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Evaluation of Zinc and Copper and Immunological Implication in Menstrual Cycle of HIV Infected Females in Nnewi, Nigeria

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Abstract
Zinc and Copper are essential for the immune system and the interaction between reproductive system and immune cells plays important immunoregulatory roles in menstrual cycle of women. It is estimated that nearly 16 million women are living with HIV/AIDS worldwide, making up approximately half of all infections. Importantly, the understanding of the burden of HIV/AIDS lies within resource limited areas particularly Sub-Saharan Africa. This was a prospective study designed to evaluate the immunological implication of Zinc and Copper in menstrual cycle of HIV infected females in NAUTH, Nnewi, Nigeria. The study composed of 120 premenopausal females with regular menstrual cycle (between 27-31 days) and aged 15-45 years. 30 were apparently healthy females recruited as Control group while 90 were HIV infected females grouped as HIV stage 1, HIV stage 2 and HIV stage 2 on ART (n=30 respectively). Blood samples were collected at follicular (7^{th} - 13^{th} day) and luteal (21^{st} -23^{rd} day) phases of menstrual cycle after obtaining their informed consent for determination of Zinc, Copper, Interleukin-6, interleukin-4 and TNFα using AAS and ELISA methods. The result showed significantly lower levels of Zn and Cu with significantly higher levels of IL-6, IL-4 and TNFα in HIV infected females with or without therapy compared to Control at both phases of menstrual cycle ($P<0.05$). The Zn levels was significantly higher in stage 2 HIV infected females on ART compared to their counterparts not on ART ($P<0.05$). The significantly lower levels of Zn and Cu with increased levels of cytokines indicate immunosuppression and active inflammatory response in HIV infected females at both phases of menstrual cycle which was improved in the participants who were placed on ART.
Keywords: Copper; Zinc; Immunological Implication; HIV; Menstrual cycle; Reproductive age.

1. Introduction

The interaction between reproductive system and immune cells plays an important immunoregulatory role in menstrual cycle of women. Zinc and Copper have been identified as being essential for proper function of the reproductive system (Bedwal and Bahuguna, 1994) and immune response (Dunlap et al, 1974, Prasad, 2007). Zinc has been associated with proper formation and maturation of T-cells and cellular response to mitogens among other immune activities (Klaus-Helge and Lothar, 2003) and Zinc deficiency has been implicated in amenorrhea, infertility and premenstrual syndrome (irritability, ache and cramping during menstruation (Canaan, 2011, Wilson, 2012) and women with eating disorders may be involved and this may affect immunity. Zinc deficiency has also been associated with high incidence of infections in the elderly (Prasad, 2007) and this was associated with increased productions of inflammatory cytokines and plasma oxidative stress markers. Thus, Zinc acts as an anti-inflammatory agent. On the other hand, Zinc supplementation was associated with lower incidence of infections, Lower generation of tumour necrosis factor alpha and oxidative stress markers. Zinc is very important in HIV-1 infection. This is because Zinc has been found to be a component of HIV-1 nucleocapsid proteins (Berthoux et al, 1997) and has also a strong affinity for binding with HIV-1 trans-activating protein (Tat) (Huang and Wang, 1996). The HIV-1 virus also utilizes Zinc for gene expression, multimerization and integration hence HIV-1 is said to be a Zinc dependent virus. This may partially explain the low levels of Zinc observed in HIV-1 infected patients. Zinc in large dosages, induces the production of cytokines such as interleukins IL-1, IL-6 and TNFα and soluble IL-2R (Mocchegiani, 1985).

Copper on the other hand, has been found to be essential for neutrophil mobilization in peripheral blood and T-cell proliferation (Percival, 1998). Copper deficiency is associated with neutrophil reduction in number, reduced ability to form superoxide anion and ingestion of microorganisms. However, the mechanism of action of copper is not yet understood. Studies have shown that copper deficiency reduces the effectiveness of the acquired immune response (Lukasewyez and Prohaska, 1990) and neutropenia has been demonstrated in humans (Williams, 1983). Copper deficiency has also been associated with arrested maturation of granulocytes and impaired phagocytic function (Heresi, 1985).

