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Mussel RL, De Sa Silva E, Costa AM, Mandarim-De-Lacerda CA (2003). Mast cells in tissue response to dentistry materials: an adhesive resin, a calcium hydroxide and a glass ionomer cement. J. Cell. Mol. Med. 7:171-178.

Booth M, Bundy DA, Albonico P, Chwaya M, Alawi K (1998). Associations among multiple geohelminth infections in school children from Pemba Island. Parasitol. 116: 85-93.0.

Fransiscus RG, Long JC (1991). Variation in human nasal height and breath, Am. J. Phys. Anthropol. 85(4):419-427.

Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A (2003). TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J. Biomed. Res. 66:476-82.

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Full Length Research Paper

A theoritical study of *Curcuma longa*'s anticancer agents, curcumin I and curcumin II, ın blood and gas by using density functional theory (DFT) and hartree–fock (HF)

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Received 4 March, 2014; Accepted 2 May, 2014

The active compounds of Curcuma longa (Curcumin I and Curcumin II) were investigated by using density functional theory (DFT) and hartree–fock (HF) in blood and gases. Curcumin II react with the radicalic forms of the molecules that cause cancer cell formation easily. On the other hand, the free energy of Curcumin I is higher than Curcumin II. Hence, hydrogen bonds formation of Curcumin II is more than Curcumin I. So, medical experimental studies can be done for these two substances.

Key words: Curcumin I, Curcumin II, density functional theory (DFT), hartree–fock (HF).

INTRODUCTİON

Curcuma longa, a plant, is a member of the Zingiberaceae family. It grows in Asia, India, China and other countries with a tropical climate. It has long leaves and bears funnel-shaped yellow flowers. The rhizome is the part of the plant used medicinally; it is usually boiled, cleaned and dried, yielding a yellow powder. Dried *Curcuma longa* is the source of the spice turmeric, the ingredient that gives curry powder its characteristic yellow color. Turmeric is used extensively in foods for both its flavor and color. It has a long tradition of use in the Chinese as tradational medicine, particularly as an anti inflammatory agent and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage and colic. The active constituents of it are the flavonoid curcumin and volatile oils including tumerone, atlantone and zingiberone. Other constituents include sugars, proteins and resins (Dobelis, 1986; Leung, 1980).

Animal studies with rats and mice, as well as in human cell lines, have demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumorpromotion, angiogenesis and tumor growth. In two studies of colon and prostate cancer, curcumin inhibited cell proliferation and tumor growth. Curcumin is also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both *in vitro* and *in vivo* studies. The anti carcinogenic effects of it is due to direct antioxidant and free-radical scavenging effects, as well as its ability to indirectly increase glutathione levels, hepatic detoxification of mutagens and carcinogens, and inhibiting nitrosamine formation (Limtrakul et al., 1997; Hanif et al., 1997; Dorai et al., 2001; Mehta and Moon, 1991; Soudamini and Kuttan, 1989; Azuine and Bhide, 1992; Pizorrno and Murray, 1999).

Turmeric has been widely used for the treatment of some diseases. Epidemiological observations, though inconclusive, are suggestive that its consumption may

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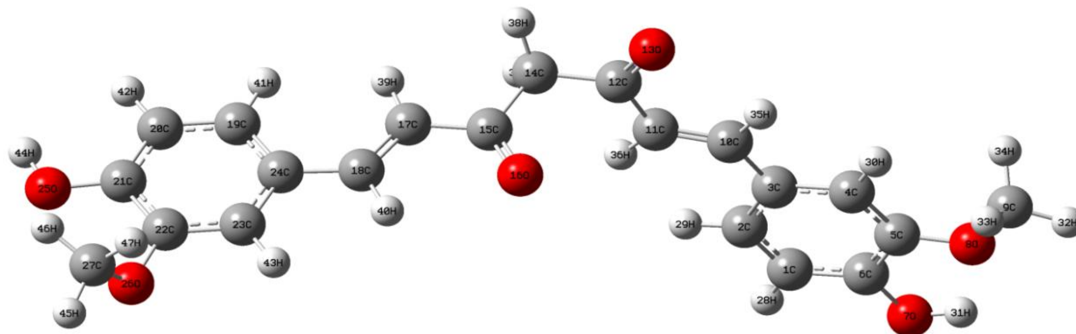

Figure 1. Moleculer structure of Curcumin I.

reduce the risk of some form of cancers and render other protective biological effects in humans. These biological effects of it have been attributed to its constituent curcumin that has been widely studied for its anti-inflammatory, anti-angiogenic, anti-oxidant, wound healing and anti-cancer effects (Maheshwari et al., 2006).

Curcumin, one of the major components of tumeric, the dried rhizome of *Curcuma longa*, has been shown to have anti-proliferating and anti-carcinogenic properties. It has anti-tumor effects when curcumin (50 to 200 mg/kg) was orally administered to nude mice transplanted with the cancer cells (Cui et al., 2006). Curcumin exhibits growth inhibitory activity against prostate, colon and breast cancer. Although the effect of curcumin on ovarian cancer cells is not known, ıt is pointed that curcumin could induce cell death in ovarian cancer cells, and enhance apoptosis induced by tumor necrosis factor-related apoptosis (Wahl et al., 2007). Curcumin, the yellow pigment in turmericis is known to decrease proliferation of cancer cells by holding them at different phases of the cell cycle and to stimulate apoptosis in tumor cells. Curcumin encourages apoptosis primarily and contains the activation of caspase-3 and mitochondria-compromise pathway in sundry cancer cells of different tissue origin (Su et al., 2006). Curcumin, an active ingredient from the rhizome of the plant, has recently been demonstrated that the chemoprotective activities might be due to its ability to restrict cell growth and stimulate apoptosis (Shi et al., 2006). Curcumin is one of the most powerful chemoprotective and anticancer agents. Its biological effects range from antioxidant, anti-inflammatory to inhibition of angiogenesis and is also shown to possess specific antitumoral activity. The molecular mechanism of it has been demostrated to have several targets and interacting macro-molecules within the cell (Singh and Khar, 2006).

Different dietary and pharmacological agents have been suggested as alternative strategies for treatment and obstruction of colorectal cancer. Curcumin, an active ingredient of turmeric, that inhibits growth of malignant tumours, has an important role in the obstruction and treatment of colorectal cancer (Reddy et al., 2006). Curcumin, a component of turmeric (*C. longa*), is one such agent that has been shown to suppress the transcription factor nuclear factor-kappaB (NF-kappaB), which is implicated in proliferation, survival, angiogenesis and chemo-resistance (Kunnumakkara et al., 2007). Curcumin retains cell proliferation and alerts apoptosis in human leukaemia, prostate cancer and non-small cell lung cancer. It has a lot of pharmacological effects and has been pointed to have anti-inflammatory and anti-tumor activities (Balcerek and Matlawska, 2005). Preclinical studies in a variety of cancer cell lines containing breast, cervical, colon, gastric, hepatic, leukemia, oral epithelial, ovarian, pancreatic and prostate have everlastingly demostrated that curcumin has anti-cancer activity *in vitro* and in preclinical animal models. Curcumin can be useful for the chemo-protection of colon cancer in humans (Johnson and Mukhtar, 2007).

