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Establishment of CD4 and CD8 Lymphocyte subsets in a healthy HIV and Toxoplasma seronegative pregnant women in Libya

RESEARCH

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ABSTRACT

Most of the diagnostic laboratories in Libya often depend on western textbooks for CD4+- and CD8+ T-lymphocyte reference values. In this paper, we established reference ranges for the Libyan *Toxoplasma*, *HIV, HBV, and HCV* seronegative healthy pregnant women in all trimesters of pregnancy, and compared them with a control group of non-pregnant women. Whole-blood samples were collected to provide normal ranges for CD4+ and CD8+ Lymphocyte subsets expressed as mean ± standard division. A total of 110 Libyan women who came from Tripoli and Zwara districts were investigated; 70 pregnant women (aged 27.8 ± 2.99, range 18-40 years old) and 40 non-pregnant women (aged 22.7±3.01, range 18-40 years old) were included as controls. All cases/controls were seronegative for toxoplasmosis, *HIV, HBV and HCV.* The CD4+ cell counts were 685±256 cell/µl at the first trimester (T1), 740±202 at T2, and 923±203 cell/µl at T3. While the CD8+ cell counts were 451±171 cell/µl at T1, 541±168 at T2, and 753±190 cell/µl at T3. The CD4:CD8 ratios were 1.5±0.64 at T1, 1.4±0.51 at T2, and 1.2±0.36 at T3. Moreover, the mean absolute CD4+ and CD8+ counts for the control group were 1001±232 cell/µl and 717±159 cell/µl respectively.

Absolute counts of CD4+ and CD8+ cells in pregnant women were significantly lower as compared to controls ($P<0.05$). Statistically significant decrease in the CD4+ and CD8+ cell counts was reported during T1 ($P<0.05$). These values increased significantly during the T2, and was comparable to the controls during T3 ($P>0.05$). The absolute CD4+ and CD8+ cell counts decreased with age for both groups. Geographical variation was reported for the cell counts between Tripoli and Zwara district at T3. We established reference ranges of CD4+ and CD8+ T-lymphocytes for the Libyan healthy pregnant women and discussed their use as prognostic markers. Further cohorts with greater sample size may be required to define the stage of the disease in relation to the normal CD4+ and CD8+ T lymphocyte count subsets in the Libyan population.

Keywords: CD4+-, CD8+ T-lymphocyte counts, Flow Cytometry, Libya, pregnancy, HIV, toxoplasmosis.

Introduction

Toxoplasmosis, caused by a protozoan intracellular parasite *Toxoplasma gondii*, is one of the most common parasitic infections among humans and other warm-blooded animals (Montoya and Liesenfeld 2004). The

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global seroprevalence of *T. gondii* in the general population varies widely and ranges between 10 and 70% (Montoya and Liesenfeld 2004, Xiao, Yin et al. 2010, Sun, Lu et al. 2013). Most cases are asymptomatic and the clinical disease is generally not recognized. However, complications may occur in immunocompromised individuals including AIDS patients, transplant recipients, and patients undergoing cancer treatment (Liu, Wang et al. 2015). These complications can range from neuropsychiatric disorders to severe and potentially fatal encephalitis due to the reactivation of latent infections in AIDS patients (Halonen and Weiss 2013).

T. gondii can pass through the placental barrier and infect the fetus (Jones, Lopez et al. 2003, Montoya and Remington 2008). Primary infections of pregnant women are associated with potential congenital infections and spontaneous abortions. The severity of congenital infections and frequency of transmission are influenced by the stage of pregnancy in which maternal toxoplasmosis is acquired (Halonen and Weiss 2013).

Transmission is relatively low (< 20%) during the first trimester resulting in spontaneous abortion, hydrocephaly and mental retardation. However, it increases up to 80% by the end of the pregnancy (Jones, Lopez et al. 2003, Ortiz-Alegria, Caballero-Ortega et al. 2010). The majority of the later cases are subclinical and resulting in asymptomatic infections or recurrent chorioretinitis that can lead to vision problems and potentially blindness (Montoya and Liesenfeld 2004).

