Genetic and reproductive toxicity of lamivudine, tenofovir disoproxil fumarate, efavirenz and their combination in the bone marrow and testicular cells of male mice

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Received 4th October, 2019, Accepted 23rd December, 2019
DOI: 10.2478/ast-2020-0001

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Abstract

The combination of antiretroviral (ARV) drugs; lamivudine, tenofovir disoproxil fumarate (TDF) and efavirenz is among the preferred first-line regimens for adolescents and adults infected with HIV. However, knowledge on in vivo genetic and reproductive toxicity of each of these drugs and their combination is limited. We evaluated the genotoxicity of lamivudine, TDF, efavirenz and their combination utilizing the mouse micronucleus (MN) and sperm morphology tests. Histopathological analysis of the testes of exposed mice was also carried out. 0.016, 0.032, 0.064 and 0.129 mg/kg bwt of lamivudine, TDF and the combination; and 0.032, 0.064, 0.129 and 0.259 mg/kg bwt of efavirenz corresponding to 0.125, 0.250, 0.500 and 1.000 x the human therapeutic daily dose (HTD) of each of the ARVs and their combinations were administered to mice for 5 consecutive days. Data on MN showed a significant increase (p < 0.05) across the tested doses of TDF, efavirenz and the combination, with the combination inducing lower frequency of MN than TDF and efavirenz. Lamivudine did not evoke significant induction of MN. Significant increase in frequency of abnormal sperm cells were observed in the tested samples, however, the combination induced the highest number of abnormal spermatozoa. The ARVs and their combination induced pathological lesions such as vacuolation and necrosis in mice testes. These findings suggest that the individual ARVs and their combination are potentially capable of activating genetic alterations in the bone marrow and germ cells of male mice thereby raising concern for long term use by HIV patients.

Keywords: HIV; Antiretroviral drugs; DNA damage; micronucleus; sperm morphology
1.0 Introduction

The human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) is a major cause of infectious disease related morbidity and mortality. HIV infection was rated the fifth leading cause of disability adjusted life years (DALY) worldwide for people aged 30 - 44 years in 2010 (Ortblad et al., 2013). Globally, 30.8 to 42.9 million people were reportedly living with HIV in 2016, with over 35 million deaths recorded due to AIDS-related illnesses since the start of the HIV epidemic (UNAIDS, 2017).

At present, no cure exists for HIV infections; however, management of the condition requires continuous daily use of antiretroviral drugs (ARVs) to prevent further weakening of a patient’s immune system by inhibiting the HIV replication process. At least thirty-nine single and combinations of ARVs have received approval of the U.S Food and Drug Administration (U.S. FDA) (AIDS, 2017) for the treatment of HIV/AIDS. These FDA-approved ARVs belong to one of five classes of drugs characterised by their molecular mechanism of action and the stage of life-cycle of the virus they inhibit. The ARV classes are: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs); protease inhibitors (PIs); integrase inhibitors (INIs); entry inhibitors (EIs); and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Calmy et al., 2009; Azu, 2012).

Antiretroviral therapy (ART) was originally administered as a monotherapy. This approach was later considered sub-optimal due to the instability of the HIV genome which allowed strains of the virus to undergo several mutations in a relatively short time span, causing them to quickly become resistant to a particular type of ARV (Broder, 2010; Kumar and Singh, 2012). To mitigate this effect, there was introduction of the highly active antiretroviral therapy (HAART), also known as combination ART (cART). The development of HAART regimens coupled with recent advancement on the development of an HIV vaccine or a cure has caused a change in the natural history of HIV infections from a progressive illness with an inevitably fatal outcome into a manageable chronic disease requiring life-long ARV treatment (Palella et al., 1998; Tozzi, 2010; Maartens et al., 2014; Aceti et al., 2015; de Moraes Filho et al., 2017).