MATERİALS AND METHODS

The thermodynamical proporties of Curcumin I and Curcumin II are studied by using density functional theory (DFT) and hartree–fock (HF). RB3-LYP methods were used for geometry optimization. Calculation method comprising the DFT method was used. RB3-LYP and HF6-31G containing polarizing functions (d, p) basis set was used. These methods and fully optimized geometric structure of the compounds using the basic cluster was determined. Geometric structures were determined by calculating the frequency accuracy. The results obtained were evaluated. Gaussıan 09 package program was used in the calculations.

RESULTS

The active compounds of *C. longa* (Curcumin I and Curcumin II)'s moleculer structures are given in the Figures 1 and 2. The hyrogen bonds of Curcumin I and Curcumin II are given in the Figures 3 and 4. Hydrogen bond formations are shown on the moleculer shapes of these two molecules in Figure 3 and 4. When we looked at the Gibbs free energy of Curcumin I and Curcumin II in blood by using DFT in Table 1, Curcumin I is higher than Curcumin II, since hydrogen bond formation in Curcumin

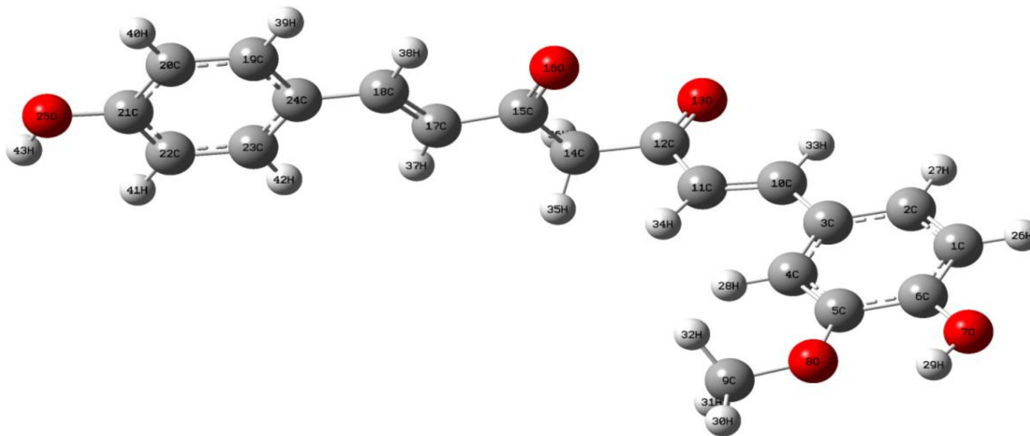

Figure 2. Moleculer structure of Curcumin II (demethoxycurcumin).

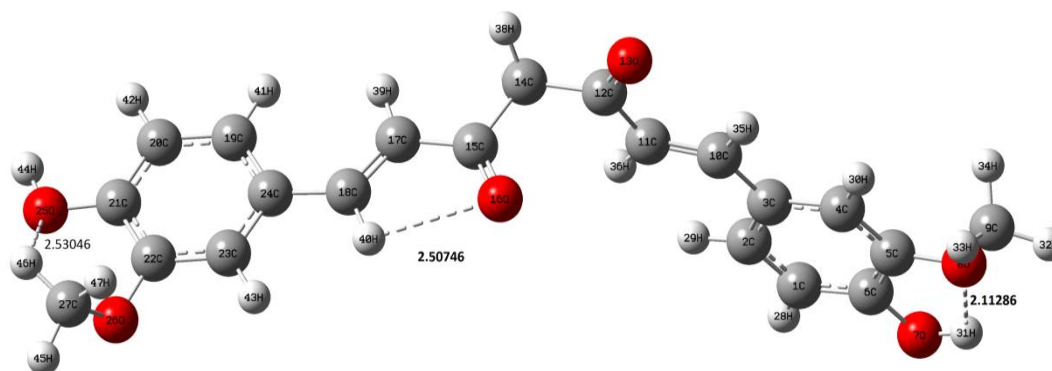

Figure 3. Hydrogen bonds of Curcumin I.

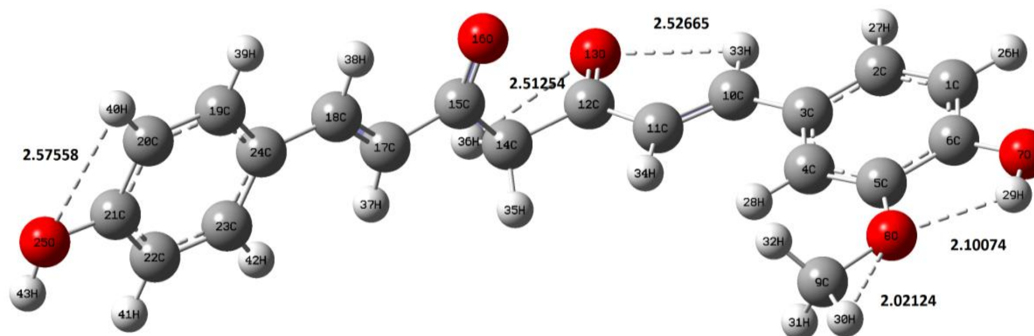

Figure 4. Hydrogen bonds of Curcumin II (demethoxycurcumin).

II is more than Curcumin I. When we looked the Gibbs free energy of Curcumin I and Curcumin II in blood by using HF in Table 2, the results are parallel to those in Table 1; Curcumin I is higher than Curcumin II, since hydrogen bond formation in Curcumin II is more than Curcumin I. When we looked at the Gibbs free energy of Curcumin I and Curcumin II in gas by using DFT in Table 3, Curcumin I is higher than Curcumin II, since hydrogen bond formation in Curcumin II is more than Curcumin I.

DISCUSSION

When we looked at the Gibbs free energy of Curcumin I and Curcumin II in gas by using HF in Table 4, the results are parallel to the results in Table 3; Curcumin I is higher

Table 1. The Curcumin I, Curcumin II values of ∆G, HOMO, LUMO, ∆ (HOMO-LUMO) and dipol moment ın blood by using DFT.

DFT (In blood)	∆G (Hartree)	HOMO	LUMO	∆ (HOMO-LUMO)	Dipol moment
Curcumin I	-1263.366036	-0.00476	0.00046	-0.00522	5.13480
Curcumin II	-1148.872154	-0.00321	0.00035	-0.00356	11.8623

HOMO: highest occupied molecular orbital; LUMO: lowest unoccupied molecular orbital

Table 2. The Curcumin I, Curcumin II values of ∆G, HOMO, LUMO, ∆ (HOMO-LUMO) and dipol moment ın blood by using HF.

HF (ın blood)	∆G (Hartree)	HOMO	LUMO	∆ (HOMO-LUMO)	Dipol moment
Curcumin I	-1255.765819	-0.30490	0.05216	0.35706	4.5055
Curcumin II	-1141.914713	-0.30169	0.05233	0.35402	9.0419

HOMO: highest occupied molecular orbital; LUMO: lowest unoccupied molecular orbital

Table 3. The Curcumin I, Curcumin II values of ∆G, HOMO, LUMO, ∆ (HOMO-LUMO) and Dipol Moment In Gas By Using DFT.

DFT (ın gas)	∆G (Hartree)	HOMO	LUMO	∆ (HOMO-LUMO)	Dipol moment
Curcumin I	-1263.286962	-0.00140	0.01354	0.13680	6.1082
Curcumin II	-1148.798758	-0.01391	0.00036	0.01427	2.3726

HOMO: highest occupied molecular orbital; LUMO: lowest unoccupied molecular orbital.