The present study was therefore designed to evaluate the immunological implication of zinc and copper in menstrual cycle of HIV infected females.

2. Materials and Methods

2.1 subjects

A total of 120 premenopausal females with regular menstrual cycle (between 27-31 days) and aged 15-45years were randomly recruited for the study. The participants consisted of 30 apparently healthy females recruited among the hospital staff who served as Control females while the remaining 90 female participants were recruited at Heart to Heart centre and HIV clinic of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State Nigeria. They were grouped according to WHO criteria for staging HIV infections as: (i) Stage 1 HIV infected females (n=30), (ii) Stage 2 HIV infected females (n=30), (iii) Stage 2 HIV infected females on ART (n=30). A structured questionnaire was administered to each of these participants to ascertain their menstrual cycle and reproductive history and other biodata.

Six mls of Blood sample was collected from each participant at follicular (7-13th day) and at luteal (21-23rd day) phases of menstrual cycle. The blood samples were collected between 8 to 10am by venepuncture and were dispensed into EDTA containers and were used immediately for malaria parasite screening, HIV screening and confirmation. The blood was centrifuged and the plasma content was separated immediately and transferred into the well labeled container and stored frozen at -20^{0}C until assayed for Zinc, copper, IL-6, IL-4 and TNFα.

The ethics committee of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra state, Nigeria approved the study design. The participants were informed about the study design and only those who gave their consent were recruited for the study.

Participants with HIV Stage –3 and 4 were excluded from the study. Only those adjudged as HIV Stage-1 (Asymptomatic HIV and Symptomatic -HIV (stage-2) were included for the study. Participants with malaria parasite infection as at the time of study were also excluded. Participants with tuberculosis were excluded and subjects with known fertility problems before contracting HIV infection were also excluded. Hence the female participants used were those with no prior fertility problems until the existence of HIV infection.

2.2 methods

Antibodies to HIV-1 and HIV-2 in Human Plasma were detected using Abbott Deterimine system, Immunoassay method [(Trinity Biotech UniGold Assay Kit (Trinity Biotech PLC, Ireland)] and imunochromatographic method [(HIV 1 and 2 STAT-PAK Assay kit (Chembio diagnostic system, INC New York, USA)] respectively as described by the manufacturers of the kits. Determination of Zinc and Copper by Atomic Absorption Spectroscopy (AAS): In the flame AAS, the principle is based on the dissociation of the element from its chemical bonds. This is then placed in an unexcited or ground state (neutral atom). Thus, the neutral atom is at a low energy level in which it is capable of absorbing radiation at a very narrow bandwidth corresponding to its own line spectrum. The amount of radiant energy absorbed at a characteristic wavelength in the flame is proportional to the concentration of the element present in the sample. Quantification is achieved by preparing standards of the element. For Zinc (Zn), the plasma was diluted 1:4 with water and aspirated to AAS. Standards and blanks were prepared by diluting with 5% glycerin (series of standards 1, 3 and 6 were recommended, however, 1 and 3 ppm were enough which have comparable concentration with sample). The instrument was set at zero with 5% glycerol in deionized water then the samples and standard were serially aspirated and analyzed at 213.8nm in AAS, unexcited zinc atom absorbs light of the same wavelength as that emitted by zinc in the excited state. The amount of light absorbed is proportional to the concentration of zinc present in solution. The results displayed digitally part per million (ppm) and converted to µg/dl by multiplying each result by 100. For copper (Cu): The plasma was diluted 1:1 with water, aspirated and read in AAS. Standards and blanks were prepared with 10% glycerin (recommended standards are 5 ppm and 15 ppm Cu; however, the lowest standard alone could be used). Standards and blanks were prepared accordingly.

