Reproductive and Oxidative Stress Toxicity of Dolutegravir-Based Combination Antiretroviral Therapy in *Drosophila melanogaster*

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Authors' contributions

This work was carried out in collaboration among all authors. Author WMI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors WMI, SO, SSG and ETA managed the analyses of the study. Authors WMI and GDB managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background/Objective: Dolutegravir-based highly active antiretroviral therapy (DTG-HAART) is the preferred regimen in the management of HIV/AIDS. However, the reproductive and oxidative stress toxicity of DTG-HAART is unknown. This study was designed to investigate the reproductive and oxidative stress toxicity of DTG-HAART in *Drosophila melanogaster*.
Materials and Methods: We performed all the experiments at the Centre of Excellence in phytomedicine Research and Development (ACEPRD), University of Jos, Nigeria, in 2019. *D. melanogaster,* (1-4 days old), were fed with ten different concentrations of DTG-HAART (range 15 mg -595 mg) or 1000 μL distilled water per 10 g food for seven days to calculate the LD_{50}, then treated with 93.11 mg, 46.56 mg, 23.28 mg, 11.64 mg or 1000 μL distilled water each per 10 g fly food for five days in five replicates. Subsequently, longevity, fly fecundity, and negative geotaxis

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evaluated. Also, activities of Acetylcholinesterase, Glutathione-S-transferase, Superoxide dismutase, Catalase, as well as Total thiol, and Malondialdehyde levels were investigated in the whole fly homogenate. Statistical values at P<0.05 were considered significant.

Results: The LD_{50} of DTG-HAART in *D. melanogaster* was 106.4 mg. The result showed significantly decrease (*P*<0.001) in mean lifespan, fly emergence, Total thiol content, Acetylcholinesterase, Glutathione-S-transferase, Catalase, and Superoxide dismutase activities in the exposed groups compared to the unexposed. Inversely, the Malondialdehyde level in the test groups was significantly (*P*<0.001) elevated compared to unexposed.

Conclusion: Collectively, our results suggest that DTG-HAART toxicity was associated with reproductive deficits and oxidative stress induction in *D. melanogaster*, here observed as reduced fly fecundity, mean lifespan, AChE activity, antioxidant parameters, and elevated MDA level. This study, thus, raised concerns for long term use of DTG-HAART by HIV patients.

1. INTRODUCTION

The Human immunodeficiency virus (HIV) infection is one of the leading causes of illness and deaths worldwide [1,2]. The antiretroviral drugs used against the HIV infection have yielded success, especially in the reduction of related incidences [2,3] and deaths [2], though at the peril of adverse drug reactions (ADRs) [4]. These ADRs sometimes result in noncompliance with the prescribed regimen leading to treatment failures [5]. Also, an estimated 37.9 million [32.7-44.0 million] people were still infected globally as of 2018 [6,7]. Since HIV infection is incurable [1], infected patients take antiretroviral medications for life to delay immune compromise, opportunistic infections, and prevent transmission [8]. Thus HAART-related ADR studies are imperative.

Antiretroviral therapy evolved from the initial monotherapy [1,9] to the current combination antiretroviral therapy (cART), also called HAART [3]. HAART drugs consist of three antiretroviral medicines: two Nucleos(t)ide Reverse Transcriptase Inhibitors (NRTIs/NtRTIs), and any of Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs), Entry Inhibitors (EIs) or Integrase Strand Transfer Inhibitors (INSTIs) [10,11]. The WHO consolidated guidelines on ARV use recommended Efavirenz (EFV 600 mg) based regimen as a preferred first-line choice for HIV-1 treatment based on efficacy and safety data at that time [11]. At the moment, however, INSTI-based HAART regimens are the most preferred for antiretroviral therapy [11,12,13] because of their potency, safety profile, and rare drug-drug interactions [13,14,15].

Dolutegravir (DTG), a new generation INSTI, acts by inhibiting the incorporation of HIV DNA into the T-lymphocyte genome of the host, thereby limiting viral propagation [13]. The use of INSTIs singly is associated with side effects like insomnia with neuropsychiatric reactions [11], diarrhea, vomiting, allergy, and increase liver enzymes [13,16] with decreased creatinine clearance [17] have been reported. Similarly, *in vitro* cell shrinkage and phospholipid scrambling of cell membranes suspected to be oxidative stress-related effects of Dolutegravir have been documented [17].

HAART drug-related ADRs include nausea, vomiting, and skin eruptions [4]. Other severe ADRs associated with HAART include induction of free radicals (reactive oxygen and nitrogen species, RONS) [18,19], reduced reproductive capacity [1,20], neurodegenerative diseases [21], and accelerated aging [22].

The reproductive and oxidative stress toxicities particularly are of serious clinical concern. Briefly, the majority of people on antiretroviral therapies are of childbearing age [9], and HAART drugs are known culprits in reproductive drug-induced anomalies [20] and oxidative stress induction [1,3,9]. The literature is replete of the fact that over 75 % of genes implicated in aging, functional senescence, and human diseases are conserved in *D. melanogaster* [23]. Thus the fly is a handy tool in toxicology [24]. We, therefore, designed the present study to investigate the reproductive and oxidative stress toxicity of DTG-HAART in *Drosophila melanogaster*.

2. MATERIALS AND METHODS

The Drosophila fly laboratory of ACEPRD, University of Jos, Jos-Nigeria, was used for this study between June to December 2019.