Table 4. The Curcumin I, Curcumin II values of ∆G, HOMO, LUMO, ∆ (HOMO-LUMO) and Dipol Moment In Gas By Using HF

HF (In Gas)	∆G (Hartree)	HOMO	LUMO	∆ (HOMO-LUMO)	Dipol moment
Curcumin I	-1255.703083	-0.29708	0.06246	0.35954	5.2579
Curcumin II	-1141.858214	-0.29072	0.06301	0.35373	1.7572

HOMO: highest occupied molecular orbital; LUMO: lowest unoccupied molecular orbital.

than Curcumin II, since hydrogen bond formation in Curcumin II is more than Curcumin I. According to highest occupied molecular orbital-lowest unoccupied molecular orbital (HOMO-LUMO) differences in Tables 1 and 2, the difference between Curcumin I is -0.00522, 0.35706 and for Curcumin II is -0.00356, 0.35402. Curcumin I is more stable than Curcumin II. The dipol moments of Curcumin I and Curcumin II are 5.13480, 4.5055 and 11.8623, 9.0419. So, Curcumin II has higher dipol moment than Curcumin II. Curcumin II dissolves in blood easily than Curcumin I. In the gas form of these two substances, Curcumin I and Curcumin II, by using DFT and HF, the dipol moment values are 6.1082, 5.2579 and 2.3726, 1.7572. They are near to each other. Therefore, we can assume that Curcumin II is eager to dissolve in blood and give reaction to radicalic forms of other mole-culues. Because of that, It slows cancer cells formation by preventing the radicalic damage. On the other hand, the free energy of Curcumin I is higher than Curcumin II. Hence, hydrogen bonds formation of Curcumin II is more than Curcumin I (5:3).

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Full Length Research Paper

Flowcytometric analysis of aldehyde dehydrogenase activity in mononuclear cells from umbilical cord blood

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Aldehyde dehydrogenase (ALDH) is a cytosolic enzyme that is responsible for the oxidation of intracellular aldehydes. Elevated levels of ALDH have been demonstrated in murine and human progenitor cells compared with other hematopoietic cells, and this is thought to be important in chemoresistance and purification techniques and an indication of the proper function of the cell. A Flowcytometric method for the assessment of ALDH activity in viable cells recently has been developed. Forty six cord blood samples from mothers which underwent normal delivery of full term infants were obtained, after informed consent. Mononuclear cells were obtained by Ficoll-Paque density centrifugation and ammonium chloride red cell lysis. Percentage of viable cells was determined by trypan blue exclusion dye. Cells were labeled with Aldefluor reagent (Vancouver Canada) as described by the manufacturer. Cells were then stained with phycoerythrin (PE)–conjugated anti-CD34 (Miltenyi Biotec, Cologne, Germany) antibodies for 30 min at 4°C. Cells were washed and re-suspended in phosphate-buffered saline (PBS) with 2% fetal calf serum. Cells were then analyzed on coulter epics flow cytometer. The mean percentage of ALDH enzyme expression among the CD34+ cells in the cord blood samples was 61.3% with a minimum of 28% and a maximum of 94.6%. Significant correlations were found between the white blood cell (WBCS) count in the cord blood samples and both the CD34+ cell count and the count of ALDH expressing cells, while no correlation was found between the CD34+ cells count or the ALDH expressing cells count in the cord blood samples and either the sex or the weight of the newborn. Identification and isolation of cells on the basis of ALDH activity provides a tool for their isolation and further analysis. In **summary, a high ALDH-1 activity identifies CD34$^+$ cells in cord blood.**

Key words: Umbilical cord blood, stem cells, aldehyde dehydrogenase (ALDH), CD34.

INTRODUCTION

Aldehyde dehydrogenase (ALDH) is a family of enzymes involved in metabolism of aldehydes to their corresponding carboxylic acids (Cheung et al., 2007). It plays an important role in metabolism of vitamin A as well as in mechanisms of resistance to alkylating agents, for example cyclophosphamide (Storms et al., 1999). For these reasons, ALDH is considered a protecting or detoxifying enzyme, able to preserve stem cells from cytotoxic effects (Storms et al., 1999; Fallon et al., 2003; Hess et al., 2004). One of the accepted technologies to

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identify human hematopoietic stem cells (HSC) is based upon flow cytometry (FCM) detection of ALDH enzymatic activity (Storms et al., 1999).

The functional role of ALDH has been studied, with specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), which was able to alter the molecular and cellular mechanisms that control self-renewal capacity of human HSC (Chute et al., 2006). The evidence of ALDH involvement in the physiology of HSC was further highlighted by a series of studies devoted to purification/analysis of highly immature progenitor cells, particularly in human cord blood (CB) as well as in murine bone marrow (BM) (Christ et al., 2007; Juopperi et al., 2007; Hess et al., 2006). The importance of ALDH in human hematopoiesis was also testified by a recent study in which the authors tried to purify HSCs by combining FCM cell sorting and Hoechst-33342 efflux ability (the so called "side population") (Pearce and Bonnet, 2007). At variance with previous findings obtained in mouse, human BM hematopoietic cells able to exclude Hoechst-33342 did not correspond to highly immature HSCs. On the other hand, the authors proposed that ALDH activity had to be considered as the reference method for the detection of immature HSCs in human BM, at the same time emphasizing the need for studies about expression pattern of ALDH in comparison with other hematopoietic cell markers in this tissue (Pearce and Bonnet, 2007).

Human HSCs have traditionally been characterized by the expression of cell surface markers such as CD34 (Civin et al., 1984; Bhatia et al., 1997a), but not all human hematopoietic repopulating cells express CD34 (Bhatia et al., 1998; Dao et al., 2003) and cell surface phenotype can be altered by cell cycle progression and *ex vivo* manipulation (Dorrell et al., 2000; Guenechea et al., 2000; Bhatia et al., 1997b; Hess et al., 2003; Nakamura et al., 1999; Sato et al., 1999). A purification strategy complementary to the use of surface phenotype involves the assessment of intracellular enzyme activities associated with the protection of primitive cells from oxidative insult during hematopoietic development. One promising purification strategy exploits cytosolic ALDH, an enzyme implicated in retinoid metabolism and the resistance of HSCs to alkylating agents such as cyclophosphamide (Sahovic et al., 1988; Takebe et al., 2001). Murine repopulating cells (Sharkis et al., 1997; Jones et al., 1995) and human hematopoietic progenitors have previously been isolated based on increased activity of intracellular ALDH (Storms et al., 1999; Jones et al., 1995).

One promising strategy is HSC isolation according to a conserved stem cell function rather than phenotype. In the murine system, lymphohematopoietic stem cells have been isolated according to the high expression of the detoxifying enzyme (ALDH) (Sharkis et al., 1997; Jones et al., 1995; Jones et al., 1995). Storms et al. (1999) described a fluorescent substrate of ALDH (termed aldefluor) that can be used to isolate cells with increased ALDH activity by fluorescence-activated cell sorting (FACS). The substrate is an amino acetaldehyde molecule conjugated to a BODIPY (4, 4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza- 5-proprionic acid) fluorochrome that is metabolized by ALDH to an aminoacetate anion was retained within the cell because of its negative charge. Thus, the amount of fluorescent product that accumulates in viable cells correlates to ALDH activity and cells with high ALDH activity can be selected from human umbilical cord blood (UCB) or mobilized peripheral blood by FACS (Fallon et al., 2003; Storms et al., 1999). UCB cells isolated by using this strategy have demonstrated to be depleted of lineage committed hematopoietic cells and are enriched for primitive hematopoietic progenitors detected in clonogenic *in vitro* cultures (Storms et al., 1999). This approach has allowed the analysis of viable murine and human ALDH+ progenitors by flow cytometry (Storms et al., 1999).