Determination of TNF-α and Interleukin-4 and 6, were done using Enzyme Linked Immunosorbent Assay (ELISA) kits (Glory Science Laboratory USA): The TNF-α, Interleukin-4 or 6 antigens and biotinylated monoclonal antibody specific for the TNF-α, Interleukin-4 or 6 were simultaneously incubated, after washing; the enzyme (streptovidin-peroxidase) was added, which reacts with antigen bound monoclonal antibody, the substrate solution acts on the bound enzyme to induce colour reaction product. The colour product is directly

proportional to the concentration of TNF-α, Interleukin-4 or 6 present in the sample.

2.3 Statistical analysis

The version 16 of SPSS package was used in statistical analysis. The variables were expressed as mean (±SD). The student t-test and analysis of variance (ANOVA) and post-hoc (LSD) were used to assess significant mean differences. Graph Pad Prism version 5.03 was used for graph presentations.

3. Results

Zinc and Copper in Tests groups and Control group at Follicular and Luteal Phases of Menstrual cycle

The mean (±SD) plasma zinc level (ppm) in stage1 HIV females was not significantly different between follicular (0.769±0.096) and luteal (0.787±0.062) phases of menstrual cycle (P>0.05). Similarly, the mean (±SD) plasma zinc level (ppm) in stage 2 HIV females was not significantly different between follicular (0.704±0.021) and luteal (0.728±0.040) phases of menstrual cycle (P>0.05). In stage2 HIV females on ART, no significant difference was also observed in the mean zinc level (ppm) between follicular (0.867±0.048) and luteal (0.845±0.072) phases of menstrual cycle (P>0.05). The mean (±SD) zinc level (ppm) in Control females was significantly higher at follicular (1.018±0.092) compared to luteal (1.014±0.090) phases of menstrual cycle (P<0.05).

However, the mean plasma zinc level (ppm) in stage 1 HIV females (0.769±0.096, 0.787±0.062), stage 2 HIV females (0.704±0.021, 0.728±0.040) and stage 2 HIV females on ART (0.867±0.048, 0.845±0.072) were significantly lower compared to Control females (1.018±0.092, 1.014±0.090) at both follicular and luteal phases of menstrual cycle (P<0.05 respectively). Furthermore, the mean (±SD) zinc level (ppm) in stage 2 HIV females on ART (0.867±0.048, 0.845±0.072) was significantly higher compared to stage1 HIV females (0.769±0.096, 0.787±0.062) and stage 2 HIV females (0.704±0.021, 0.728±0.040) at both follicular and luteal phases of menstrual cycle (P<0.05 respectively).

The mean (±SD) copper concentration (ppm) in stage 1 HIV females was not significantly different between follicular (0.892±0.129) and luteal (0.929±0.128) phases of menstrual cycle (P>0.05). The mean (±SD) copper concentration (ppm) in stage 2 HIV females was not significantly different between follicular (0.856±0.086) and luteal (0.870±0.121) phases of menstrual cycle (P>0.05). No significant difference was observed in the mean copper level (ppm) in stage 2 HIV females on ART between follicular (0.951±0.134) and luteal (0.980±0.117) phases of menstrual cycle (P>0.05). There was no significant difference in the mean copper level (ppm) in Control females between follicular (1.138±0.187) and luteal (1.145±0.215) phases of menstrual cycle (P>0.05 respectively).

The mean (±SD) copper concentration (ppm) in stage 1 HIV females (0.892±0.129, 0.929±0.128), stage 2 HIV females (0.856±0.086, 0.870±0.121) and stage 2 HIV females on ART (0.951±0.134, 0.980±0.117) were significantly lower compared to Control females at (1.138±0.187, 1.145±0.215) phases of menstrual cycle (P>0.05). Similarly, The mean (±SD) copper concentration (ppm) in stage 2 HIV females on ART (0.951±0.134, 0.980±0.117) was significantly higher compared to stage 1 HIV females (0.892±0.129, 0.929±0.128) and stage 2 HIV females (0.856±0.086, 0.870±0.121) at follicular and luteal phases of menstrual cycle (P<0.05) (See Fig 1).