2.1 Reagents and Antiretroviral Drugs

Reagents used were of analytical grade. Distilled water, reduced glutathione (GSH), Hydrogen peroxide, 1-chloro-2,4-dinitrobenzene, (CDNB), 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB) were purchased and supplied by the fly laboratory, ACEPRD, University of Jos. Trichloroacetic acid (TCA) 30 %, Thiobarbituric acid (TBA) 0.75 %, 0.15 M Tris-KCl buffer (pH 7.4), 0.1M HCl, 0.05M carbonate buffer (pH 10.2), and 0.3M Adrenaline were made available by the Biochemistry Division, National Veterinary Research Institute (NVRI), Vom, Jos Nigeria.

The HAART drug [DTG mg (CAS 501051375-16-6) + TDF 300 mg (CAS 202138-50-9) + 3TC 300 mg (CAS 134678-17-4) mg per tablet, Batch number 3075041, Batch No.:25/1/2014, Mfd. Oct. 2018, Exp: Sept. 2020 and manufacture: Mylan] was contributed by the General Hospital Gboko, Benue State Nigeria.

Thirty (30) tablets of DTG-HAART were weighed, average weight calculated, and then powdered using porcelain mortar and pestle. The quantity of powder equivalent to each of the predetermined drug concentration was calculated and weighed out using an analytical balance (Meltlar Model No. MT-200B). The distilled water (1000 µL) water designated as the control in this study was also used to dilute the powdered concentrations before incorporation into cold fly food.

2.2 Animal Model

The *D. melanogaster (*Harwich strain) was sourced at the ACEPRD, University of Jos. The fly stock was inbred and maintained at standard environmental conditions, as previously described [20]. The flies were fed on Drosophila food composed of yellow cornmeal (100 g), brewer's yeast (20 g), agar (16 g), and the preservative methylparaben (1 g) [25].
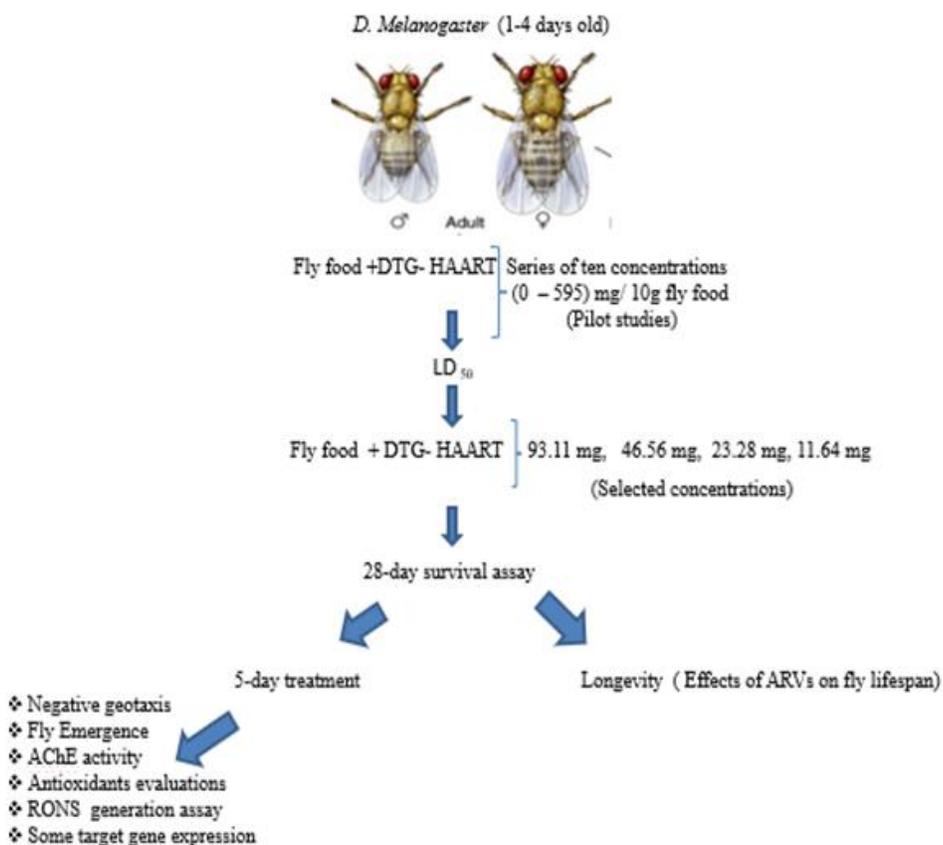
Scheme 1. Summary of experimental design

2.3 Experimental Design

The investigation on DTG-HAART-induced toxicities was carried out using different arrays of a nutritional regimen. *D. melanogaster* (1 to 4-day-old) were divided into five groups of 60 flies per plastic vial (height, 11 cm, diameter, 2.5 cm) each in five replicates per group and exposed to graded DTG-HAART concentrations (0 mg -595 mg) first for 7 days to determined LD_{50}, then 93.11 mg, 46.56 mg, 23.28 mg, 11.64 mg or 1000 µL distilled water (control) per 10 g food for longevity assay, and five-day treatment for determination of negative geotaxis, fecundity, and biochemical assays. We used young flies (1-4 day old) because growing animals are most susceptible to toxic insults [26]. The five days treatment period chosen for this study in *D. melanogaster* corresponds to fly survival of 70 % and above in all test groups on the 28-day Kaplan-Meier survival curve (result not shown).

2.4 Median Lethal Dose (LD_{50}) Determination

The median lethal dose (LD_{50}), defined as the concentration in normal fly food resulting in 50 % death in seven days [27], was determined as previously described [20]. Sixty (60) flies (1- 4 days) were fed ten different concentrations of DTG-HAART (15 mg, 25 mg, 45 mg, 95 mg, 145 mg, 195 mg, 295 mg, 395 mg, 495 mg, 595 mg) or 1000 µL distilled water (as control) each per 10 g fly food separately in five replicates for 7 days. Deaths were recorded every 24 hours interval during this period. The percentage survival versus log concentrations was plotted in dose-response simulation using Graphpad prism 8.0.2. In all experiments, the highest concentration of DTG-HAART used was 94.11 (12.5 % below LD_{50} based on our pilot studies), and 46.56 mg, 23.28 mg, 11.64 mg (representing 50 % of every preceding concentration respectively).