The overall incidence of congenital toxoplasmosis in most populations, based upon serological methods, is from 1 in 1000 to 1 in 10,000 live births (Tenter, Heckeroth et al. 2000, Dubey and Jones 2008). However, a study measuring vertical transmission rates in humans using PCR detection of the umbilical cord found transmission rates of 19.8% (Hide, Gerwash et al. 2007). This suggests that human vertical transmission of Toxoplasma may be under reported when measured by serological methods (Hide, Morley et al. 2009).

Pregnancy is considered as physiological immunosuppression condition and can be associated with suppression of several immunological functions in order to accommodate the fetus (Dayama, Pandit et al. 2003). This includes the cell-mediated arm of the immune system which is conferred by the lymphocytes. These lymphocytes arise from hematopoietic stem cells in the bone marrow and have two major types: the B-cells, which when activated, differentiate into plasma cells and they secrete specific antibodies; and the T-cells, which rise in the thymus and have two main types: the first type differentiates on activation into cytotoxic T-cells, which directly kill cells infected with viruses, whereas the second type differentiates into cells that activate other cells such as B-cells and macrophages. The later includes the helper T- lymphocytes which assist the B-cells in antibody response and express the cluster determinant 4 (CD4+) molecules, while the cytotoxic T-cells express cluster determinant 8 (CD8+) molecules (Janeway Jr, Travers et al. 2001).

In several countries, prenatal screening of women is performed with the goals of early diagnosis and treatment of *T. gondii* infections (Roberts, Hedman et al. 2001). Moreover, serological diagnosis is routinely used to determine the immune status with regard to *T. gondii* infection (Jenum and Stray-Pedersen 1998). Estimation of CD4+ and CD8+ T-lymphocytes counts are used to measure the strength of an individual's immune response and continues to be an important aspect for monitoring of immune function (Yang, Wu et al. 2015). They are also used as indicators to begin prophylactic therapy against opportunistic infections (Force 1994). Other parameters that can be used to monitor the immune status include CD4+/CD8+ ratio, CD4+ and CD8+ percentages (Dayama, Pandit et al. 2003).

Available evidences suggest that the variations in CD4+ and CD8+T-lymphocytes could depend on certain important factors namely environment, ethnicity, genetic differences and dietary patterns in addition to age and gender (Tollerud, Clark et al. 1989, Lebranchu, Thibault et al. 1991, Tsegaye, Messele et al. 1999, Fahey, Schnelle et al. 2000, Uppal, Verma et al. 2003). Moreover, CD4+ and CD8+T-lymphocytes counts are influenced by viral infections such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) (Zhou, Zhang et al. 2014), and parasitic infections such as toxoplasmosis which is highly prevalent in Libya. Seroprevalence among Libyan pregnant women ranges between 44.8% and 50% (Kassem and Morsy 1991, Magrhi, Abudher et al. 2003, Mousa, Mohammad et al. 2011). Moreover, 17.6% of women who suffered from spontaneous apportion in Tripoli were *Toxoplasma* seropositive (Gashout, Lazrag et al. 2008), and the prevalence of congenital toxoplasmosis was found to be 44% (Alkhunfas 2008). The diagnosis of *Toxoplasma* is based on serology. Recent introduction of molecular detection of the parasite was reported in Tripoli (Gashout, Amro et al. 2016). The development of monoclonal antibodies and flow cytometry technology has made possible new approaches to leucocytes subset identification. However, no studies on T-lymphocyte subsets were conducted and no country-specific immunohaematological reference values are available in Libya. Most of the diagnostic laboratories often depend on western text books for CD4+- and CD8+ T-lymphocyte reference values, therefore interpretation of these values in pregnant women is inappropriately based on reference values established for healthy nonpregnant women. Hence, we conducted this study to established reference ranges for the Libyan healthy Toxoplasma and *HIV*, *HBV*, and *HCV* seronegative pregnant women in all trimesters of pregnancy and compared them with a non-pregnant group before using these levels as prognostic marker.

Methods

Study settings and samples

Between 2008-2009, 70 pregnant women attended to antenatal Rahama clinic were selected for this study. Control group of 40 non-pregnant women were included for comparison. Women with a history of any disease (e.g., complications in pregnancy, cough, cold or fever in the past one month) were excluded.

