Health System and Medical Journal

E-ISSN: XXXX-XXXX
P-ISSN: XXXX-XXXX

(Research/ Review) Article

Histological and Oxidative Stress Toxicity of Dolutegravir-Based Antiretroviral Therapy on the Placenta of Adult Wistar Rats

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ABSTRACT. Tenofovir Disoproxil Fumurate/Lamivudine/Dolutegravir (TLD) is a fixed dose combination antiretroviral therapy recommended by World Health Organization (WHO) as preferred first and second-line antiretroviral therapy for people living with HIV. However, a relationship between placenta histology and oxidative toxicity of TLD is unknown. This study was designed to investigate the histological and oxidative stress toxicity of TLD on the placenta of adult Wistar rats. Ten rats weighing between 154-194g were randomly assigned into two groups; a control and TLD-treated. After pregnancy was achieved, the treated group was still administered combination drugs of Tenofovir Disoproxil Fumurate in 5mg/kg, Lamivudine in 5mg/kg and Dolutegravir in 0.8mg/kg body weight daily for 90 days. Dams were weighed on gestational day (GD) 0, 7, 14 and 21. At GD21, the dams were sacrificed, and placenta were harvested, counted, weighed, measured, and submitted for biochemical oxidative stress assessments and a section taken for histological evaluation. The findings revealed significant decrease in the body weight, placenta weight, diameter, and thickness of the treated group. Also, there was a significant decreased in placenta tissue reduced glutathione, glutathione peroxidase, superoxide dismutase, and catalase was decreased with insignificant difference, the concentration of malondialdehyde increased with insignificant difference in the treated compared to the control group. There was no significant difference in the number of number of placenta between the control and treated groups. The placenta histology of the TLD treated revealed vacuolation, congestion, and dilatation of the fetal-maternal vascular bed. These findings suggest TLD administration during pregnancy have negative effects on the placenta and could lead to abortion or cause serious negative consequences for the developing foetus. Concerns over long-term usage; especially during pregnancy, cannot be overemphasized.

Key words: *HIV, TLD, Wistar rats, placenta histology, oxidative stress*

Received: April 15th, 2025
Revised: April 29 th, 2025
Accepted: May 13 th, 2025
Online Available: May 15 th, 2025
Curr. Ver.:May 15 th, 2025

1. INTRODUCTION

World Health Organization (WHO) recommends Tenofovir Disoproxil Fumurate/Lamivudine /Dolutegravir (TLD) as the preferred first and second-line HIV treatment regimen for all populations, including pregnant women and breastfeeding mothers (WHO, 2018; WHO, 2019; WHO, 2021). TLD is a fixed dose combination therapy, once-daily oral drug that can be taken with or without meal, classified as a Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) and Integrase Strand Transfer Inhibitor (INSTI). TLD has been known to have a high genetic resistance barrier, improved efficacy, a shorter time to viral suppression, a good tolerability profile, lower toxicity, fewer drug-drug interactions, improve regimen adherence, and improve the quality of life for people living with HIV (PLWH). Despite its benefits, TLD has been linked to potential adverse effects, including skin rashes, headaches, anxiety, nausea, diarrhoea, and insomnia (WHO, 2018).

The placenta is a vital feto-maternal organ that attaches the developing embryo to the uterine wall during pregnancy, providing immunity and protection (Agarwal *et al.,* 2012). It releases steroids, hormones, and cytokines, acting as a barrier against infections and xenobiotics (Furukawa *et al.,* 2014). The placenta adjusts morphologically and/or functionally by changing its size, shape, or efficiency to maintain fetal growth, control gas exchange and nutrients (Vrooman *et al.,* 2016). The placenta functions as a barrier to effectively reduce, but not completely eradicate, HIV transmission during the last 14 days before delivery, which is the

most common window for HIV transmission in utero (Minkoff, 2003). The mechanism by which HIV crosses the placenta is uncertain (Al-Husaini, 2009). Pregnancy can result in the transmission of one or more substantial maternal HIV variants (Dickover *et al.,* 2001).
HIV-1 is mostly transmitted from mother to child (MTCT) by the transcytosis of HIV-infected cells (Lagaye *et al.,* 2001), it can also enter the placenta through damaged villous surfaces (Al-Husaini, 2009). Fetal-placenta macrophages called Hofbauer cells have the ability to transmit HIV across the placenta (Lewis *et al.,* 1990) and infect the developing fetus with certain HIV variants (Lagaye *et al.,* 2001).