Cytokines (IL-6, IL-4 and TNFα) in Tests groups and Control group at Follicular and Luteal Phases of Menstrual cycle

The mean (±SD) plasma IL-6 level (pg/ml) in stage 1HIV females was not significantly different between follicular (387.4±148.1) and luteal (347.4±172.6) phases of menstrual cycle (P>0.05). The mean (±SD) plasma IL-6 level (pg/ml) in stage 2 HIV females was significantly lower follicular phase (474.1±153.2) compared to luteal (583.3±271.3) phases of menstrual cycle (P<0.05). However, the mean plasma IL-6 level (pg/ml) in stage 2 HIV females on ART was not significantly different between follicular (224.9±54.6) and luteal (296.6±143.7) phases of menstrual cycle (P>0.05). Similarly, the mean plasma IL-6 level (pg/ml) in Control females showed no significant difference between follicular (217.6±64.9) and luteal (204.6±36.7) phases of menstrual cycle (P>0.05).

On the other hand, The mean (±SD) plasma IL-6 level (pg/ml) in stage 1 HIV females (387.4±148.1, 347.4±172.6) and stage 2 HIV females (474.1±153.2, 584.3±271.3) were significantly higher compared to Control females (217.6±64.9, 204.6±36.7) at both phases of menstrual cycle (P<0.05 respectively). Furthermore, the mean (±SD) plasma IL-6 level (pg/ml) in stage 2 HIV females on ART (224.9±54.6, 296.6±143.7) was significantly lower compared to stage 2 HIV females (474.1±153.2, 584.3±271.3) at both phases of menstrual cycle (P<0.05 respectively).

The mean (±SD) plasma IL-4 level (pg/ml) in stage 1 HIV females was significantly lower at follicular phase (327.1±116.2) compared to luteal (440.9±126.9) phases of menstrual cycle (P<0.05). However, the mean (±SD) plasma IL-4 level (pg/ml) in stage 2 HIV females was not significantly different between follicular (627.2±231.3) and luteal (660.2±254.2) phases of menstrual cycle (P>0.05). However, the mean (±SD) plasma

IL-4 level (pg/ml) in stage 2 HIV females on ART was significantly lower at follicular phase (354.9±207.7) compared to luteal (497.6±216.1) phase of menstrual cycle were compared (P<0.05). The mean plasma IL-4 value (pg/ml) in Control females was also significantly lower at follicular phase (210.7±71.2) compared to luteal phase (334.8±76.5) of menstrual cycle (P<0.05).

Furthermore, the mean plasma IL-4 level (pg/ml) in stage 1 HIV females (327.1±116.2, 440.9±126.9), stage 2 HIV females (627.2±231.3, 660.4±254.2) and stage 2 HIV females on ART (354.9±207.7, 497.6±216.1) were significantly higher compared to Control females (210.70±71.2, 334.8±76.5) at follicular and luteal phases of menstrual cycle (P<0.05 in each case). Similarly, the mean plasma IL-4 level (pg/ml) was significantly lower in stage 2 HIV females on ART (354.9±207.7, 497.6±216.1) compared to stage 2 HIV females (627.2±231.3, 660.2±254.2) at both phases of menstrual cycle (P<0.05).

The mean (±SD) plasma TNFα level (pg/ml) in stage 1 HIV females was significantly higher at follicular (843.1±359.2) compared to luteal (504.2±270.8) phases of menstrual cycle (P<0.05). The mean (±SD) plasma TNFα level (pg/ml) in stage 2 HIV females was significantly higher at follicular phase (949.6±335.7) compared to luteal (788.2±171.7) phase of menstrual cycle (P<0.05). However, the mean (±SD) plasma TNFα level (pg/ml) in stage 2 HIV females on ART showed no significant difference between follicular (483.6±160.0) and luteal (519.2±197.8) phases of menstrual cycle (P>0.05). However, the mean plasma TNFα value (pg/ml) in Control females was significantly lower at follicular phase (211.8±57.6) compared to luteal (333.0±72.2) phase of menstrual cycle (P<0.05).