The World Health Organisation (WHO) currently recommends a three-drug regimen consisting of two NRTIs/NtRTIs in combination with any of NNRTIs, PIs, EIs or INIs for HIV infections treatment. Combination of tenofovir disoproxil fumarate (TDF), lamivudine and efavirenz is one of the preferred first-line regimens for adolescents and adults (WHO, 2016). Lamivudine, an NRTI, is a prodrug analogue of dideoxycytidine that needs three phosphorylation steps by intracellular kinases to produce its pharmacologically active triphosphate anabolite, lamivudine triphosphate (Anderson and Rower, 2010; Eke et al., 2012). TDF is an acyclic nucleoside phosphonate diester analogue of deoxyadenosine 5’-monophosphate that needs stepwise diphosphorylation by intracellular kinases to be converted into its bioactive metabolite, tenofovir (Ng et al., 2015). It is the only currently approved NtRTI and has long-term action against Hepatitis B virus and HIV. On the other hand, efavirenz is a first-generation NNRTI that was endorsed by the U.S. FDA for HIV-1 infections treatment in 1998. It is an important component of several first-line HAART combinations for the treatment of HIV patients worldwide (de Moraes Filho et al., 2017).

Despite the clinical benefits of managing HIV infections with ARVs, concerns exist about the potential effect of long-term usage of these chemical agents on the genetic material of patients. These concerns have been further exacerbated by the need to combine ARVs in HAART, as the combination may enhance the individual toxic effects they have on the DNA. Administered as monotherapy or in combination, lamivudine, TDF and efavirenz have been shown in in vitro studies to possess genotoxic, mutagenic, carcinogenic and/or teratogenic abilities (Bayram and Topkaş, 2008; Fang et al., 2009; Franchi et al., 2009; Guimarães et al., 2013). However, only few studies (Lourenço et al., 2010; de Oliveira et al., 2014; Ng et al., 2015; Vivanti et al., 2015; de Moraes Filho et al., 2016) have evaluated the genotoxic effects of these drugs and their combination using animal bioassays. There is therefore need for more studies using different assays and endpoints to obtain information which can help in making informed decisions on the potential DNA damage by lamivudine, TDF and efavirenz individually and in combination.

In this study we assessed the genotoxicity of lamivudine, TDF and efavirenz individually and compared the results with the genotoxic effects of their combination using the mouse bone marrow micronucleus (MN) and sperm morphology tests. In addition, we carried out testicular histopathological examination of exposed male mice.

2.0 Materials and Method

Biological model

Healthy young male Swiss mice (Mus musculus; 6-7 weeks old for MN assay and 11-13 weeks old for sperm morphology assay) procured from the animal breeding facility of the Department of Zoology, University of Ibadan, Nigeria, were housed in transparent plastic cages lined with wood shavings, and provided with standard food pellet and water ad libitum. They were subjected to the natural day-night cycle, ambient temperature and relative humidity. Animals were handled according to standard guidelines prescribed in the Guidelines for Biomedical Research Involving Animals (CIOMS, 2012). The University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC) (UI-ACUREC/App/2015/031) approved the study.

Test drugs and preparation

Lamivudine (300 mg) and TDF (300 mg) were obtained from Mylan® and Sunrise® laboratories, India, respectively, while efavirenz (600 mg) and the combination of lamivudine, TDF and efavirenz were obtained from Hetero Labs Limited, India. Each ARV and the combination of the three ARVs were separately dissolved in normal saline for 24 hours and used as the stock. Four doses: 0.016, 0.032, 0.064 and 0.129 mg/kg bwt of lamivudine, TDF and the combination; and 0.032, 0.064, 0.129 and 0.259 mg/kg bwt of efavirenz were prepared from the stock suspensions which correspond to 0.125,
Sperm morphology test

Histological examination of the testes

The testes of treated and control mice in the sperm abnormality test were surgically removed and preserved in Bouin’s fluid for 48 hours prior to tissue preparation. The preserved testes were thoroughly rinsed using 50 % ethanol to remove the yellow colouration of picric acid. They were then dehydrated in graded alcohol, cleared using two changes of xylene and embedded in paraffin wax, using tissue embedding system (Leica EG 1160). Haematoxylin and eosin were used to stain 4 µm thick serial sections of the testes for pathological examination at x400 magnification.