MATERIALS AND METHODS

We obtained forty six cord blood samples from mothers attending Suez Canal University Hospital, after informed consent. All cord blood samples (each sample = 30 ml) were stored overnight at room temperature before ALDH analysis. The protocol was approved by the institutional research ethics committees. Mononuclear cells (MNCs) were obtained by Ficoll-Paque density centrifugation and 0.8% ammonium chloride red cell lysis. Percentage of viable cells was determined by trypan blue exclusion dye.

Cell labeling

Cells were labeled with Aldefluor reagent (Vancouver Canada) as described by the manufacturer. Cells were then stained with phycoerythrin (PE)–conjugated anti-CD34 (Miltenyi Biotec, Cologne, Germany) antibodies for 30 min at 4°C. Cells were washed and re-suspended in phosphate-buffered saline (PBS) with 2% fetal calf serum. Cells were then analyzed on coulter epics flow cytometer. Aldefluor reagent was excited at 488 nm. Gates were set up to exclude nonviable cells and debris. The negative fraction was determined using appropriate isotype controls (Figures 1 and 2). For consistent results, Aldefluor-stained cells must be analyzed within 2 h of labeling. However, cells retain their ability to convert the ALDH substrate for at least 24 h after collection. We stored cord blood samples overnight before Aldefluor labeling and analysis without any detectable effect on the ALDH profile. DEAB tubes or negative control tubes were done to confirm that cellular fluorescence was the result of the activity of cytosolic ALDH; cells were incubated with DEAB which is a specific, competitive inhibitor of cytosolic ALDH that is nontoxic to cells *in vitro* and *in vivo* for 15 min at 37°C. These steps were repeated for each sample to be tested.

Set-up analyzer

1. In set-up mode, a DEAB control sample was placed on the cytometer; on the FSC vs. SSC plot, the R1 region was adjusted to encompass the leukocyte population of interest based on scatter. 2. On the FL1 vs. SSC plot, the FL1 photo-multiplier tube (PMT)

Table 1. Count and percentage of CD34+ cells to the total leucocytic count (TLC) in the cord blood samples.

Parameter	Mean	Maximum	Minimum	Standard deviation
% of CD34+ cells to the TLC	3.60	8.10	1.40	1.40
Count of CD34+ cells × 103/µl	0.48	1.12	0.12	0.23

TLC: thin layer chromatography.

Table 2. Count of ALDHbr cells × 103/µl in cord blood samples.

Parameter	Mean	Maximum	Minimum	Standard deviation
Count of ALDHbr cells × 103/µl	0.29	0.89	0.05	0.19

Table 3. Comparison between means of CD34+ cells count and ALDHbr cells count in cord blood samples.

Cd34+cells count × 10^3/µl		ALDHbr cells count × 10^3/µl		t-test	Significance (2-tailed)
Range	M±SD	Range	M±SD		
0.12-1.16	0.49±0.23	0.05-0.89	0.29±0.23	14.07	0.000**

**Difference is statistically significant.

voltage was adjusted so that the right edge of the stained population was placed at the 2nd log decade on the dot plot. The tube was removed. All cells were fluorescent due to the intracellular substrate.
3. The corresponding ALDH test sample was placed on the cytometer. The R2 region was adjusted to encompass the cell population that was side scatter-low and ALDH-bright. The tube was removed.
4. For data acquisition of test samples, the analyzer was put in acquisition mode and 100,000 events were collected on each ALDH and DEAB sample using the same instrument settings. DEAB control regions might need to be adjusted for each sample. ALDH-bright, SSC-low stem cells appeared in the R2 region.
5. Analysis for double expression of ALDH bright cells and CD34 +ve cells was done.

Data handling

1. FSC vs. SSC dot plot and region R1 that would encompass the leukocyte population of interest based on scatter were created.
2. Two FL1 vs. SSC dot plots were created gated on R1. A region R2 was created in both plots that began at the 2nd log decade of FL1 and was within the range of 200-400 on side scatter.
3. An ALDH positive sample data file was opened. The R1 region was adjusted in FSC vs. SSC dot plot to encompass the "viable" leukocyte population.
4. On the FL1 vs. SSC dot plot, the R2 region was adjusted to encompass the SSC low, ALDH-bright cells
5. Using the corresponding DEAB control tube, placement of the R2 region on the ALDH sample was verified by making sure that there were few or no events in the R2 area.
6. Region statistics were added to the plots.
7. The percentage of the ALDH-bright population was found from the percentage gated in R2 from the FL1 vs. SSC dot plot.

Statistical analysis

Statistical package for the social sciences (SPSS version 10.0) software was used for data analysis. The Student's paired t-test for significance of no difference was used throughout this report.

RESULTS

The study population included 46 umbilical cord blood samples collected from immediate newborns, 22 males (47.8%) and 24 females (52.2%). The mean weight of the newborns was 3258.7 g, with a minimum of 2,300 g and a maximum of 3,900 g. The mean white blood cells count in the tested cord blood samples was 13.6 × 10^3/µl, with a range of 5.7 × 10^3/µl to 27.3 × 10^3/µl, while the mean blood hemoglobin of the tested cord blood samples was 14.5 g/dl, with a range of 10.6 to 17.2 g/dl.

The mean percentage of the CD34 cells to the total leucocytic count was 3.6%; with a minimum of 1.4% and a maximum of 8.1% (Table 1); while the mean count of the CD34 cells × 10^3/µl in cord blood samples was 0.48 × 10^3/µl with a minimum of 0.12 and a maximum of 1.16 × 10^3/µl (Table 1). The mean count of the cells expressing the ALDH enzyme × 10^3/µl in cord blood samples was 0.29 × 10^3/µl, with a minimum of 0.05 and a maximum of 0.89 × 10^3/µl (Table 2). We found a statistically significant difference when we compared between the mean count of CD34+ cells and the mean count of ALDH expressing cells (Table 3). Also, there was a significant positive correlation between the count of CD34+ cells and the count of ALDH expressing cells (Figure 2). The mean percentage of ALDH enzyme expression among the CD34+ cells in the cord blood samples was 61.3% with a minimum of 28% and a maximum of 94.6% (Table 4 and Figure 3). Significant correlations were found between the WBCs count in the cord blood samples and both the

Table 4. Percentage of ALDHbr cells to the CD34^{+} cells in cord blood samples.