However, the mean (±SD) plasma TNFα level (pg/ml) in stage 1 HIV females (843.1±359.2, 504.2±270.8), stage 2 HIV females (949.6±335.7, 788.2±171.7), stage 2 HIV females on ART (483.8±160.0, 519.2±197.8) were significantly higher compared with the values in Control females (211.8±57.6, 333.0±72.2) (P<0.05 respectively). Furthermore, the mean (±SD) plasma TNFα level (pg/ml) in stage 2 HIV females on ART (483.8±160.0, 519.2±197.8) was significantly lower compared to stage 1 HIV females (843.1±359.2, 504.2±270.8) and stage 2 HIV females (949.6±335.7, 788.2±171.7) at follicular and luteal phases of menstrual cycle (P<0.05) (See Fig 2)

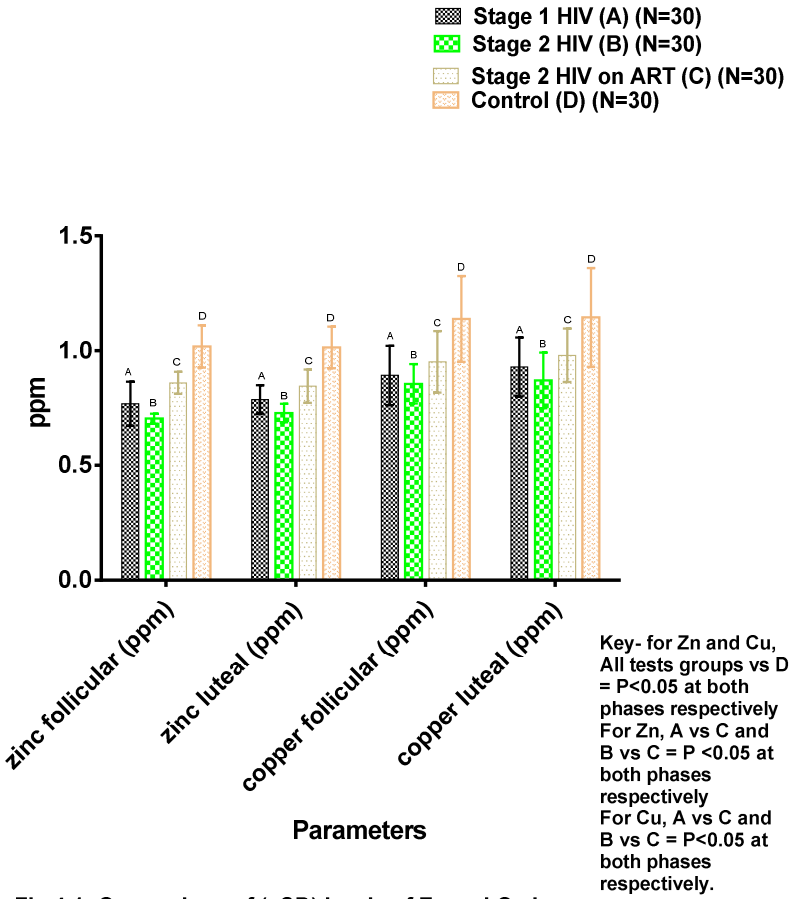
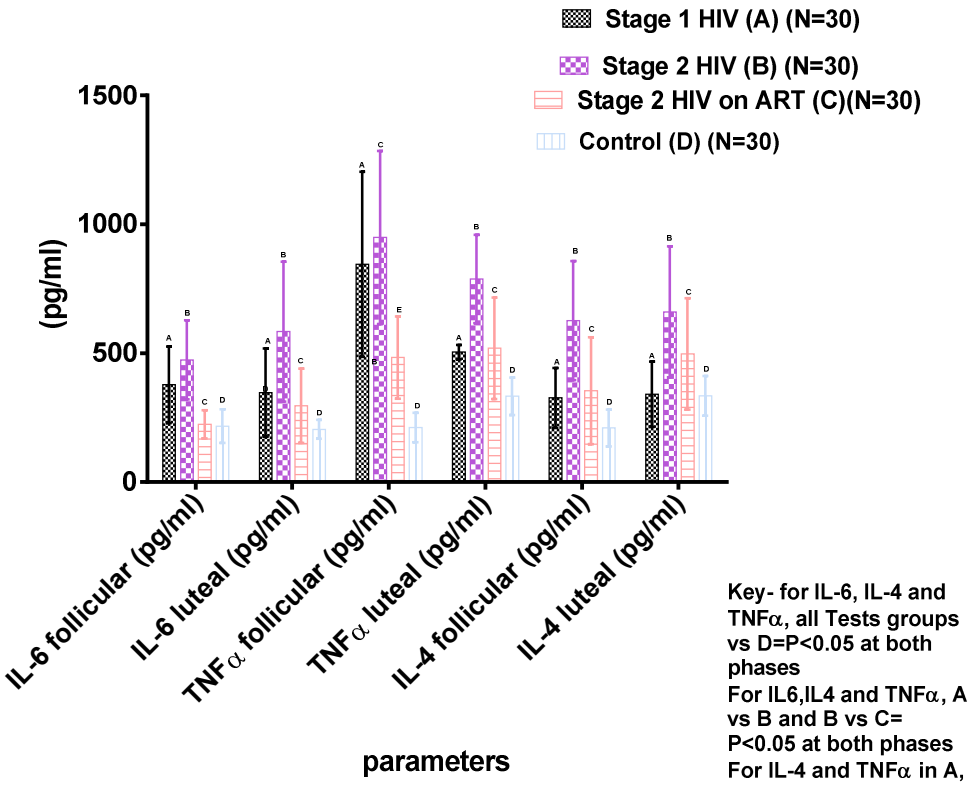