Analysis of Data

Data were analysed using GraphPad Prism 5.01 and presented as mean ± SE. Statistical comparisons were performed using the one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test at 0.05 level of significance. The combination index (CI) of the three ARV drugs was calculated using the CompuSyn software (CompuSyn, Inc). A CI = 1 depicts additive effect, while CI > 1 and < 1 depicts antagonism and synergism, respectively (Chou, 2010).

3.0 Results

Micronucleus assay

Table 1 presents the mean value of MNPCES induced in mice treated with different doses of lamivudine, TDF, efavirenz and their combination, as well as the drug CI. Compared with the negative control, TDF and efavirenz individually induced significant increase in MNPCES at the tested doses. The induction of MN by TDF was dose-dependent with the highest dose of 0.129 mg/kg bwt inducing the most frequency of MN (16.75 ± 2.46). Induction of MN by efavirenz on the other hand was not dose-dependent as the lowest dose (0.032 mg/kg bwt) induced the highest frequency of MN (12.25 ± 2.14). At low doses (0.032 and 0.064 mg/kg bwt), efavirenz was more genotoxic than TDF, inducing a higher frequency of MNPCES, however, at high doses (0.064 and 0.129 mg/kg bwt), TDF showed higher genotoxicity than efavirenz. There was no significant increase in MNPCES by lamivudine at the tested doses. The combination of the three ARVs induced significant dose-dependent MNPCES compared to the negative control. At low (0.016 and 0.032 mg/kg bwt) and high (0.064 and 0.129 mg/kg bwt) doses, the combination was less genotoxic compared to efavirenz and TDF individually. The CI of greater than 1 was obtained at the tested doses for the combination. Figure 1 shows representative images of MNPCES and PCE in the treated mice.

Analysis of sperm abnormalities

A summary of the frequency of sperm abnormalities induced in mice treated with lamivudine, TDF and efavirenz alone and in combination is presented in Table 2. Lamivudine induced a significant increase in sperm abnormalities only at 0.129 mg/kg bwt compared to the negative control. Efavirenz and TDF caused a dose-dependent significant increase in abnormal sperm shape, except at 0.016 mg/kg bwt of TDF. In comparison to TDF, efavirenz induced more sperm abnormalities at the tested doses. The combination of the three ARVs also induced a significant, dose-dependent increase in sperm abnormalities compared to the negative control. However, the induction was highest at all the doses of the combination than with...
lamivudine, TDF and efavirenz individually. Figure 2 presents the observed sperm shape anomalies in mice exposed to lamivudine, TDF, efavirenz and their combination.

**Histological examination of mice testicular sections**

Normal tissue cellular architecture was observed in the testes of mice in the negative control group (Figure 3a), but in the groups treated with lamivudine, TDF and efavirenz individually and in combination, there was disruption of the normal testicular cellular architecture. These disruptions include tubular deformation, increased luminal width, sloughing of the germinal epithelium, shrunken seminiferous tubules, interstitial cell proliferation, seminiferous epithelium vacuolisation and necrosis of the spermatogonia (Figure 3b - d). The lesions were most severe in the efavirenz-treated mice.

**Table 1:** The mean (± SE) of MNPCes induced in the bone marrow of mice treated with different doses of lamivudine, tenofovir disoproxil fumarate (TDF) and efavirenz and their combination for 5 days.