Parameter	Mean	Maximum	Minimum	Standard deviation
% of ALDHbr cells to CD34+ cells	61.30	94.60	28	19.80

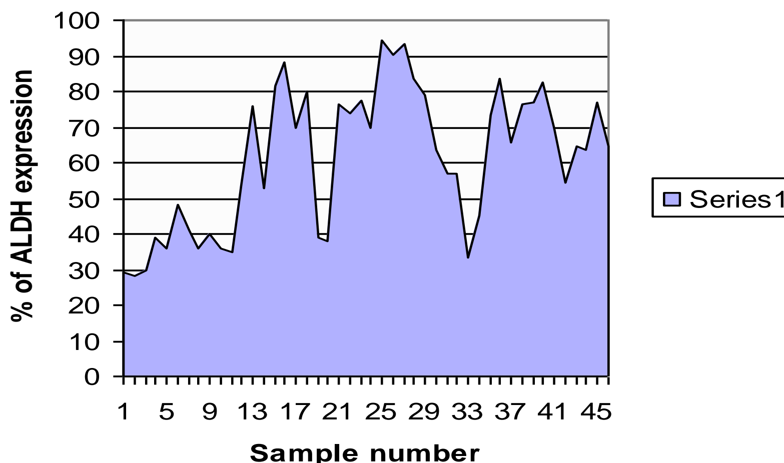

Figure 1. Percentage of ALDHbr cells to the CD34+ cells in cord blood samples

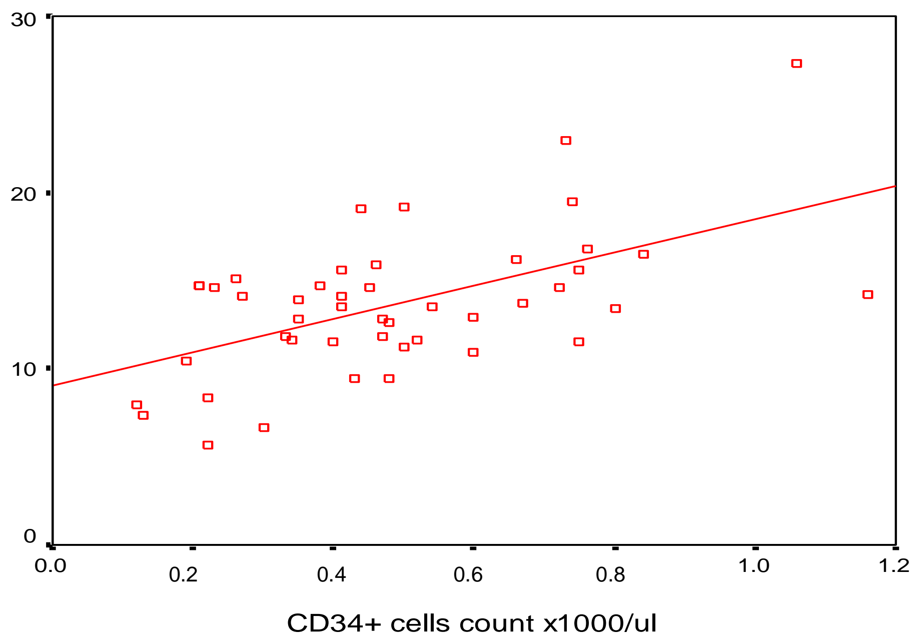

Figure 2. Correlation between WBCs count and CD34+ cells count. Pearson correlation: 0.557. Significance: 0.000; correlation is significant.

CD34+ cell count and the count of ALDH expressing cells (Figures 4 and 5). No correlation was found between the sexes or the weight of the newborn and neither the CD34+ cells count nor the ALDH expressing cells count in the cord blood samples.

DISCUSSION

Human cord blood hematopoietic cells with high ALDH activity are highly enriched for primitive CD34+ cells and depleted for lineage-positive (Lin+) cells (CD3, CD14,

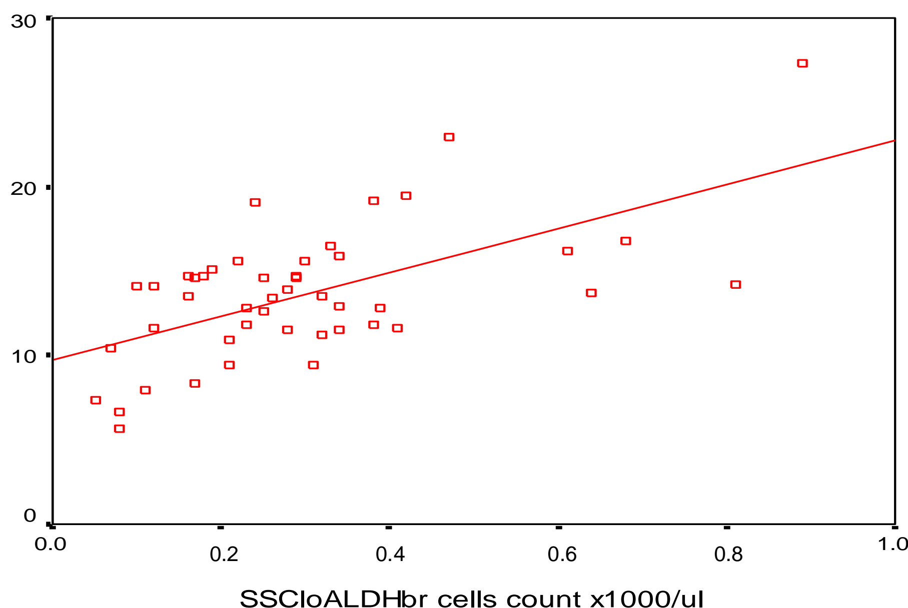

Figure 3. Correlation between WBCs count and ALDHbr cells count. Pearson correlation: 0.602. Significance: 0.000; SSClo ALDHbr: cells with low side scatter and bright ALDH. Correlation is significant.

CD20, and CD56), indicating that they do indeed represent a primitive hematopoietic cell population (Storms et al., 1999).

Methods to safely identify primitive HSCs with enhanced repopulating function are constantly sought for clinical stem cell transplantation. Conventionally, HSCs are purified using a single isolation strategy, such as the selection of cells based on cell surface phenotype (CD34 expression) or efflux of metabolic markers such as Hoechst dye by membrane pumps (Civin et al., 1984; Bhatia et al., 1997a; Sharkis et al., 1997; Gallacher et al., 2000; Guenechea et al., 2001; De Wynter et al., 1998; Goodell et al., 1997; Handgretinger et al., 2003). However, cell phenotype, such as CD34 surface expression, can vary depending on micro-environmental factors or cellular activation (Dao et al., 2003; Hess et al., 2003) and clinical procedures are incompatible with the use of toxic or DNA-intercalating dyes. Nontoxic cell-sorting strategies based on conserved stem cell function, in combination with cell surface phenotype, are necessary for clinical cell purification and may be useful for the study of complex developmental processes such as self-renewal versus the sequential transition from primitive HSCs to restricted progenitors. Our laboratory and others have demonstrated that cells with high intracellular ALDH activity from human UCB comprise a heterogeneous population of clonogenic progenitors and are enriched for NOD/SCID repopulating cells (Storms et al., 1999; Fallon et al., 2003; Hess et al., 2004). This isolation strategy uses a nontoxic, fluorescent substrate of ALDH, safely and effectively labeling cells with ALDH activity for selection by flow cytometry.

Many studies are describing ALDH expression in human CB (Hess et al., 2004; Christ et al., 2007; Hess et al., 2006; Gentry et al., 2007), peripheral blood stem cells (Fallon et al., 2003) and even acute myeloid leukemia (AML) BM cells (Cheung et al., 2007) data aiming to characterize normal BM ALDH+ cells are still limited to a few recent reports (Pearce and Bonnet, 2007; Gentry et al., 2007). Our interest in ALDH detection relies on the consideration that FCM based ALDH activity assessment is exploited in order to evidence a conserved stem cell function, rather than to merely identify a stem cell antigen (Morita et al., 2003).