Numerous studies have demonstrated that HIV and antiretroviral therapy (ART) exposure has been linked to low placenta weight, area, and irregular shape (Dos Reis *et al.,* 2020; Yampolsky *et al.,* 2021), maternal vascular malperfusion (Ikumi *et al.,* 2021), antiangiogenic state (Conroy *et al.,* 2017), and changes to placenta transporters (Kojovic *et al.,* 2020). Some of these changes have been linked to placenta growth disorders (Conroy *et al.,* 2017; Obimbo *et al.,* 2019; Dos Reis *et al.,* 2020; Kojovic *et al.,* 2020).

Oxidative stress during pregnancy is a physiological state brought on by the placenta's increased oxygen intake and metabolism. Even though oxidative stress is necessary for cellular signaling and embryonic development, its effects are not always detrimental. When the equilibrium is upset, oxidative stress can cause problems like preeclampsia, intrauterine growth restriction (IUGR), and premature birth, but under normal conditions, it can support essential physiological functions (Agarwal *et al.,* 2012; Pereira and Martel, 2014; Ibrahim *et al.,* 2024). This dual role must be understood in order to evaluate how external factors, including as viral infections and antiviral medications, affect the health of the mother and fetus (Grzeszczak *et al.,* 2023).

The placenta's metabolic load is supported by an increase in mitochondrial biogenesis during pregnancy (Holland *et al.,* 2017). Fetal and placental development may be impacted by mitochondrial dysfunction at this critical period (Wakefield *et al.,* 2011). According to research, redox imbalance is made worse by ART and HIV infection; pregnant women with HIV have higher redox indicators in the early stages of pregnancy compared to those who are HIV-negative and not pregnant (Martinez *et al.,* 2021).

HIV/ART has been linked to changes in the mother's and fetus mitochondrial function in addition to redox imbalance. Infants from mothers with HIV who were exposed to ART had higher levels of mitochondrial DNA in their peripheral blood and lower levels of mitochondrial expression in their umbilical cord blood (Ross *et al.,* 2012). According to other studies, children who are HIV-exposed but not infected have dysregulated mitochondria and altered cell metabolic activities as a result of early life exposure to HIV/ART (Mataramvura *et al*., 2023; Du Toit *et al*., 2023). Overall, a wealth of clinical evidence has demonstrated that women with HIV using ART and their children experience increased oxidative stress as a result of redox imbalance, which leads to mitochondrial dysfunction.

Although the importance of TLD among women of childbearing age, including pregnant and breastfeeding mothers, to reduce transmission of HIV to their unborn child has been recognized, little is known about its effect on animal models at this time. This study aimed to investigate the histological and oxidative stress toxicity of TLD of the placenta of adult Wistar rat.

2. MATERIALS AND METHODS

Drugs

Tenofovir Disoproxil Fumarate 300 mg/Lamivudine 300 mg/Dolutegravir 50 mg (TDF/3TC/ DTG) (Lot:3125312, manufactured 11/2019, expired in 10/2023, manufactured by Mylan Laboratories Limited, India), were purchased from Alpha Pharmacy and Stores Ltd., located at #59 Ogbunabali Road, Port Harcourt, Rivers State, Nigeria.

Experimental Animals

Adult Wistar rats used for the study were bred at the animal house of the Department of Anatomy, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, Edo State, Nigeria. The animals were kept in appropriate cages with wired open tops at room temperature and saw dusts were used as beddings for the cages. The animals were given distilled water and grower mash obtained from Sa-Vee Livestock Feed Service,

Isihor, Benin City. They were weighed before the commencement and also weekly throughout the duration of the experiment using electronic weighing scale balance (manufactured by Kern & Sohn GmbH, D-72336 Balingen, Germany), calibrated in gram and recorded to the nearest whole number. Protocols for these experiments were in accordance with the guidelines for the care and use of laboratory animals (National Research Council of the National Academies, 2011).