2.5 Fly Treatment for Negative Geotaxis, Reproduction Ability and Biochemical Assays

After five-day treatment, DTG-HAART exposed flies or control were immobilized in ice, weighed, and homogenized in phosphate buffer [0.1 M pH 7.0, (1 mg: 10 µL)], then centrifuge (Eppendorf AG, 5227 R, Germany), at temperature 4 °C for 10 min at 4000 rpm. The supernatant of the whole fly homogenate was used to determine Total thiol content, the activities of Acetylcholinesterase (AChE), Catalase (CAT), Glutathione-S-transferase (GST), Superoxide dismutase (SOD) and Malondialdehyde (MDA) levels.

2.5.1 Negative geotaxis

The negative geotaxis (Climbing performance) of DTG- HAART exposed and unexposed flies were evaluated as previously described [28]. Ten DTG-HAART exposed and control flies were anesthetized using ice, sorted into sexes, and placed in separate labeled glass tubes (length, 15 cm; diameter, 2 cm). The flies were allowed to recover (20 minutes), then the tube gently tapped to the bottom. After 6 s, the number of flies that moved up to the 6 cm mark was recorded. A group score is the average of three trials. Data plotted as percentage climbing against concentration.

2.5.2 Reproductive ability

After 5-day exposure to DTG-HAART, flies were evaluated for fecundity as previously described [20] with modifications. Ten flies (5 males and 5 females) each per group in five replicates were pair mated using two strategies: (1) DTG-HAART exposed flies (males and female) were transferred into vials containing fly food for 24 hours, the eggs laid in each vial within this period were observed daily for 14 days for eclosion. (2) DTG-HAART naïve flies (male and female) were pair mated in fly food treated with different cART (93.11 mg, 46.56 mg, 23.28 mg, 11.64 mg) or 1000 µL distilled water as a control. The flies were maintained in these vials for 24 hours, where they mated and lay eggs, then the flies were removed. The eggs were kept for 14 days in the treated food or the control and observed daily for the emergence of adult flies. The cumulative mean number of flies hatched represents a measure of reproductive capacity.

2.5.3 Acetylcholinesterase (AChE) activity

The AChE (EC 3.1.1.7) activity was determined by slightly modifying the method of Ellman et al. [28]. The mixture consisted of 285 µL of distilled water, 180 µL of 100 mM PBS (pH 7.4), 60 µL of 10 mM DTNB, 15 µL of the sample, and 60 µL of 8 mM acetylthiocholine was added. The absorbance was monitored spectrophotometrically (UV-VIS spectrophotometer, Jenway 7315) at 412 nm for 2 min at intervals of 10 s. Data was calculated

against blank and sample blank, and the activity expressed as µmol/min/mg protein.

2.5.4 Total thiol content

Total thiol content was determined using the method of Ellman, as previously described [20]. Briefly, the reaction mixture comprised of 510 µL potassium phosphate buffer (0.1 M, PH 7.4), 25 µL of the sample, and 30µL of DTNB (10 mM). The mixture was incubated for 30 min at room temperature, the absorbance measured at 412 nm and used to calculate the sample total thiol levels (in micromol/mg protein). GSH (35µl) served as standard.

2.5.5 Glutathione-S-transferase (GST) activity

The GST (EC 2.5.1.18) activity was determined by the method of Habig and Jacoby as earlier described [26] using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Briefly, the assay mixture consisted of 600 µL of solution A (20 µL of 0.25 M potassium phosphate buffer, pH 7.0 with of 2.5 mM EDTA, and 510 µL of 0.1 M GSH at 25 °C), 60 µL of the sample (1:5 dilution) and 30µL of 25 mM CDNB. Increased absorbance was measured at 340 nm for 2 min at 10 s interval using a spectrophotometer (Jenway). Data were expressed in mmol/min/mg of protein utilizing the molar extinction coefficient (ε) of 9.6 mM1 cm1 of the coloured GS–DNB conjugate formed by GST.

2.5.6 Catalase (CAT) activity

The CAT (EC 1.11.1.6) activity was determined, as described by Abolaji et al. [24]. The reaction mixture contained 100 µL of potassium phosphate buffer, pH 7.0, 194µL of 300 mM H_2O_2 as solution A. 10 µL of the sample was made to react with 590 µL of solution A and the clearance of H_2O_2 was monitored at 240 nm at 25 °C. The decrease in H_2O_2 was observed for 2 min (10 s intervals), at 240 nm using a UV–visible spectrophotometer (Jenway) and expressed as mmol of H_2O_2 consumed/min/mg of protein.

2.5.7 Determination of Superoxide Dismutase (SOD) activity

The SOD activity was determined by the method of Misra and Fridovich [20]. Briefly, a volume of 200 µL of the sample was diluted with 800 µL of distilled water (1:5). Then 200 µL of the diluted sample was added to 2500 µL of 0.05 M carbonate buffer (pH 10.2) in the cuvette and allowed to equilibrate in a spectrophotometer. Then, 300 µL freshly prepared 0.3 M adrenaline was added to start the reaction. The cuvette used as standard contained 2500 µL buffer, 300 µL adrenaline, and 200 µL of water. The absorbance was monitored every 30 seconds for 150 seconds at 480 nm.

