Purified protein derivative has been reported to stimulate CD4+ T-lymphocytes (Turner and Dockrell, 1996), which exert regulatory activity on macrophage function for bacterial elimination. This response is activated by IFN-γ in synergy with TNF-α (Cavalcanti et al., 2012). Although, some authors reported reduction in lymphoproliferative response stimulated by PPD antigen (Sanchez *et al*., 1994, Torres *et al*., 1998), a study by Moura and Colleagues, however reported no reduction in lymphoproliferative response (Moura *et al*., 2004). In this present study, higher TNF-α and IFN-γ levels were found as the tuberculin diameter increases. This finding is further corroborated by the positive associations observed between the TST and these inflammatory cytokines. Thus, indicating the presence of tuberculosis stimulating antigen in these participants. However, 3-NT and iNOS levels were highest at indurations between 10 and 14 mm with no association between these oxidative stress biomarkers and TST. This finding supported the report of de Andrade *et al*. (2008), where PPD positively correlate with TNF-α.

In conclusion. in this study, higher levels of tumour necrosis factor-alpha and interferon-gamma were found as tuberculin diameter increases in the healthcare workers. Almost half of the healthcare workers had tuberculin diameter of indurations ≥10 mm, which may indicate higher likelihood of progression to active tuberculosis from latent tuberculosis. Therefore, it would be recommended that Nigerian healthcare workers be made to undergo further diagnostic procedures and be screened intermittently for tuberculosis. This is to establish that the positive tuberculin skin test is due to development of antibodies to *Mycobacterium* species but not as a result of actual infection. Also, fruit and vegetables, which can help in boosting the immune system, be included in the daily diet of all healthcare workers.

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