In the current study, only 61.3 ± 19.8% of CD34+ cells were found to express ALDH activity. Robert et al. (1999) found that 74% ± 20 of CD34+ cells express ALDH activity, while David et al. (2004) found that the percentage was 91 ± 1.4%. Christ et al. (2003) found that the percentage was 95 ± 1%. These data strongly suggest that there is functional heterogeneity within the CD34+ cell population and that further purification of human stem or progenitor cells may be achieved through the analysis of ALDH activity. ALDHbr UCB cells was found to be 2% of the thin layered chromatography (TLC) while it was about 1% in the studies done by Christ et al. (2003) and David et al. (2004).

In the current study, no relation could be found between sex or weight of the newborn and expression of ALDH or CD34+. It was reported that birth weight of the neonate did not affect the mono-nucleated cell count (MNC) and subsequently CD34+ cell count. Hiett et al.

(1995) reported that there was no significant difference in the mean number of progenitors/UCB unit according to newborn weight. On contrary to these results, Arovita et al. (2005) reported that the correlation between birth weight and CD34+ cell concentration was statistically clearly significant. In this study, they tested 1368 CB samples for associations of selected factors as birth weight. Another study included 3838 CB units analyzing CD34+ cell contents only on units with a volume > 80 ml, a correlation analysis of CD34+ count and weight, revealing that baby weight was associated with higher CD34+ cell content in UCB (P = 0.0001). In this same study, a correlation analysis of CD34+ count and sex revealed that male newborns was associated with higher content of CD34+ cells (P = 0) (Guenechea et al., 2000).

Arovita et al. (2005) reported also that male infants had significantly higher median CD34+ cell concentration than female infants (31.8 × 103/µl vs. 30.2 × 103/µl, respectively (P = 0.03). There are several potential applications to this strategy for identifying and isolating HSCs. Enumerating ALDHbr cells may be a more reliable means for quantitating the transplantable stem cells in bone marrow, peripheral blood and UCB. Isolating ALDHbr cells also may be an effective method for purging autologous bone marrow or peripheral blood stem cell collections of tumor cells (Colvin et al., 1999). According to the manufacturers, the Aldefluor kit is active against the ALDH-1 isoform but not the ALDH-3 isoform. Both ALDH1 and ALDH3 are reportedly involved in chemoresistance (Civin et al., 1984; Bhatia et al., 1998; Bhatia et al., 1997b). In this study, we confirmed the use of the ALDH substrate kit to identify cord blood stem/progenitor cells expressing CD 34 via multicolor flow cytometry of cord blood ALDH+ cells.

A study done by Schuurhuis et al. (2013) showed marked difference between ALDH activity of HSC and LSC with the AML BM indicating the importance of ALDH activity as a functional stem cell biomarker and its usefulness in identification and purification of HSC and LSC with the aim of treatment decision making, relapse prediction and development of LSC specific therapies. Although HSC and LSC can, in a considerable part of AML cases, be distinguished using aberrancies of marker expression (van Rhenen et al., 2007a; van Rhenen et al., 2007b; Jordan et al., 2000) and scatter properties (Terwijn et al., 2007; Janssen et al., 2011), assessment of ALDH activity enables such discrimination in all AML cases even in the absence of aberrancies.

ALDH has received considerable attention as a functional marker for identification of cells with enhanced tumorigenic/metastatic potential and elevated therapeutic resistance in several cancers of epithelial origin (Ginestier et al., 2007; Jiang et al., 2009; Tanei et al., 2009). A possible application of ALDH detection by FCM to the field of acute leukemia may derive from the study of Cheung et al. (2007), in which the authors described ALDH expression in AML. They noted that in AML patients in complete remission, a relevant population of cells characterized by high ALDH activity remained (Cheung et al., 2007). So their data about multidimensional expression profile of ALDH combined with other hematopoietic antigens in normal BM precursors could represent the basis to distinguish by FCM leukemic from normal ALDH+ cells.

Overall, the ALDH kit is quick (1 h in total), easy to use and does not significantly affect cell viability or repopulation ability. The fluorescent substrate may be analyzed in conjunction with other common fluorochromes on a standard benchtop flow cytometer equipped with a 488 nm laser line. These properties suggest that this is a technique more suitable for the clinic than alternative techniques that are toxic and require expensive analytical equipment (for example, a UV laser) (Goodell et al., 1997).

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Full Length Research Paper

Occurrence of hepatitis 'B' and 'C' amongst patients on antiretroviral drug therapy (ART) in a treatment centre in Calabar, Nigeria

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The occurrence of hepatitis 'B' and 'C' virus amongst patients on antiretroviral drug therapy (ART) was studied. Two hundred (200) human immunodeficiency virus (HIV) positive subjects on ART and 100 apparently healthy HIV negative subjects (control) were recruited for the study. The subjects aged 1 to 75 years were screened for hepatitis B and C viral antibodies using hepatitis B and C test strips manufactured by ACON Laboratories. Questionnaire were also administered. CD4 counts of the subjects were determined using CyFlow Counter manufactured by GEM Laboratories, Germany. Fourteen (7%) of the subjects were positive for hepatitis B virus (HBV) infection, 6 (3%) for hepatitis C virus (HCV) and 2 (1%) for mixed infections. In the control group, a prevalence of 6 (6%) was recorded for HBV, 4 (4%) for HCV and none for mixed infections. Among the test group, subjects in age group 51 to 60 years had the highest prevalence rate for HBV (25%), 31 to 40 years for both HCV (7.3%) and mixed infection (3.6%). There was no statistically significant difference in infection according to age P=0.475. Males had a higher prevalence rate (9.1%) than the females (5.4%) for HBV, but there was no statistically significant difference in HBV infections according to gender P=0.404. In HCV infection, males had a higher prevalence rate (5.7%) than females (0.9%), but there was no statistically significant difference in HCV infection according to gender P=0.089. Subjects with CD4 counts in the range of 1401 to 1600 had the highest infection rate (50%) for HBV and 201 to 400 for HCV (7.7%) and mixed infection (5.1%). This work has shown that HBV and HCV are common among patients on ART and the need for routine screening of this category of patients in order to aid in the effective management of co-infections.

Key words: Hepatitis B, Hepatitis C, antiretroviral therapy, HIV, Calabar.

INTRODUCTION

In Nigeria and other developing countries, human immunodeficiency virus/acquired immunodeficiency virus (HIV/AIDS) disease is a major public health problem, and a serious threat to development. Since the introduction of potent antiretroviral drug therapy (ART), HIV/AIDS has been successfully converted from a uniformly fatal illness

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to a manageable chronic infection. Correspondingly, during the past years, the opportunistic infections that complicate profound immunosuppression have been replaced with newer forms of morbidity and even mortality. Chief among these has been the development of progressive liver disease due to hepatitis C virus (HCV) and hepatitis B virus (HBV). Due to their shared routes of transmission, HCV and HBV are frequently found in the HIV-infected host, while HCV co-infection has deservedly gained considerable attention as a major cause of mortality in the post-highly active antiretroviral therapy era (Bonacini et al., 2001). Complications of HBV-related liver disease are being increasingly recognized especially as drug-resistant forms of HBV have become nearly universal (Saravanan et al., 2007).