Fig 4.1: Comparison of (+SD) levels of Zn znd Cu in Tests groups and Control at follicular and luteal phases of menstrual cycle



Key- for IL-6, IL-4 and TNFα, all Tests groups vs D=P<0.05 at both phases
For IL6,IL4 and TNFα, A vs B and B vs C= P<0.05 at both phases
For IL-4 and TNFα in A, P<0.05 between follicular and luteal phases
For IL-6 and TNFα in B, P<0.05 between Follicular and Luteal phases
For IL-4 in C, P<0.05 between Follicular and Luteal phases.

Fig 2:Comparison of mean (±SD) plasma levels of IL6,IL4 and TNFα in Tests groups and Control group at follicular and luteal phases of menstrual cycle

4. Discussion

The insignificant difference in the levels of zinc and copper in HIV infected females with or without ART between the follicular and luteal phases of menstrual cycle shows that there was minimal or no variation in immune response between the two phases of menstrual cycle. This is inconsistent with the observation in the Control subjects where there was significantly higher level of zinc at follicular phase compared to luteal phase. This has been documented previously in apparently healthy women (Das and Chowdhury, 1997). The insignificant variations noted in HIV infected females could be attributed to hormonal and metabolic changes during menstrual cycle. However, the significantly lower levels of Zn and Cu in HIV infected females compared to Control females at follicular and luteal phases of menstrual cycle is consistent with previous reports (Akinola, 2012). This implies immunosuppression since these two elements have been associated with effective immune function (Lukasewyez and Prohaska, 1990, Prasad, 2007). The role of Zn and Cu in the ovarian cycle cannot be overemphasized. For instance, copper is closely associated with estrogen receptors and function and deficiency states may manifest with menstrual irregularity (Bedwal and Bahuguna, 1994). Recent report by Clancy documented that reproductive function of women may be tied to their immune status and leftover energy is dedicated to reproduction. The implications of zinc and copper deficiencies in HIV infected women may include the impairment of immune status of the affected women considering the immunological roles of these elements (Prasad, 2000). Further implications of zinc deficiency include Hypogonadism, (a condition in which the ovaries do not produce enough sex hormones), amenorrhea and increased prevalence of menstrual and reproductive dysfunction considering the roles of zinc in menstruation (Bedwal and Bahuguna, 1994). Reduced zinc level may also be associated with rapid atrophy of the thymus, lymphopenia and increased cell death (apoptosis) (Elmes and Jones, 1980). These may worsen the progression of HIV disease in affected women. Zinc deficiency has been reported to be associated with reduction of primary and secondary antibody responses especially those

involved in T-cell development like heterologous red blood cell. This also reduces antibody responses and generation of splenic cytotoxic T-cells after immunization (Baum *et al,* 2000). Other implications of the significantly low levels of plasma zinc and copper in HIV infected females could be HIV disease progression as a result of increased opportunistic infections which is always common in affected individuals. This is substantiated by previous reports (Baum *et al*, 1997).