2.5.8 Lipid peroxidation assay

Lipid peroxidation, as an index of reactive oxygen species (ROS) generation, was determined by measuring the level of Malondialdehyde using the method of Varshney and Kale as previously described [20]. Briefly, 400 µL of the sample was first mixed with 1600 µL of tris-KCl buffer followed by 500 µL of 30% TCA. 500 µl of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^0C. Ice was used to cool the mixture, then centrifuged at 3000 g for 5 minutes. The clear supernatant obtained was collected, and absorbance measured against distilled water as reference blank at 532 nm. Lipid peroxidation was expressed as MDA formed/mg protein using a molar extinction coefficient of 1.56×10^5 M^{-1} Cm^{-1}

2.6 Statistical Analysis

We expressed data as mean±SEM (standard error of mean). One-way analysis of variance (ANOVA) was used with Tukey's post hoc test using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The *P*-values (*P*<0.05) were regarded as statistically significant.

3. RESULTS

3.1 Seven (7) Day LD$_{50}$

We selected the experimental concentrations of the DTG-HAART from the LD$_{50}$. Flies were first exposed to graded concentrations of the cART (15 mg -595 mg) for seven days. The result from the exposed flies during this period showed 100 % mortality at 295 mg, 345 mg, 495 mg, and 595 mg per 10 g food. The mortality rate of 2.33 % was recorded in the unexposed group. The LC$_{50}$ was determined to be 106.4 mg (Fig. 1).

### 3.2 Lifespan (Longevity) Studies

Longevity studies showed significantly (*P*<0.001) reduced lifespan of flies in the exposed groups

compared to the unexposed. The flies in the highest experimental concentration (93.11 mg/10 g food) survived up to 32 days representing 49.21 % lifespan reduction compared to the 63 days survival in the unexposed group. The median lifespan (ML) with a corresponding hazard ratio (HR) for the experimental concentrations or control was: 93. 11 mg (ML=10.5 days, HR=4.10±2.91), 46.56 mg (ML= 11 days, HR = 3.11±2.58), 23.28 mg (ML = 22 days, HR = 2.36±1.97), 11.64 mg (ML = 24 days, HR = 1.9±1.60) and control (ML = 27 days, HR = 0.12±0.10) as shown in Fig 2.
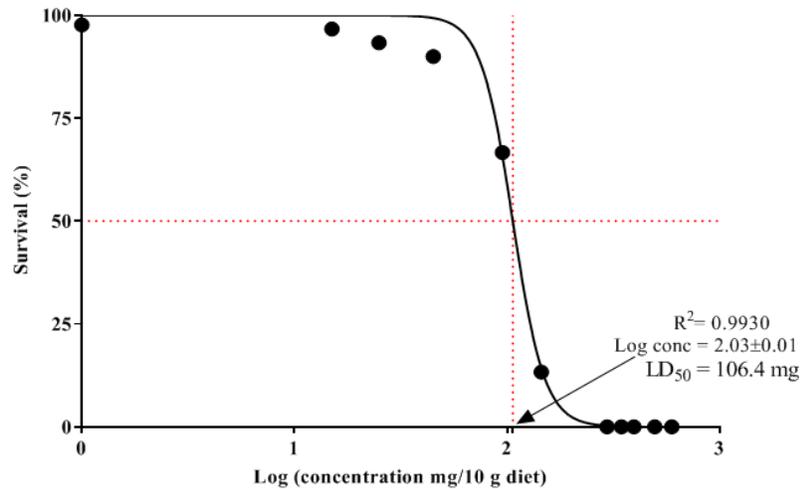
Fig. 1. LC$_{50}$ of Dolutegravir-based highly active antiretroviral therapy (DTG-HAART) in *D. melanogaster.* Data are presented as mean±SEM of five independent biological replicates carried out in two separate experiments
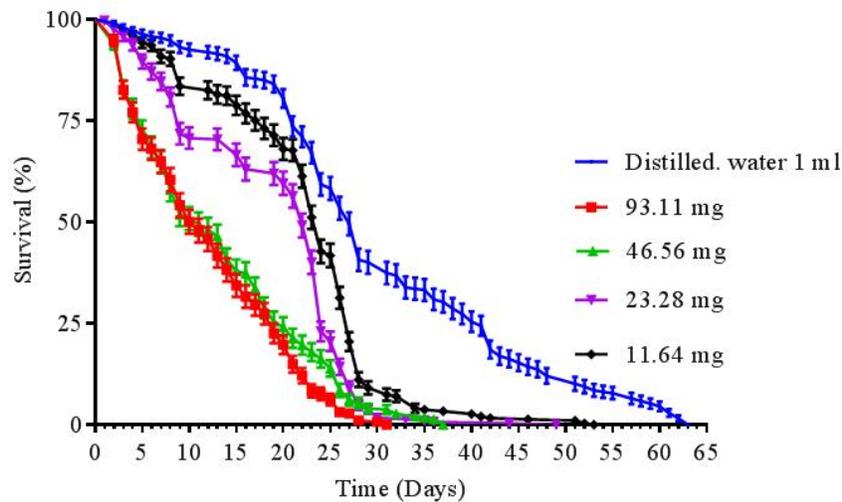
Fig. 2. DTG-HAART exposure significantly (p<0.05 reduced the life span of *D. melanogaster* in a dose-dependent manner. Data are present as mean±SEM of five independent biological replicates carried out in duplicates. *p<0.05. DTG-HAART= Dolutegravir based highly active antiretroviral therapy, 3TC= Lamivudine, TDF=Tenofovir disoproxil fumarate

3.3 Reproductive Ability

The reproductive ability of *D. melanogaster* exposed to DTG-HAART was significantly ($P<0.001$) reduced after five days of exposure compared to the unexposed group (Fig 3A). The two higher concentrations 93.11 mg and 46.56 mg each per 10 g food showed 0 % emergence (or 100 % emergence failure). The eclosion ability reduced in the range of 20.80±2.08 – 0 flies along the concentration gradient compared to 66 flies in the unexposed group. We sought to understand further whether the result of cART post-exposure emergence affected the adult reproductive capacity, or the eclosion or both. Thus, we exposed DTG-HAART naïve flies (male and females) to fly food containing cART for 24 hours, where they mated and laid eggs. The result after 14 days showed significantly ($P<0.001$) reduced fly emergence in all the experimental concentrations along the concentration gradient compared to the unexposed group (Fig 3B).