Serologic tests

The ELISA test was used to assess Toxoplasmosis sero negativity for both groups. Approximately 5 ml of blood were collected from each subject. Serum was separated from the whole blood by centrifugation at 3000 rpm for 5 min and screened for anti-*Toxoplasma* IgG and IgM antibodies by using standard ELISA commercial kits (Human Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) in accordance with the manufacturer's instruction. All samples were analyzed in the Libyan National Centre for Infectious Diseases Prevention and Control (LNCDC). Moreover, all serum samples were tested for *HIV* (anti-*HIV* Tetra ELISA, Biotest Co. Dreieich, Germany), *HBV* hepatitis B antibody (Anti-HBc EIA WELL, Radim, Italy), and *HCV* antibody (HCV-Ab ELISA, DRG Co., Marburg, Germany),

Flow cytometry Immunophenotyping

To provide normal ranges for CD4 and CD8 Lymphocyte subsets, whole-blood samples (1 ml) were collected using sterile EDTA Vacutainer tubes. Three EDTA blood samples were collected from each pregnant woman and the test was performed every trimester of pregnancy. Samples were collected in triplicates between 9:00 -12:00 AM to avoid any error in subset counts due to diurnal variations (Carmichael and Abayomi 2006). Immunocytometry was done using CyFlow Counter machine (Partec, Münster, Germany) with two monoclonal antibodies; CD4 Antibody (MEM-241) [Phycoerythrin] and CD8 Antibody (MEM-31) [Phycoerythrin]. In brief, 20 µl of whole blood in EDTA was mixed with 20 µl of CD4 mAb PE or CD8 mAb PE and incubated for 15 min at room temperature protected from light. Red blood cells were then lysed by adding 800 µl of fluorescence-activated cell sorter lysing solution. After vortex, tubes were incubated in the dark at room temperature for 15 min then measured. The CA3 software provides instrument control, data acquisition, data analysis, and True Volumetric Absolute Counting (Greve, Cassens et al. 2003).

Statistical Analysis

Measurement data among groups were expressed as mean ± standard division. Statistical analysis was carried out using GraphPad Prism version 5 for Windows. Comparison of the means was carried out using the student–t test. AP < 0.05 indicated that the difference was significant.

Results

A total of 110 Libyan women were investigated and divided into two groups, 70 pregnant women (aged 27.8 ± 2.99, range 18-40 years old) and 40 non-pregnant women (aged 22.7±3.01, range 18-40 years old) included as controls. All cases/controls came from Tripoli district except 10 pregnant women who came from Zwara district. To ensure that all cases/controls are free from infections, participants were screened for anti-Toxoplasma IgG and IgM antibodies and were seronegative. Moreover, all cases/controls were seronegative for, *HIV*, *HBV* and *HCV*.

Immunophenotyping of Peripheral Blood lymphocytes

The mean absolute CD4+ and CD8+ cells count ± SD and their ranges were calculated and compared for the two groups. Table 1 summarizes the results of the pregnant women group during the three trimesters of pregnancy. The CD4+ cell counts range from 685±256 cell/µl at the first trimester (T1) and 923±203 cell/µl at T3. While the CD8+ ranges from 451±171 cell/µl at T1 and 753±190 cell/µl at T3. The CD4:CD8 ratio ranges from 1.5±0.64 at T1 and 1.2±0.36 at T3 (Table 1). Moreover, the mean absolute CD4+ and CD8+ counts ± SD for the control group (Non-pregnant women) were found to be 1001±232 cell/µl and 717±159 cell/µl respectively (Table 1). Absolute counts of CD4+ and CD8+ cells in pregnant women were significantly lower as compared to non-pregnant group (*P*<0.05). A wide variation of CD4+ and CD8+ counts was reported between both groups (Table 1). During the first trimester of pregnancy, there were statistically significant decrease in the CD4+ and CD8+ cell counts compared to the control group (P<0.05). These values increased significantly during the T2 compared to T1. At the third trimester, CD4+ and CD8+ cell count increased to 923±203 and 753±190 which were comparable to the values of the control group and did not show statistically significant differences (*P > 0.05*) (Table 1).