The significantly higher levels of TNFα in HIV infected females not on ART at follicular phase compared to luteal phase of menstrual cycle implies significant inflammatory reaction at follicular phase of menstrual cycle which may be due to some hormonal changes in HIV infection. Increased level of pro-inflammatory cytokines at the follicular phase when compared to the luteal phase in HIV infected female subjects has been previously reported (Al-Harthi et al., 2007). The significantly higher level of TNFα in HIV female subjects at the follicular phase when compared with the luteal phase is inconsistent with findings in healthy women. It has been documented that in apparently healthy women, TNFα is generally higher at the luteal phase during which the population of activated monocytes, which secret TNFα, are reported to be higher (Brannstrom, 1999). Reports of IL-6 levels during the menstrual cycle phases have been controversial. Whereas Angstwurm *et al* reported higher levels of IL-6 during the follicular phase of menstrual cycle, (Konecna *et al*, 2000) reported higher levels during the luteal phase which is consistent with the present study.

Further implication of zinc and copper deficiency in HIV infected women is exaggeration of dangerous inflammatory cytokines including tumor necrosis factor alpha (TNFα) (Klaus-Helge and Lothar, 2003). This may be related to the high incidence of neoplasms in HIV infected people. These reports are consistent with the present study. HIV is a chronic inflammatory disease and is said to induce a Th-1type immune response which is characterized by the production of harmful pro-inflammatory cytokines such as IL-6, IL-10, and TNFα (Chowdhury *et al*, 2010). The implications of elevated cytokines on endocrine and metabolic functions have been previously reported (Gatner, 2009) and this has far reaching effects on menstrual and reproductive functions of the affected women (O' Brien, 2007). Zinc plays a role in inhibiting Tumor necrosis factor alpha (Flieger *et al*, 1989) which has been implicated in the pathophysiology of HIV/AIDS induced wasting and cachexia (Beutler B and Cerami, 1987). Deficiency states therefore, lead to over production of pro-inflammatory cytokines such as IL-6 and TNFα (Rosenberg and Fauci, 1989). Zinc deficiency also is associated with reduction in the production of anti-inflammatory cytokines particularly IL-4, a growth factor for T-cell helpers (Dowd *et al*. 1986). IL-4 is said to inhibit the production of TNFα by monocytes (Hart *et al*, 1989). This highlights the potential role of adequate Zinc status in preventing HIV progression (Rosenberg and Fauci, 1990).

The significantly higher levels of zinc and copper in HIV infected females on ART compared to their counterparts without treatment at both follicular and luteal phases of menstrual cycle imply that administration of antiretroviral therapy had some beneficial effects on these patients. This has been previously reported (Kassu *et al*, 2006). However, Silberry *et al* noted that administration of high or very low zinc concentrations may interfere with HIV replication. The author noted that selective blockade of active zinc finger site of HIV may inhibit the virus and spare the immune cells while progressive depletion of intracellular zinc may compromise the host cell thereby leading to apoptosis. Further reports indicate that excessive administration of zinc may contribute to HIV disease progression as a result of interference of copper and iron utilization which affects HDL cholesterol concentration and monocytes function (Schlesinger *et al*, 1993). The improved concentrations of zinc and copper in HIV infected women on ART has far reaching effects in reversing menstrual irregularities and restoring fertility or reproductive function . This highlights the beneficial effects of early diagnosis and treatment which probably improves the immunological response in the affected individuals with consequent reduction of opportunistic infections.

5. Conclusion

In conclusion, zinc and copper deficiencies occur in HIV infected women which may affect disease progression and worsen the prognosis. The introduction of ART seems to improve this condition.

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