3.4 Acetylcholinesterase (AChE) Activity and Negative Geotaxis

The AChE activity in *D. melanogaster* exposed to DTG-HAART (Fig 4A). was significantly ($P<0.001$) reduced in the first three concentrations: 93.11 mg, 46.56 mg, and 23.28 mg (15.91±4.46 – 3.68±1.36 units/mg protein)

compare to the unexposed (26.90±3.10 units/mg protein) along concentration gradient except the lowest concentration (11.64 mg) where the reduction in AChE activity (14.91±3.10 units /mg protein) was statistically non-significant (P = .11). Similarly, the result of negative geotaxis (Fig. 4B) showed significantly ($P<0.05$) decreased climbing ability in the first three higher concentrations except for the lowest concentration that showed non-significant (P = 0.99) climbing deficits compare to the control.

## 3.5 Total Thiol Content and Glutathione-S-transferase (GST) Activity

Total thiol contents significantly ($P<0.05$) decreased (range: 1.44±0.18 – 0.31±0.08 units/mg protein) at 23.28 mg, 46.56 mg, or 93.11 mg of the *D. melanogaster* exposed groups compared to unexposed group (3.01±0.08 units/mg protein). However, a non-significant (P = .32) decrease was observed at the lowest concentration (11.64 mg, 2.24±.038 units/mg protein) (Fig. 5A). Similarly, the GST activity significantly ($P<0.001$) decreased at 46.56 mg (0.22±0.47 units/mg protein) and 93.11 mg (0.29±0.06 units/mg protein) compared to unexposed group (1.07±0.05 units/mg protein). However, the reduction in GST activity at 11.64 mg (0.67±0.19 units/mg protein), or 23.28 mg (0.65±0.26 units/mg protein,) was statistically non-significant (P >0.05) (Fig. 5B).
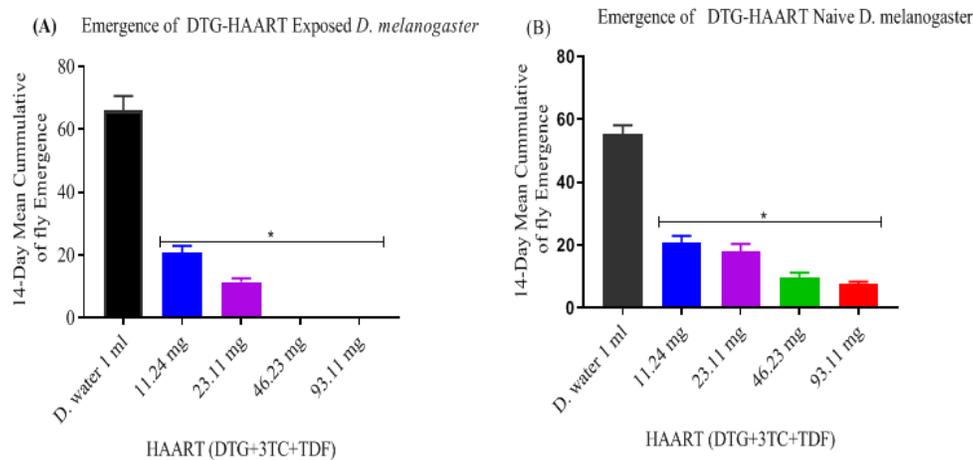
Fig. 3 (A-B). DTG-HARRT significantly (p0.05) reduced D.melanogaster eclosion. (A) Fly eclosion after 5 days treatment of adult flies of both sexes with DTG-HAART (eggs/larva unexposed for 14 days). (B) Fly eclosion of DET-HAART naïve D. Melanogaster (eggs/larva exposed for 14 days). Data are presented as mean±SEM of five independent biological replicates of each concentration. Each assay was carried out in two independent experiments. *p<005 vs. control
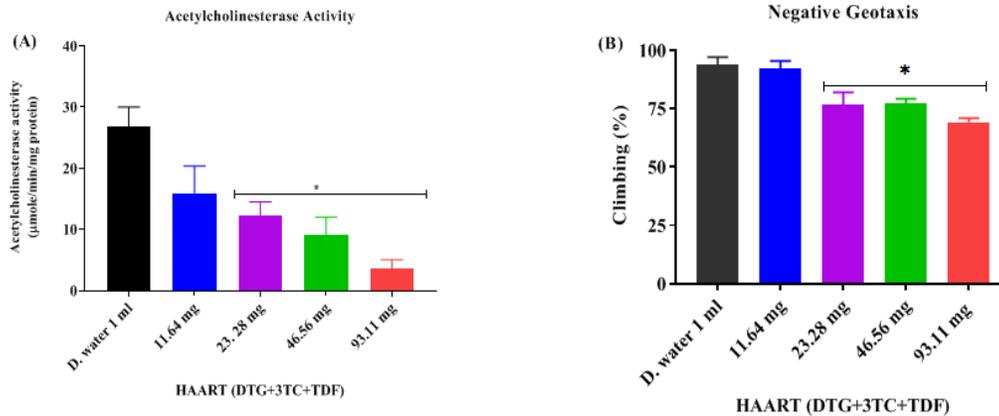
Fig. 4 (A-B). HAART significantly (P 0.05) decreased the level of AChE and Negative geotaxis in *D. melanogaster* after 5 days of exposure. (A) AChE levels after treatment of flies with DTG-HAART. (B) Negative geotaxis after DTG-HAA exposure. Data are presented as mean±SME of five independent biological replicates for each drug concentration. *p<0.05 vs control
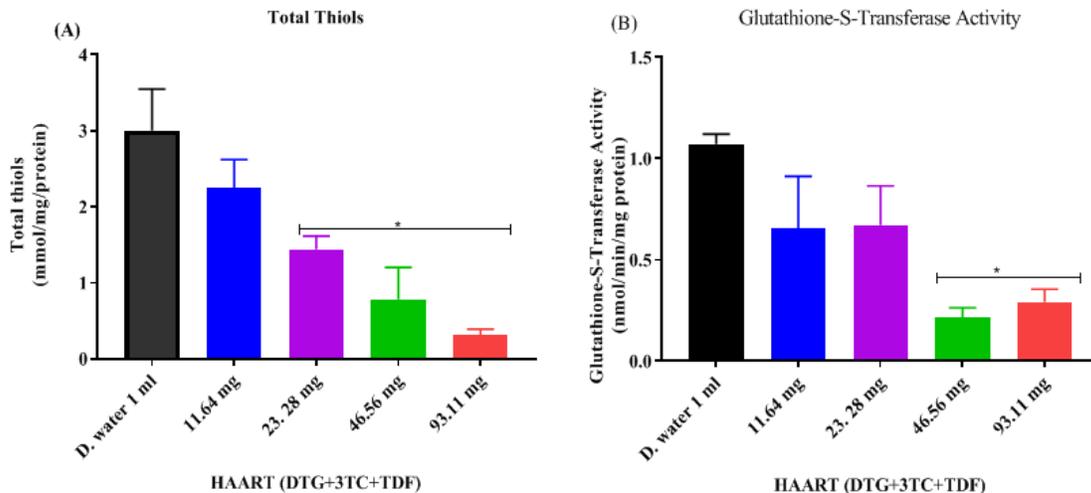
Fig. 5. Significantly decreased total thiol content and GST activity in D. Melanogaster after 5 days. (B) GST activity of DET-HAART treated flies after 5 days of exposure. Data are presented as mean±SME of five independent biological replicates for each drug concentration. *p<0.05 vs control. DTG-HAART= Dolutegravir based highly active antiretroviral therapy, 3TC=Lamivudine, TDF=Tenofovir disoproxil fumarate