Design of Study

A total of 10 adult female Wistar rats weighing between 154g-194g, aged between 90 days and 120 days were used for this study. The animals were randomly selected and assigned into two groups, control and treated, comprising five rats each. Group 1 served as the control and was fed growers mash and distilled water only. Group 2 serves as the treated group, and was fed growers mash, distilled water and was orally administered the combination of Tenofovir Disoproxil Fumurate in 5mg/kg, Lamivudine in 5mg/kg and Dolutegravir in 0.8mg/kg body weight. Human exploratory dose was calculated based on animals weight.

Sample Collection

Following ninety days (90) of oral administration of TLD, the animals in proestrus phase were selected and were paired overnight with a male in the ratio of 2:1. Pregnancy was confirmed the following morning (between 9 am to 10 am) by the presence of or sperm cells in the vaginal fluid. The day pregnancy was confirmed was recorded as gestational day 0 (GD0). The administration TLD continued, the dams were weighed at day GD0, GD7 and then at GD14 and thereafter at GD21. At GD21, the dams were anesthetized under chloroform, sacrificed, and the ventral abdominal walls were opened uterine horns were harvested cleared of connective tissue, implanted fetuses and placenta were counted, separated and placenta were weighed using digital weighing balance calibrated in gram (manufactured by ECOSTAR, China), measured in milimeter using vernier caliper, recorded to the nearest two decimal places, preserved in 10% formal saline contained in sample bottles with the appropriate labels and was taken for histological evaluation. While some placentas for oxidative stress were preserved in normal saline (0.9% NaCL) contained in sample bottles with the appropriate labels and was taken for oxidative stress assessments.

Histological Assessment

The tissue was prepared according to the protocol described by Drury and Wallington, (1980). The placenta tissue samples were fixated, dehydrated, cleared, filtered, embedded, sectioned, and stained in order to be examined histologically. To ensure correct fusing, the tissues were sliced to a thickness of roughly 5 mm. The tissues were fixed with 10% formal saline and then immersed in 50% alcohol at 70%, 80%, 85%, 95%, and 100% for two hours. To get rid of the alcohol, the treated tissues were titrated through an equal mixture of 100% (absolute) alcohol and xylene for one hour each. Every tissue was infiltrated twice by subjecting it to molten paraffin wax in an oven preheated to 40 ^{o}C for one and a half hours each time. The tissues were soaked in molten paraffin wax, then placed on a wooden block and cut to size. 10 μm thick serial sections were made using a rotatory microtome. Once sliced, the pieces were put on slides and cooked in a warm water bath to 40 ^{o}C. Six parts were removed from each treated organ. Three samples were presented on each slide. Using varying magnifications of 10, 40, 100, and 400, a microscopic analysis was performed to verify that the samples were properly mounted on the slide. Microscopic investigation was carried out after sections were stained and mounted using dimethyl paraffinate xylene (DPX) as a mounting agent.

Anti-oxidants Stress

The tissues were homogenized in a porcelain mortar and pestle using acid-washed sand and PBS after two cold phosphate buffered saline (PBS) washes. For ten minutes, the tissue homogenate was centrifuged at 10,000 rpm and 40 ^{o}C. In order to analyze the endogenous antioxidant enzymes, the supernatant was treated immediately. Malondialdehyde (MDA) activity was measured using the Buege and Aust, (1978) technique. Glutathione peroxidase

(GPx) activity was measured using the Nyman, (1959) technique. Ellman's, (1959) method was used to estimate glutathione (GSH) levels. Superoxide dismutase (SOD) activity was measured using the Misra and Fridovich, (1972) method. The catalase (CAT) activity was measured using the Cohen *et al.* (1970) technique.