Table 1. CD4 and CD8 cell counts and ratios for cases and controls [mean ± SD (range)]

Counts	Pregnant women (n = 70) (study group) [Data are mean ± SD (range)]			Non-pregnant women (n= 40) control group
	1st Trimes.	2nd Trimes.	3rd Trimes.	
CD4+ (count/µl)	*685±256 (182-1639)	*740±202 (209-1458)	923±203 (214-1720)	1001±232 (420-1718)
CD8+ (count/µl)	*451±171 (126-924)	*541±168 (144-907)	753±190 (150-2008)	717±159 (310-966)
CD4:CD8 ratio	1.5±0.64 (0.19-3.13)	1.4±0.51 (0.16-3.38)	*1.2±0.36 (0.22-2.4)	1.4±0.25 (1.07-2.14)

* *P* < 0.05 compared to control

There was no statistically significant change in the CD4:CD8 ratio during the first and second trimesters, however, it decreased significantly from 1.5 at T1 and 1.4 at T2 to 1.2 at T3 (P<0.05). Moreover, significant decrease of CD4:CD8 was seen between T3 and control group (Table 1). Further analysis of our results showed that the absolute numbers of the CD4+ and CD8+ T-lymphocyte cell counts decreased with age for both groups except the CD4+ cell counts for the pregnant group (Table 2).

T-lymphocyte subsets values were compared between Tripoli and Zwara districts. Significant increase in CD8+-cell counts was predicted in Zwara (869± 50 cell/µl) compared to Tripoli district (722± 61 cell/µl) only at the 3rd

Table 2. Age distribution of CD4 and CD8 subset values among pregnant and non-pregnant Libyan women.

	Women Pregnant			Non Pregnant women		
Age group	Number of cases	CD4	CD8	Number of cases	CD4	CD8
18-22	5	163±602	250±590	5	1138±379	769±177
23 – 27	29	216±863	157±623	15	1022±138	770±119
28 –32	21	139±684	118±508	12	1013±160	739±127
33 – 37	12	206±848	174±645	6	999±297	644±179
38 - 40	3	142±730	63±449	2	754±86	508±7
Total	70	< 0.05). However, no		40		

significant differences in CD4+-cell counts were found between the two districts for pregnant and non-pregnant groups.

Discussion

In this study, we established reference ranges of CD4+ and CD8+ T-lymphocytes for the Libyan healthy, HIV, HBV, HCV, and Toxoplasmosis seronegative pregnant women and discussed their use as prognostic markers. The absolute CD4+ and CD8+ cell counts in pregnant were significantly lower than non-pregnant women. This is consistent with the findings of most of studies that examined the effect of pregnancy on T-lymphocytes counts. Clinical data on pregnant women shows a deviation of the immune system consistent with a weakening of the cell-mediated immunity and strengthening of humeral immunity (Wegmann, Lin et al. 1993), which is important for the success of pregnancy (McIntire and Hunt 2005, Aagaard-Tillery, Silver et al. 2006, Hunt 2006, Munoz-Suano, Hamilton et al. 2011).

An Indian study among HIV-negative people found absolute CD4+ cell counts to be significantly lower in pregnant than in non-pregnant women (Dayama, Pandit et al. 2003). An earlier study among Africans demonstrated reduced absolute values of CD4+, CD8+ and total lymphocytes during pregnancy (Vassiliadou and Bulmer 1998). However, the mean absolute values of CD4+, CD8+ counts in our study were higher than those reported from Nigerian (Aina, Dadik et al. 2005) and Chinese reports (Jiang, Kang et al. 2004) and were comparable to the Indian reports (Murugavel, Balakrishnan et al. 2009). The relationship between gestational age and T-lymphocyte levels in pregnant women varies in the literature. A study in Kenya found no relationship between gestational age and any immunological variable in both HIV-positive and negative women (Temmerman, Nagelkerke et al. 1995). Ibitokou et al. found significant decrease of CD4+ cell counts between the second trimester and delivery of the sub-Sahara African women (Ibitokou, Brutus et al. 2013). Conversely, Tuomala et al., found an increase in CD4+ cell counts of 2.76 cells/uL per week of pregnancy during serial measurements in pregnant women (Tuomala, Kalish et al. 1997). In our study, significant decrease of CD4+ and CD8+ cell counts were predicted during the first trimester followed by significant increase during the second trimester, and were comparable to the control group in the third trimester. These discrepancies could be related to the significant geographical and racial differences described in earlier studies. For example, CD4+-cell counts for Asian was found to be lower than that recorded for Caucasians (Lee, Yap et al. 1996), and among African populations, healthy Ethiopians (Tsegaye, Messele et al. 1999) have markedly lower counts than those recorded in Uganda (Tugume, Piwowar et al. 1995) and Tanzania (Levin, Brubaker et al. 1996). Hence, this establishment of country-specific immunehaematological reference values in Libya is crucial.