3.6 Superoxide Dismutase (SOD) and Catalase (CAT) Activity

The SOD activity in the *D. melanogaster* groups exposed to 23.28 mg, 46.56 mg or 93.11 mg were significantly ($P<0.001$) reduced (range: 4.11±1.70 – 2.28±0.46 units/mg protein) compared to the unexposed group (8.19±0.78) except for the 11.64 mg (6.91±0.67 units/mg protein) group (P = .88) as presented in figure 6A. Likewise, the result of CAT activity significantly ($P<0.001$) decreased at concentrations 23.28 mg, 46.56 mg, or 93.11 mg/10 g food (range:37.73±3.86 - 30.49±0.26 units/mg protein) compared to the unexposed ground (80.94±12.73). The decreased activity was non-significant (P =0.22) at 11.64 mg (51.24±14.44) (Fig. 6B).

3.7 Malondialdehyde (MDA) Level

The result of the MDA level showed significant ($P<0.025$) increase at 23.28 mg, 46.56, and 93.11 mg/10 g food; however, the 11.64 mg /10 g food was non-significant ($P = .10$) compared the unexposed group (Fig. 7A). The MDA levels increased along the concentration gradient (11.64 mg -93.11 mg (range 2.86±0.13 – 4.42±1.02 units/mg protein) compared to the unexposed group (0.79±0.10 units/mg protein).

The observed increase in the MDA level was in a hundredfold from low to highest experimental concentrations (range 361.40±16.92 % – 558.80±128.70 %) compared to unexposed group 100±12.75 %) (Fig. 7B).