Statistical Analysis

The data was analysed using graphpad prism Version 9 (manufactured by Graphpad Software Inc., California). The parameters for each group were compared using Paired-Samples T-Tests, and the data was presented using Mean±SEM. P<0.05 were considered significant.

3. RESULTS

Table 1: Effect of TLD on the body weight of the Experimental Rats

Body Weight (g)	Control Group	Treated Group	P-Value
Initial body weight	173.4±6.592	171.6±8.524	0.8715
Final body Weight	270.0±4.336	233.4±5.163*	0.0006

Values are represented as Mean±SEM; *indicates significant difference (p<0.05) in the final mean body weight of the treated group of the experimental animals compared to the control.

As show in table 1, daily oral administration of Tenofovir Disoproxil Fumurate in 5mg, Lamivudine in 5mg and Dolutegravir in 0.8mg/kg body weight caused significant increase (p<0.05) in the final mean body weight in both the TLD-treated and control groups compared to the initial mean body weights of the experimental animals. However, there was significant decrease (p<0.05) on the final mean body weight of the TLD-treated group compared to the control (Table 1).

Table 2: Effect of TLD on the number of placenta, placenta weight, diameter and thickness of the Experimental Rats

Parameters	Control Group	Treated Group	P-Value
Number of placenta (n)	6.00±1.43	6.00±1.21	0.917
Placenta weight (g)	0.45±0.02	0.37±0.02*	0.002
Placenta diameter -major axis (mm)	14.25±0.19	13.61±0.19*	0.039
Placenta diameter –minor axis (mm)	11.59±0.19	11.13±0.13*	0.021
Placenta thickness (mm)	3.11±0.07	2.82±0.06*	0.002

Values are represented as Mean±SEM; *indicates significant difference (p<0.05) of the control group.

From the table 2 above, There was insignificant differences (p>0.05) in number of placenta in TLD-treated group when compared to the control group. However, there was a significant decrease (p<0.05) of placenta weight, placenta diameter (major and minor axis) and placenta thickness in the TLD-treated group, when compared to the control group (Table 2).

Table 3: Evaluation of placenta oxidative stress of the Experimental Rats

Parameters	Control Group	Treated Group	P-Value
MDA (mole/mg protein) *10^2	5.691±0.67	9.07±0.634	0.120
GPx (U/mg protein)	1.615±0.13	1.181±0.046	0.058
GSH (µM)*10^{-1}	2.155±0.008	1.963±0.024*	0.016
SOD (U/mg protein)	3.583±0.288	2.446±0.077	0.072
CAT (U/mg protein) *10	1.207±0.093	0.894±0.025	0.105

Values are represented as Mean±SEM; *indicates significant difference ($p<0.05$) from the control group. Total Protein (TP), Malondialdehyde (MDA), Glutathione Peroxidase (GPx), Reduced Glutathione (GSH), Superoxide Dismutase (SOD), Catalase (CAT).

From the table 3 above, It was observed that daily oral administration of TLD caused an increase with insignificant difference ($p>0.05$) in the concentration of malondialdehyde, causes a decrease with insignificantly difference ($p>0.05$) in the concentration of glutathione peroxidase, superoxide dismutase and catalase ($p<0.05$) compared to the control except in the concentration of reduced glutathione which was significantly reduced ($p<0.05$) in the treated group compared to the control (Table 3).

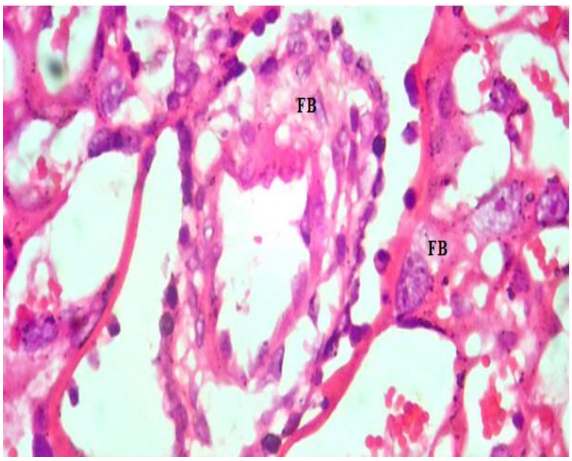

Plate 1: Photomicrograph of the placenta (control groups) of adult Wistar rat showing histological features: fetal blood vessel (FBV) filled with fetal blood (FB) (H&E X400).