HBV and HCV co-infection in HIV positive individuals is of utmost importance due to the underlying cones-quences such as the hepatological problems associated with these viruses, which have been shown to decrease the life expectancy in the HIV-infected patients (Koziel and Peters, 2007; Major, 2009). Moreover, among the HIV-infected patients, 2 to 4 million are estimated to have chronic HBV co-infection, while 4 to 5 million are co-infected with HCV (Soriano et al., 2009). In Nigeria, the average carrier rate of hepatitis B in the general population is estimated to be 4% (Taylor et al., 2006). This study was an attempt to investigate the current prevalence of HBV and HCV among patients on ART in the study centre.

MATERIALS AND METHODS

Study location

The study centre was ART Laboratory, General Hospital, Calabar located in Calabar Municipality in Cross River State. Cross River is a coastal state in South Eastern Nigeria, bordering Cameroon to the east and it is located in Nigeria's Delta region.

Patients' recruitment

The study subjects were 200 consecutive patients aged 1 to 75 years on ART, while 100 HIV negative apparently healthy control subjects who were randomly selected from members of the general hospital community whose HIV status were negative at the time of study. The study subjects and the controls were age and sex matched. The study was conducted between November 2011 and June 2012. Questionnaires were administered to obtain the demographic data of the subjects.

Ethical clearance was sought and obtained from the State Ministry of Health. Prior to specimen collection, verbal consent from each of the subjects and/or their guardians were sought and obtained. Those who declined participation were excluded from the study.

CD4 T lymphocytes count

Partec CyFlow Counter was the machine used for analysis of CD4 count with serial No. 090746022 manufactured by GEM Laboratories, a biotechnological company in Germany (Pantec GmbH Am flugplatz 13 D-02828 Glorilitz Germany).

The Partec CyFlow Counter which is a fully equipped portable/mobile flow cytometry system (FCM) was used for the identification and the enumeration of the CD4 T lymphocytes which is the first point of attack by the HIV virus.

HBV screening test

Sample from each subject was screened serologically for hepatitis B surface antigen. The test was done using ACON hepatitis B surface antigen rapid test strip manufactured by ACON Laboratory Inc 4108 Serrente Valley Boulevard, San Diego, CA 92121 in United States of America.

The HBsAg one step hepatitis B surface antigen test strip (serum/plasma) is a qualitative, lateral flow immunoassay for the detection of HBsAg in serum or plasma. The membrane is pre-coated with anti-HBsAg antibodies on the test line region of the strip. During testing, the serum or plasma specimen reacts with the particle coated with anti-HBsAg antibody. The mixture migrates upward on the membrane chromatographically by capillary action to react with anti-HBsAg antibodies on the membrane and generate a colour line. The presence of this coloured line in the test region indicates a positive result, while its absence indicates a negative result. This test strip has been compared with a leading commercial HBsAg EIA test and the correlation between this two is over 90%. The relative sensitivity, specificity and accuracy are 99, 97 and 98.5%, respectively (Blumberg et al., 1971).

HCV screening test

Hepatitis C virus antibodies was screened using ACON one strip hepatitis C virus test strip manufactured by ACON Laboratory Inc 4108 Serrente Valley Boulevard, San Diego, CA 92121 in United States of America. This HCV one step hepatitis C test strip (serum/plasma) is a qualitative, membrane based immunoassay for the detection of antibody to HCV in serum or plasma. The membrane is coated with recombinant HCV antigen on the test line region of the strip. During testing, the serum or plasma specimen reacts with the protein A coated particles. The mixture migrates upward on the membrane chromatographically by capillary action to react with recombinant HCV antigen on the membrane and generate a coloured line. The presence of this coloured line indicates a positive result, while its absence indicates a negative result. To serve as a procedural control, a coloured line will always appear at the controlled line region indicating that proper volume of specimen has been added and membrane wicking has occurred. This test strip has been compared with a leading commercial HCV EIA test. The relative sensitivity, specificity and accuracy are >99.0, 98.6 and 99.3%, respectively (van der Poel et al., 1991; Wilber, 1993).

HIV screening test

HIV screening was done using the serial algorithm of screening with determined and confirmed result with Unigold (WHO, 1993).

Alere determined HIV1/2 is an immunochromatographical test for the qualitative detection of antibodies to HIV-1 and HIV-2.

HIV confirmatory test using uni-gold

All the test samples that were positive were confirmed with a second test using uni-gold. For testing, two drops of whole blood from the pricked finger were allowed to fall into the sample port, followed by two drops of wash buffer and allowed to react.

Table 1. Prevalence of HBV and HCV amongst subjects examined according to age.

Age group	Test subject				Control subject			
	No. examined	No. (%) with HBV infection	No. (%) with HCV infection	No. (%) with both infections	No. examined	No. (%) with HBV infection	No. (%) with HCV infection	No. (%) with both infections
1-10	6	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)
11-20	7	0 (0)	0 (0)	0 (0)	7	0 (0)	0 (0)	0 (0)
21-30	58	2 (3.45)	0 (0)	0 (0)	37	4 (10.8)	1 (2)	0 (0)
31-40	55	5 (9.1)	4 (7.3)	2 (3.6)	32	1 (3.1)	1 (3.1)	0 (0)
41-50	57	4 (7.0)	2 (3.5)	0 (0)	16	0 (0)	1 (6.25)	0 (0)
51-60	8	2 (25)	0 (0)	0 (0)	8	1 (12.5)	1 (12.5)	0 (0)
61-70	8	1 (12.5)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)
71 above	1	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)
Total	200	14 (7)	6 (3)	2 (1)	100	6 (6)	4 (4)	0 (0)

Table 2. Prevalence of infection among subject examined according to gender.

Gender	Test subject				Control subject			
	No. examined	No. (%) with HBV infection	No. (%) with HCV infection	No. (%) with both infections	No. examined	No. (%) with HBV infection	No. (%) with HCV infection	No. (%) with both infections
Female	112	6 (5.4)	1 (0.9)	1 (0.9)	46	4 (8.7)	1 (2.2)	0 (0)
Male	88	8 (9.1)	5 (5.7)	1 (1.1)	54	2 (3.7)	3 (5.6)	0 (0)
Total	200	14 (7)	6 (3)	2 (1)	100	6 (6)	4 (4)	0 (0)

Antibodies of HIV-1 or HIV-2 proteins were bound to the colloidal gold linked antigens. The antibody protein colloidal gold complex moves chromatographically along the membrane to the test and control regions of the test device. A positive reaction is visualised by a pink band in the test region of the device and in the control line. A negative reaction occurs in the absence of human immunoglobin antibodies to HIV in the analysed specimen. Consequently, no visually detectable band develops in the test region of the device.

Data analysis

Variables were analysed using Statistical Package for Social Sciences (SPSS) software.

RESULTS

Two hundred (200) HIV positive subjects on ART and 100 apparently healthy HIV negative subjects (control) were recruited into the study.

Table 1 shows the prevalence of HBV and HCV amongst subjects examined according to age. In the test subjects group, those in the age group 51 to 60 years had the highest prevalence rate of infection with HBV (25%), but there was no statistically significant difference in the prevalence of HBV infection by age (χ^2 = 6.9, df (7), P > 0.05). Those in age group 31 to 40 years had the highest HCV rate infection (7.3%). But there was no statistically significant difference in HCV infection by age (χ^2 = 6.2, df (7), P >0.05).

Amongst those with mixed infection, subjects in age group 31 to 40 years had the highest prevalence of infection (3.6%), but there was no statistically significant difference in mixed infection (χ^2 = 5.3260, df (7), P >0.05).