3.8 Correlation Matrix

Correlation among the study parameters was evaluated to quantify the degree to which they related and the result presented in Table 1.
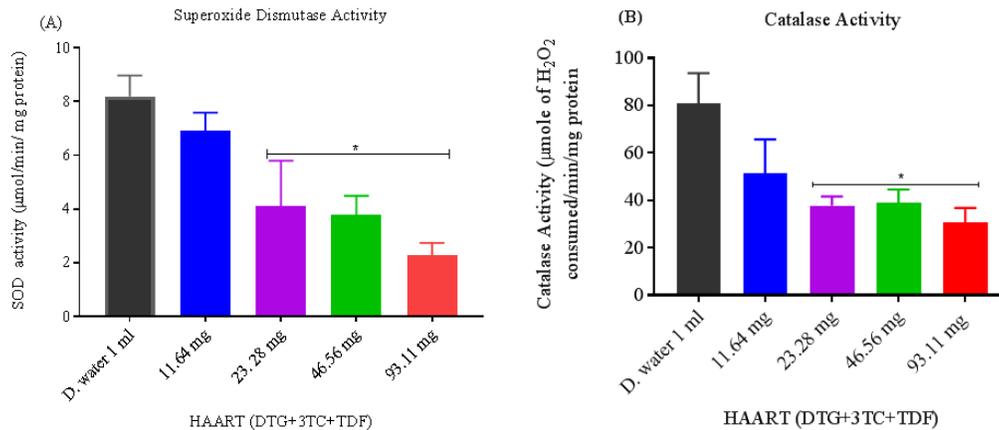
Fig. 6. DTG-HAART significantly decreased SOD and CAT activities in *D. melanogaster* after 5 days of exposure. (A) SOD activity of DTG-HAART exposed *D. melanogaster* after 5 days of exposure. (B) CAT activity of DTG-HAART exposed *D. melanogaster* after 5 days of exposure. Data are presented as mean±SME of five independent biological replicates for each drug concentration (n=60). *p<0.05 vs control
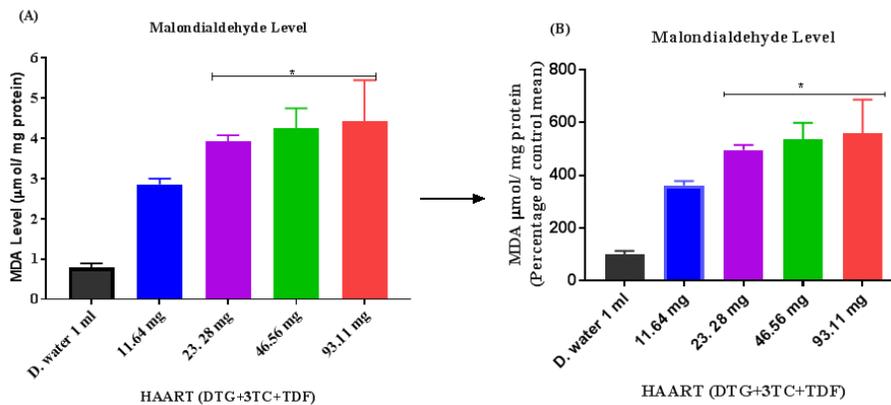
Fig. 7. DTG-HAART exposure significantly (p 0.05) increased the Malondialdehyde levels in *D. melanogaster*. (A) MDA level in the whole fly homogenate of *D. Melanogaster* after 5 days of exposure. (B) Malondialdehyde concentration (as a percentage of control mean) in the whole fly homogenate of DTG-HAART exposed *D. melanogaster* after 5 days of exposure. Data presented as mean±SME of five independent biological replicates for each drug concentration (n=60). * P<0.05 vs. control
HAART=highly active antiretroviral therapy, 3TC+Lamivudine, EFV+ Efavirenz, TDF= Tenofovir disoproxil fumarate

Table 1. Correlation among different parameters measured in DTG-HAART exposed *D. Melanogaster*

	MS	EM	AChE	TOT	GST	SOD	CAT	MDA
MS	1.00							
EM	0.81	1.00						
AChE	0.88*	0.96*	1.00					
T.Thiol	0.95*	0.91*	0.97*	1.00				
GST	0.95*	0.93*	0.73*	0.93*	1.00			
SOD	0.85	0.98*	0.99*	0.95*	0.93*	1.00		
CAT	0.77	0.98*	0.97*	0.92*	0.87	0.99*	1.00	
MDA	-0.82	-0.99*	-0.97*	-0.94*	-0.91*	-0.99*	-0.99*	1.00

Data are presented as mean±SME of five independent biological replicates for each drug concentration (n=60).
*P<0.05 vs control. MS= Mean Survival, EM = Emergence, AChE = Acetylcholinesterase, TOT – Total thiols,
GST = Glutathione-S-transferase, SOD = superoxide dismutase, CAT= Catalase, MDA= malondialdehyde

4. DISCUSSION

Combination Antiretroviral Therapies (cART), also called HAART, are very useful in the management of HIV/AIDS. However, several reports have implicated HAART-induced oxidative stress and reproductive deficits in human and animal models [1,3,9,20]. Nevertheless, to the best of our knowledge, reproductive decline and oxidative stress-related toxicities associated with DTG-HAART exposure in *D. melanogaster* have not been reported. In the present study, we evaluate if reproductive deficits and oxidative stress events accompanied DTG-HAART exposure in *D. melanogaster*.

The LD_{50} of DTG-HAART (650 mg/tablet) in this study was 106.40 mg (Fig. 1), implying that the drug combination might be toxic to the flies. DTG-HAART toxicity further manifested as a significant ($P<0.05$) reduction in the mean lifespan of the exposed flies. It concomitantly increased hazard ratios along the concentration gradients in our longevity studies (Fig. 2). We earlier reported a similar effect on lifespan using efavirenz-based HAART[EFV-HAART: Efavirenz / Lamivudine / Tenofovir disoproxil fumarate)] in *D. melanogaster* [20]. Our result further validates another previous report that linked the increased mortality rate as a toxic event in cART use [22].

Free radical generation occurs in the normal metabolic processes in all living things [29]. It may play a vital role in therapeutics [30], spermatozoan maturation [31], promotion of adaptive responses, and signaling mechanisms at low concentrations [32]. However, ROS production beyond the natural antioxidant capacity leads to oxidative stress [33]. Some of the ROS-related damage in a living system include the destruction of amino acids, enzymes,

peroxidation of unsaturated fats, gene mutations, apoptosis, aging, and death. [33,34]. The significant ($P<0.05$) reduction in the lifespan of DTG-HAART exposed flies compared to the unexposed group in the present study (Fig. 2) might be due to ROS generation and reactions that resulted in accelerated aging and premature deaths.

Evaluation of reproductive capacity in the 5-day DTG-HAART exposed *D. melanogaster* showed significant ($P<0.001$) reduction in fly emergence at the two lower concentrations (11.64 mg or 23.28 mg) with 100 % emergence failure at the higher experimental concentrations (46.56 mg or 93.11 mg) (Fig.3A). The exposure of DTG-HAART naïve flies showed significant ($P<0.001$) reduction in fly emergence without eclosion failure. This observation implies that DTG-HAART may have altered more overwhelmingly the reproductive capacity in the exposed adult flies than the developmental toxicity at the eclosion stage. HAART (Efavirenz / Lamivudine / Tenofovir) exposure was earlier reported to have adversely changed reproductive hormones, sperm parameters, and the number of ovarian follicles in rodents [3]. In this study, we observed a significant (P = 0.02) positive correlation (r =0.81) between decreased mean survival and reproductive deficits (Table 1). The correlation result suggests that the cause of increased mortality in *D. melanogaster* in our study was 81 % likely to cause emergence failure.