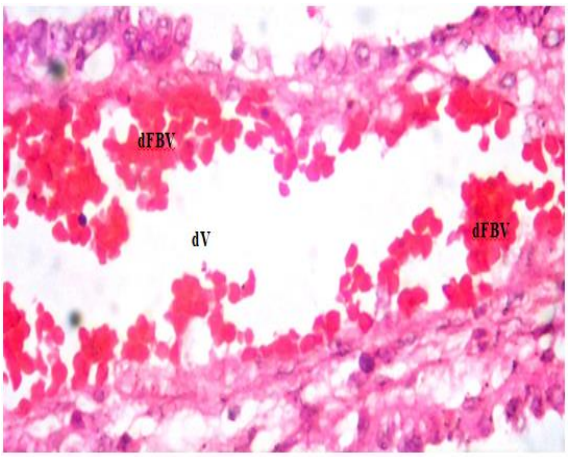

Plate 2: Photomicrograph of the placenta (TLD-treated groups) of adult Wistar rats of showing histological features: dilated fetal blood vessel (dFBV) filled with fetal blood and dilated vacoulation (dV) (H&E X400).

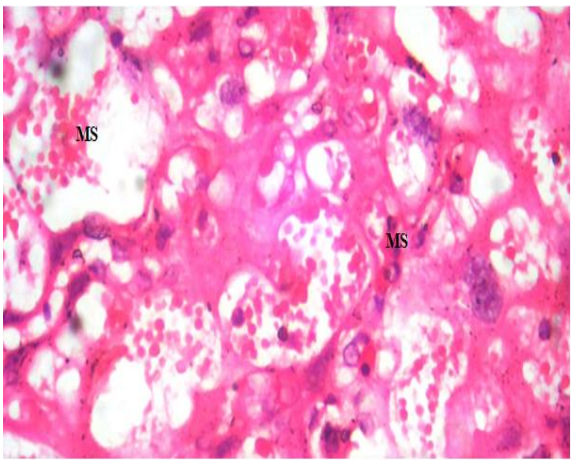

Plate 3: Photomicrograph of the placenta (control groups) of adult Wistar rats showing histological features: maternal sinusoids (MS) filled with maternal blood (H&E X400).

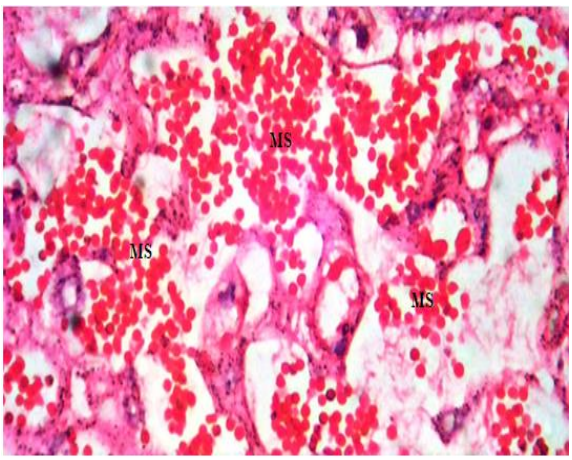

Plate 4: Photomicrograph of the placenta (TLD-treated groups) of adult Wistar rats showing histological features: maternal sinusoids that are dilated and congested with maternal blood (H&E X400).

From the photomicrograph above, the histological features of the placenta showed presence of fetal vessel filled with fetal blood in the control groups (Figure 1) as compared to the TLD-treated groups which shows the presents of dilated fetal blood vessel filled with fetal blood and dilated vacoulation (Figure 2). Also, there were presents of maternal sinusoids filled with maternal blood in the control groups (Figure 3) as compared to the TLD-treated groups which shows maternal sinusoids that are dilated and congested with maternal blood (Figure 4).