The CD4:CD8 ratio was comparable to the control group during T1 and T2. This indicates uniform decrease of the tow lymphocytes subsets during these periods. However, the significant decrease of the ratio during T3 indicating faster increase of CD8 compared to that for CD4 cells during this period.

The CD4+ and CD8+ T-lymphocyte cell counts decreased with age for both groups except the CD4+ cell counts for the pregnant group. This is in agreement with a previous study concluded that maternal age had no significant effects on CD4+ cell count levels in pregnancy (Akinbami, Gbadegesin et al. 2015), and disagrees with the results from Abimiku et al. which reported that low CD4+ cell count was significantly associated with older age(Abimiku, Villalba-Diebold et al. 2009). Moreover, Lugada et al reported that CD4+ cell count is highest during the early years of life, declines steadily to stable adult values, and it is lowest in the elderly (Lugada, Mermin et al. 2004). However, our results are limited with smaller sample and need to be validated with larger cohorts.

Geographical variation in the CD8+-cell counts has been reported through Zwara district compared to Tripoli district at the 3rd trimester of pregnancy. Though our sample size is very small, these results emphasize the need for further investigation on T-lymphocyte counts variation between different districts in Libya. There are many factors affecting the levels of circulating lymphocytes and may lead to differences across regions. This includes genetic makeup, altitude, dietary patterns, body mass index and smoking habits (Schaberg, Theilacker et al. 1997, Feldman, Minkoff et al. 2006, Mair, Hawes et al. 2008).The influence of these factors point out to the fact that the T-lymphocytes reference ranges of one population might not be precisely used as a reference range for another, and might give an inaccurate interpretation of the immune status.

The findings of the present study though important, are limited with small sample size and restricted to two districts and hence cannot be generalized. Another limitation is that the samples were collected during 2008-2009 and that current values can vary according to environmental and nutritional conditions. Nevertheless, our study design was unique since we evaluated the T-lymphocytes counts during the three trimesters, while most of studies in the literature were conducted only on the first trimester of pregnancy.

The lymphocytes reference values have been established by many studies throughout the world and have shown some variability according to geographical locations and methodology (Bosire, Nyamache et al. 2013). The validity of comparison of CD4+ and CD8+ cell counts depend on the comparability techniques, duration and temperature of sample storage which could differ significantly between studies.

Moreover, assay variations may be attributed largely to processing methods, monoclonal antibodies, analysis methods and lag period between drawing blood and processing of the specimen (Landay and Muirhead 1989). Hence, the use of single – platform flow cytometry, and quality control of this platform are recommended to eliminate some of the variability between different studies and making them more comparable. This platform should be used for future studies in order to ensure correct comparability and interpretation of the results (Chng, Tan et al. 2004).

Conclusions

In conclusion, the establishment of normal ranges for T-lymphocytes with the local population is a helpful tool to clinicians for the better clinical management of pregnancy specific-diseases in Tripoli and other surrounding areas, and they are used for clinical classification, to determine prognosis, and to decide whether to prescribe prophylaxis for opportunistic infections especially Toxoplasmosis which is highly prevalent in Libya. Further cohorts with greater sample size may be required to define the stage of the disease in relation to the normal CD4+ and CD8+ T lymphocyte count subsets in the Libyan population.

Declarations

Ethics approval and consent to participate

All aspects of the study were revised and approved by the ethics committee of the Libyan National Centre for Disease and Control (LNCDC). Prior to starting the study, study objectives and procedures were explained for each participant. Informed written consent was obtained from all participants. Confidentiality was ensured through secure data management, and no personal identifiers were in the computer system. Data and samples were labelled with anonymous identification numbers. Test results were confidentially disclosed to the subjects following post-test counselling.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

AG and AA designed the study, AG, HAD, ME, AAl, and AAb investigated the patient and have done the clinical evaluation and laboratory tests. AG and AA analysed the data. AG and AA have written the manuscript. All authors have read the manuscript and approved its content

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