Among the control subjects, age group of 51 to 60 years had the highest infection with HBV and HCV (12.5 and 12.5%), respectively. There was no statistically significant difference in infection according to age (χ^2 = 0.1199, df (7), P >0.05) and (χ^2 = 1.813, df (7), P >0.05), respectively. There was no mixed infection in the control subjects.

The prevalence of HBV and HCV infection according to gender is as shown in Table 2. In HBV infection, males (9.1%) were more infected than the females (5.4%), but there was no statistically significant difference between infections in males and females (χ^2 = 1.055, df (1), P = 0.404). Males were also more infected (5.7%) than females (0.9%), and there was also no statistical significant difference in HCV infection (χ^2 = 3.884, df (1), P > 0.05). Among those with mixed infection, males (1.1%) were more infected than females (0.9%), but there was also no statistically significant difference in mixed infection according gender (χ^2 = 0.030, df (1), P = 0.864).

In the control subjects, females were more infected

Table 3. Distribution of infection according to CD4 count of subject examined.

CD4 count range	Test subject				Control subject			
	No. examined	No. (%) with HBV infection	No. (%) with HCV infection	No. (%) with both infections	No. examined	No. (%) with HBV infection	No. (%) with HCV infection	No. (%) with both infections
0-200	21	2 (9.5)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)
201-400	39	4 (10.2)	3 (7.7)	2 (5.1)	0	0 (0)	0 (0)	0 (0)
401-600	49	3 (6.4)	1 (2.0)	0 (0)	22	1 (4.5)	2 (9.1)	0 (0)
601-800	32	3 (9.4)	0 (0)	0 (0)	24	3 (12.5)	0 (0)	0 (0)
801-1000	19	1 (5.3)	1 (5.3)	0 (0)	26	2 (7.7)	2 (7.7)	0 (0)
1001-1200	14	0 (0)	1 (7.1)	0 (0)	15	0 (0)	0 (0)	0 (0)
1201-1400	6	0 (0)	0 (0)	0 (0)	5	0 (0)	0 (0)	0 (0)
1401-1600	2	1 (50)	0 (0)	0 (0)	4	0 (0)	0 (0)	0 (0)
1601-1800	18	0 (0)	0 (0)	0 (0)	4	0 (0)	0 (0)	0 (0)
Total	200	14(7)	6(3)	2(1)	100	6(6)	4(4)	0(0)

(8.7%) with HBV than males (3.7%), but there was no statistically significant difference in infection according to gender (χ^2 = 1.098, df (1), P = 1.410), while males were more infected (5.6%) with HCV than females (2.2%), but there was also no significant difference in the infection (χ^2 = 0.740, df (1), P > 0.05).

Table 3 shows the distribution of infection according to CD4 count. Subjects with a CD4 count of 1401 to 1600, had the highest infection rate (50%) with HBV. In HCV infection, the CD4 count of 201 to 400 had the highest prevalence rate of infection (7.7%). In the control group, subjects with CD4 count of 601 to 800 had the highest infection rate (12.5%) with HBV.

Among those with HCV infection, subjects with CD4 count of 401 to 600 had the highest infection rate (9.1%). No mixed infection was recorded in the control group.

DISCUSSION

From the results obtained from the test subjects, those in age group 51 to 60 years had the highest prevalence rate of infection for HBV (25%). This can be compared to the work done by Denue et al. (2011) at medical wards of University of Maiduguri Teaching Hospital, Nigeria on the survey of hepatitis B and C virus prevalence in HIV positive patients, who had a prevalence rate of 12.3% for HBV and 0.5% HCV infection with no mixed infection obtained. Similarly, this work can be compared to the work done by Adewole et al. (2009) at the Department of Medicine, Obafemi Awolowo University, Ile-Ife, Nigeria, on hepatitis B and C virus co-infection in Nigeria patients with HIV infection. Adewole et al. (2009) had 11.5% prevalence rate for HBV, 2.3% prevalence rate for HCV and 1.5% for mixed infections. This result can also be compared with the work carried out by Soriano et al. (2009) on hepatitis B and C in HIV/AIDS, Hong Kong, who had 23% for HBV, 16% for HCV and 5 to 10% mixed infections. Those in age group 31 to 40 years had the highest prevalence rate with HCV infection (7.3%). Among those with mixed infection, subjects in age group 31 to 40 years had the highest prevalence of infection (3.6%), but there was no statistically significant difference in mixed infection (P > 0.05). Among the control subjects, age group of 51 to 60 years had the highest infection with HBV and HCV (12.5 and 12.5%), respectively. There was no mixed infection in the control subjects.

The prevalence of HBV and HCV infection according to gender showed that in HBV infection males (9.1%) were more infected than females (5.4%), but there was no statistically significant differences between infections in males and females (P > 0.05). Among those with mixed infection, females were more infected (8.9%) than males (5.7%) and there was a statistically significant difference in mixed infection according to gender P < 0.05. In the control subjects, female were more infected with HBV (8.7%) than males (3.7%), but there was no statistically significant difference in infection according to gender P > 0.05, while males were more infected with HCV (5.6%) than females (2.2%), but there was no statistically significant difference in the infections P > 0.05. This can be compared to the work done by Denue et al. (2011), where blood donors were used as their controls, with a percentage prevalence of 5.2% for HBV and 1.4% for HCV. The distribution of CD4 count showed that CD4 count group of 1401 to 1600 had the highest infection rate (50%) with HBV. In HCV infection, the CD4 count group of 201 to 400 has the highest prevalence rate of infection (7.7%). In the control group, those with CD4 count of 601 to 800 CD4 group had the highest infection with HBV (12.5%). Among those with HCV infection subjects in CD4 count group of 401 to 600 had the highest infection rate (9.1%). No mixed infection was recorded in the control group. This can also be compared with the work done by Denue et al. (2011) at medical wards of University of Maiduguri, Nigeria on the survey of

hepatitis B and C virus prevalence in HIV positive patients. The mean CD4 count of the control group was significantly higher (181 cell/μl) than the test subjects (117 cell/μl).

According to WHO estimates, the global burden of HIV, HBV and HCV is 33.2, 400 and 170 million, respectively. Knowledge of the prevalence and distribution of blood borne viruses and sexually transmitted disease (STDs) in different part of the world, and particularly in Africa it is important for the planning of prevention measures and the development of vaccination programmes. More females than males were presented for care during the study period, but majority of males in the control subjects were blood donors. The gender inequality in presentation for therapy is consistent with the sex distribution documented in majority of treatment centres, particularly in the first decade of ART. The reason for more females at the study centre is that women present for care after positive HIV test on their sick children, death of their husbands or perhaps they are more sensitive to changes in their health and may be socially conditioned to seek and receive assistance for their sickness. This however does not mean that more women are infected with HIV in study centre, as study in Nigeria actually found that more men were afflicted with HIV/AIDS (Ola et al., 2005).

HIV has been shown conclusively to be an independent risk factor for more rapid CD4 decline, although it has been associated with increased occurrence of HBV, but HCV has not been known to decline CD4 count. The limitation of this study has been the availability funds for serotyping of these viruses, that is, confirmation of HBV and HCV, respectively. This work has shown that HBV and HCV are common amongst patients on ART. It is therefore advisable to screen for these viruses in all the HIV infected individuals and their sexual partners as a routine management and check up in order to aid in the proper management of the disease.

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