Acetylcholinesterase activity and negative geotaxis were significantly ($P<0.001$) reduced after 5-day DTG-HAART exposure in *D. melanogaster*. Correlation between decreased AChE activity and climbing deficits has been reported after manganese [26], or HAART exposures in *D. melanogaster,* respectively.

Acetylcholinesterase enzyme metabolizes endogenous acetylcholine, and inhibition of the enzyme raises the neurotransmitter levels resulting in sustained action potential and hence desensitization/impairment at the neuromuscular junction [20,28]. Also, AChE mutants of *D. melanogaster* exhibits short lifespan, and direct administration of acetylcholine agonist results in abnormalities in target cells [35]. Precisely, decreased AChE activity concomitantly increases ROS generation, impairs spermatogenesis, promotes oocyte damage [36,37], and reduces locomotor ability [38]. Our investigation suffers a lack of certainty that DTG-HAART acted exclusively on AChE and the involvement of the presumed acetylcholine increase against reproductive decline as well as ROS generation.

The DTG-HAART-induced decrease in AChE activity in the current study might have directly or indirectly induced oxidative stress-related toxic effects on the fly sperm, oocyte integrity, or eclosion. We found a significantly ($P = 0.01$) positive correlation (r = 0.96) between AChE activity and Fly emergence (Table 1), implying that the decreased activity of the former had a 96 % likelihood to cause the decrease in the later.

The correlation between AChE activity and fertility in this study is at variance with that of Urra et al. [39]. They observed increased follicular development and ovulatory events in rats following Huperzine-A induced AChE inhibition. A possible explanation could be that the AChE inhibitor, Huperzine-A, is an antioxidant molecule [40]; thus, its fertility-enhancing effects might be an antioxidant derived benefit as earlier proposed [31] rather than AChE inhibition.

Data on antioxidant parameters showed significantly ($P < 0.05$) decreased total thiol content and antioxidant enzyme (GST, SOD, and CAT) activities, respectively. Total thiols are non-enzymatic antioxidants with sulfhydryl (-SH) moiety that conjugates xenobiotics and other RONS *in vivo* [26,41]. GST, an enzymatic antioxidant, destroys electrophilic pro-oxidants by catalyzing the transfer of sulfhydryl (-SH) from glutathione to these reactive species during conjugation reactions [26]. At the onset of oxidative stress induction, the antioxidant system abates the accumulation of RONS build-up through the action of enzymatic antioxidants SOD or CAT [42,43]. SOD first catalyzes the dismutation of two superoxide anions to hydrogen peroxides (H_2O_2) and water. CAT

finally converts the H_2O_2 to water and oxygen quickly to prevent hydroxyl radical formation via Fenton reaction [42]. Thus, deficits in antioxidant systems could increase RONS and cause cell death [26].

Our investigation showed a state of oxidative stress in *D. melanogaster* exposed to DTG-HAART, evidenced by a significant ($P<0.05$) decrease in total thiol levels, GST, SOD, and CAT activities, respectively. The depleted defense antioxidant enzymes in the present study suggest that the DTG-HAART exposed *D. melanogaster* may have suffered diminished antioxidant capacity thus became vulnerable to oxidative stress-induced damage on fly reproductive capacity. We observed significant ($P <0.05$) positive correlation [r: 0.91 – 0.98] among total thiols ($P = 0.01$), GST ($P = 0.02$), SOD ($P = 0.003$) or CAT ($P = 0.003$) and fly emergence (Table 1). Furthermore, our work agrees with earlier reports that HAART regimens induce oxidative stress or reproductive decline in humans [44,45,46], rodents [1,9], and *D. melanogaster* [47].

To understand further the involvement of oxidative stress against reproductive capacity in DTG-HAART exposed *D. melanogaster*, we evaluated the level of MDA in the exposed flies. MDA is an endogenous DNA-derived toxic product of RONS-induced polyunsaturated fatty acids (a key component of cell membranes) [48,49]. Our result showed a hundredfold increase in MDA levels after five days of fly exposure to DTG-HAART (Fig. 7A & B), similar to earlier *D. melanogaster* exposure to Efavirenz-based HAART [47]. Our result also agreed with a previous report [50] that rats exposed to Tenofovir (a component of the HAART in our study) caused marked MDA elevation. The correlation matric (Table 1) showed a high but negative correlation (r = 0.84 – 0.99) between MDA and all test parameters in this study. Notably, our finding showed that MDA elevation was 99 % (r = 0.99, $P =0.001$), likely to decreased reproductive capacity in DTG-HAART exposed *D. melanogaster*.

The elevated MDA levels in our study confirm that DGT-HAART has the potential to induce oxidative stress, thereby causing reproductive deficits in *D. melanogaster*. It is worthy to note that all the DTG-HAAT-induced toxic endpoints investigated in the current study were previously reported of EFV-HAART by our team in the same model [20,47]. This similarity might not be

a mere coincidence since two drugs (Lamivudine and Tenofovir) are present in both regimens.

5. CONCLUSION AND RECOMMENDA-TION

5.1 Conclusion

Our study suggests that DTG-HAART has the potentials to induce reproductive deficits and oxidative stress-related toxicities in *D. melanogaster,* buttressed by decreased mean survival, fly emergence, AChE activity, antioxidant parameters and markedly increased MDA level. This study, thus, raised concerns for long term use of DTG-HAART by HIV patients.

5.2 Recommendation

More research should focus on DTG-based HAART using appropriate human-based models for possible intervention against these toxic effects in antiretroviral therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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