| Dose levels (mg/kg bwt) | Lamivudine | TDF | Lamivudine + TDF + Efavirenz | Dose levels (mg/kg bwt) | Efavirenz | CI |
|-------------------------|------------|-----|-----------------------------|-------------------------|-----------|----|
| NC                      | 3.75 ± 0.25| 3.75 ± 0.25| 3.75 ± 0.25 | NC                     | 3.75 ± 0.25 |
| 0.016                   | 6.25 ± 0.75| 8.50 ± 1.71*| 8.75 ± 0.85* | 0.032                  | 12.25 ± 2.14** | 1.29 |
| 0.032                   | 7.50 ± 0.87| 8.00 ± 0.82*| 9.70 ± 1.11** | 0.064                  | 10.75 ± 1.31* | 1.58 |
| 0.064                   | 6.50 ± 1.32| 13.50 ± 1.85** | 10.25 ± 1.38** | 0.129                 | 9.75 ± 2.49* | 2.43 |
| 0.129                   | 6.00 ± 1.08| 16.75 ± 2.46** | 12.50 ± 0.65** | 0.259                 | 11.00 ± 0.41* | 32.7 |
| PC                      | 20.25 ± 2.53** | 20.25 ± 2.53** | 20.25 ± 2.53** | PC                  | 20.25 ± 2.53** |

Data are shown as Mean ± SE (n = 4). * p < 0.05; ** p < 0.01 compared with negative control (NC): normal saline. Positive control (PC): Cyclophosphamide (20 mg/kg bwt).

**Table 2:** The mean (± SE) of abnormal spermatozoa induced in mice intraperitoneally exposed to different doses of lamivudine, tenofovir disoproxil fumarate (TDF), efavirenz and their combination for 35 days.

| Dose levels (mg/kg bwt) | Lamivudine | TDF | Lamivudine + TDF + Efavirenz | Dose levels (mg/kg bwt) | Efavirenz |
|-------------------------|------------|-----|-----------------------------|-------------------------|-----------|
| NC                      | 73.50 ± 9.04| 73.50 ± 9.04| 73.50 ± 9.04 | NC                     | 73.50 ± 9.04 |
| 0.016                   | 96.20 ± 18.24| 101.20 ± 6.22| 192.40 ± 5.51* | 0.032                  | 116.80 ± 7.00* |
| 0.032                   | 100.40 ± 8.58| 115.60 ± 18.31* | 230.00 ± 16.45** | 0.064                 | 169.20 ± 7.64* |
| 0.064                   | 107.00 ± 16.58| 125.60 ± 16.19* | 250.00 ± 3.62** | 0.129                 | 175.00 ± 15.22* |
| 0.129                   | 117.80 ± 21.91* | 168.60 ± 26.71* | 284.60 ± 19.00** | 0.259                 | 189.60 ± 13.67* |
| PC                      | 244.60 ± 2.98** | 244.60 ± 2.98** | 244.60 ± 2.98** | PC                  | 244.60 ± 2.98** |

Data represent Mean ± SE (n = 5). * p < 0.05; ** p < 0.01 compared with negative control (NC): normal saline. Positive control (PC): Cyclophosphamide (20 mg/kg bwt). 1000 sperm cells per mouse were assessed.
Figure 1: Representative photomicrographs of normal polychromatic erythrocyte (PCE) and micronucleated polychromatic erythrocyte (MNPCE) in the bone marrow of mice intraperitoneally exposed to lamivudine, tenofovir disoproxil fumarate and efavirenz, and their combination. (a) PCE; (b and c) MNPCE. Magnification: x1000.

Figure 2: Abnormal sperm cells induced in mice intraperitoneally exposed to different doses of lamivudine, tenofovir disoproxil fumarate and efavirenz individually and their combination. (a) normal sperm cell (b) knobbed hook (c) amorphous head and atrophied midpiece (d) no hook (e) folded sperm (f) short hook (g) sperm with two tails (h) sperm with three tails (i) wrong tail attachment. Magnification: x1000.
Figure 3: Representative photomicrographs of histoarchitecture of testicular sections of mice in the negative control and those treated with different doses of lamivudine, tenofovir disoproxil fumarate and efavirenz, individually, and their combination. (A) cross-section of normal testicular tissue showing seminiferous tubule with no visible lesions (B) necrosis of spermatogonia (black arrow); apical sloughing of germinal epithelium (red arrow) (C) decrease in diameter of the seminiferous epithelium (double ended arrow) and increase in luminal width (green arrow) (D) seminiferous epithelium vacuolation (blue arrow) and interstitial cell proliferation resulting in congestion of the interstitial space (black star) (Magnification: x400).

4.0 Discussion

The use of lamivudine, TDF, efavirenz and their combination for ameliorative treatment of HIV/AIDS is of immense benefit to the patients, as the disease can now be managed and the diagnosis is no longer a death sentence unlike it was considered to be previously. However, since these drugs are administered continuously throughout the life of the patient, there is need to maximize their tolerability and safety. There is a growing concern about the possible toxicity of this class of drugs especially on the DNA both in the short- and long-term use. This study showed that lamivudine, TDF and efavirenz, individually and in combination caused genetic damage in somatic and gonadal cells of mice as assessed by the mouse bone marrow MN and sperm morphology assays.

The in vivo MN assay is widely utilised to evaluate the ability of test agents to induce chromosomal damage and/or mitotic disturbances (Hwang et al., 2013; Oyeyemi et al., 2015). The result of the MN assay showed that TDF, efavirenz and their combination are clastogenic and/or aneugenic at the tested doses. This shows that TDF and efavirenz probably have the ability to alter DNA as suggested by Vivanti et al. (2015), thereby generating primary and/or secondary DNA damage, resulting in accentric chromosome fragments or chromosome loss. The mechanism(s) by which ARVs exert their genotoxicity are essentially unknown, though a number of different mechanisms have been suggested. One possible mechanism that has been postulated is the disturbance of the intracellular nucleotide pool by NRTIs and NtRTIs, like lamivudine and TDF, which could ultimately result in mutations by increasing the rate of errors in nucleotide incorporation by cellular DNA polymerases (Wutzler and Thust, 2001). Most HAART regimens require the addition into cells of at least two nucleoside analogues which may create genomic instability within the cells thereby contributing to increased risk of nuclear and mitochondrial DNA damage. The genotoxic effect of TDF and efavirenz reported herein is in concert with the observations of Vivanti et al. (2015) and de Moraes Filho et al. (2017).

The combination of the drugs showed induction of MN lowered than what was observed with efavirenz and TDF individually. This suggests that interactions of these drugs probably reduce their genotoxic effect in the bone marrow cells of exposed mice. This suggestion was further
butressed by the CI which showed values greater than one, indicating an antagonistic effect for the induction of MN relative to the individual ARVs at the tested doses. This antagonism could be the consequence of a non-competitive inhibition between the two NRTI/NtRTIs and the NNRTI drugs. Antagonistic genotoxic effect of lamivudine, TDF and efavirenz have also been reported by de Moraes Filho et al. (2017) in a study that utilised the somatic mutation and recombination test (SMART) and comet assay. Data of the MN test further showed that lamivudine alone was not genotoxic at the tested doses. This is an indication that lamivudine is probably not a clastogenic or aneugenic agent. Weak/no induction of genotoxicity by Lamivudine was reported by de Moraes Filho et al. (2016) and Fang et al. (2009) in mice bone marrow and in NIH 3T3 cells, respectively. Perhaps, this is why lamivudine is categorised as weak inducer of MN formation and gross mutation (Bayram and Topaktas, 2008).

Result of the sperm shape test revealed that lamivudine, TDF, efavirenz and their combination are capable of altering the process of spermatogenesis and damaging mouse germ cells. The murine sperm shape test is among the most widely utilized genetic toxicity assays (Rasgele, 2014). It is a sensitive and reliable endpoint for evaluating and identifying xenobiotics capable of increasing spermatogenic dysfunction (Wyrobek and Bruce, 1975) and has been successfully employed in biological monitoring, environmental pollution assessment and in drug toxicity (Utulu and Bakare, 2010; Alabi et al., 2019). In our study, lamivudine induced abnormal sperm cells only at the highest dose of 0.129 mg/kg bwt, indicating weak genotoxic effect on the sperm cells similar to its observed effect in the bone marrow cells. High frequency of abnormal sperm cells induced by TDF and efavirenz herein showed that both drugs can induce DNA damage during sperm cell formation. Induction of abnormal sperms is assumed to result from point mutation (Bruce and Heddle, 1979), chromosomal aberration (Bruce et al., 1974) and small deletions in testicular DNA (Giri et al., 2002). This is therefore an indication of the ability of TDF and efavirenz to induce sperm cell abnormalities in other animals, including humans, by inducing chromosomal aberrations or gene mutation during the packaging of DNA in the sperm head (Bruce et al., 1974; Bruce and Heddle, 1979).

The combination of the ARVs provoked more abnormal sperm cell formation than the individual ARV. This is an indication that exposure of cells to two or more types of ARV may cause disruption in the natural deoxynucleotide pool, while such imbalances may lead to the triggering of genomic instability thus increasing the risk of repairable or permanent injuries for the nuclear and mitochondrial DNA (Morris et al., 2009; Guimaraes et al., 2013). The genotoxicity of lamivudine, TDF, efavirenz and their combination using sperm morphology assay suggests potential induction of infertility in male animals. There is a strong association between sperm abnormalities, male infertility and sterility in most species and the sperm structure plays a crucial role in both fertilization and pregnancy outcome (Kumar and Singh, 2015). Aside infertility, this result raises concern about the possibility of these ARVs and their combination to elicit genetic damage which might be transmissible from one generation to another generation. This is because spermatozoa with damaged DNA may introduce damaged genome into the oocytes causing deleterious effects on fertilization, embryonic, foetal and post-natal development (Lewis and Aitken, 2005; Zini, 2011). This therefore calls for more study and special attention to patients of reproductive age using these drugs and their combination to forestall potential side effects in long term use.

To further understand the ability of lamivudine, TDF, efavirenz and their combination to induce testicular toxicity, histopathological examination of the testes of exposed mice was carried out. Histopathological alterations are widely used as biomarkers for chemical and other xenobiotic induced toxicity (Rashid et al., 2012) and are acknowledged as the most sensitive means to detect effects on spermatogenesis (Creasy, 2002; Vidal and Whitney, 2014). The individual ARVs and their combination induced various pathological lesions in the testes of exposed mice including tubular deformation, increased luminal width, sloughing of the germinal epithelium, interstitial cell proliferation, seminiferous epithelium vacuolisation and necrosis of spermatogonia. Induction of histological damage in the testes of exposed mice indicates that ARVs are capable of passing through the blood-testis barrier; thus, providing direct evidence to support the conclusion that ARVs disrupted spermatogenesis in mice, causing elevated frequency of abnormal sperm cells when compared with the negative control.

In conclusion, our study showed that while lamivudine is a weak genotoxic agent, TDF, efavirenz and the combination of the three ARVs demonstrated significant potentials to cause germ and somatic cell genetic damage in male mice. Since genetic damage to cells has been implicated in the aetiology of cancers and congenital abnormalities, it is important that constant monitoring and intervention strategies are established to ensure that patients receiving treatment with these drugs continue to enjoy the quality of life that HIV treatment is supposed to bestow. More studies are required to shed light on the mechanism through which these ARVs induce genetic and testicular toxicity in vivo.

Acknowledgement

The authors are grateful to authorities of Osogbo state specialist hospital, Asubiaro, Osun State, and the President’s Emergency Plan for Aids Relief (PEPFER) at University College Hospital, Ibadan Oyo State for providing some of the drugs used in this study.

Declaration of Conflict of Interests

The authors declare no conflict of interests.

Funding

This study was partly supported by the postgraduate vote of Department of Zoology, University of Ibadan

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Execution: [KMA, BAA, FCO, SOA, SA, OMF, and OIO]

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Unauthentifiziert | Heruntergeladen 15.02.20 09:10 UTC
Writing the paper: [AAB, CGA, OAA, ITO, OMA, KMA, BAA, FCO, SOA, SA, OMF and O10]

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