4. DISCUSSION

HIV/AIDS can be effectively managed with combination antiretroviral therapy (cART). However, a number of studies have linked oxidative stress brought on by cART to both human and animal models (Awodele *et al.,* 2018; Oyeyipo *et al.,* 2018). However, to the best of our knowledge, no toxicities linked to oxidative stress or placenta histopathology have been reported in adult female Wistar rats using TLD. In this study, we investigate the possible relationships between placenta histology and oxidative stress in adult female Wistar rats receiving TLD.

Daily oral administration of Tenofovir Disoproxil Fumurate in 5mg, Lamivudine in 5mg and Dolutegravir in 0.8mg/kg body weight caused no mortalities on the experimental animals.

TLD caused significant increase in the final mean body weight in both the treated and control groups compared to the initial mean body weights of the experimental animals. However, there was significant decrease on the final mean body weight of the TLD-treated group compared to the control. The finding of this study is consistent with previous studies. For instance Wagner *et al.* (2011) reported significant reduction in body weight of female rats treated with a Zidovudine/Lamivudine/Ritonavir or oral treatment with Zidovudine-Stavudine (Antonio *et al.,* 2012). The weight loss can be attributed to mechanisms such as mitochondrial dysfunction, hormonal alterations, and systemic metabolic effects. However, compared to TLD or other nucleoside reverse transcriptase inhibitors, a research has shown an increase with regimens based on Dolutegravir (DTG), particularly when DTG is paired with Tenofovir Alafenamide/Lamivudine (3TC/TAF) (Kanters *et al.,* 2022). Although the exact mechanism is still unknown, some studies have shown that ART causes weight gain since patients who underwent ART for up to three years have been shown to be overweight or obese over time (Crum-Cianflone *et al*., 2010; Koethe *et al*., 2016). Accordingly, it was proposed that the distribution of adipocytes may have been linked to the increase in body weight as a result of exposure to these medicines, which hinder adipocyte metabolism and cause accumulation (Couturier *et al.,* 2018). Additionally, the results of this study is in contrast with the some reports that found no significant changes on the body weight of rats given Lamivudine (Pontes *et al.,* 2005), Zidovudine/Lamivudine/Nevirapine (Oluwaseun *et al*., 2013; Ayeni *et al*., 2013), or Lopinavir/Ritonavir combination regimen (Kulay *et al.,* 2013). The duration of time or dosage of therapies utilized in the various studies may be the reason for the differences between our study and those of earlier researchers. This claim is supported by a study that found that while weight increase is a common post-ART presentation of HIV/AIDS patients, it is not in all cases (Sattler *et al.,* 2015).

The number of placenta was not significantly affected by daily administration of TLD in this study. Several studies have also reported no significant difference with administration of Indinavir (Quintino *et al*., 2011), Zidovudine/Stavudine (Antonio *et al.,* 2012) and Zidovudine/Lamivudine/Nevirapine (Ayeni *et al.,* 2013). The drug in

question may not have an impact on the drug's intercompartmental clearance from the placenta to maternal plasma, which could explain its non-significant effect on placenta number. According to the mechanism proposed by this study findings, trophoblastic proliferation and vascularization are likely maintained during early placenta development, resulting in an unaltered placenta number.

Tenofovir Disoproxil Fumurate/Lamivudine/Dolutegravir (TLD) administered daily in this study caused significant decrease placenta weight, diameter and thickness. The observed decrease in placenta weight is consistent with previous study by Wagner *et al.* (2011) that reported that administering Zidovudine/Lamivudine/Ritonavir decreased placenta weight. Through the placenta, pregnant rats and their fetuses form a close bond. In contrast to this study, a human investigation by Ikumi *et al.* (2020) found no significant differences in placenta weight, diameter, or thickness between HIV-positive groups on ART and HIV-negative control groups. Fetal resorption can therefore be caused by medications or chemicals that cause abnormal fetal development. Furthermore, placenta growth is inhibited by mitotic suppression, apoptosis, trophoblast deterioration, and/or destruction brought on by direct placenta damage or nonspecific effects brought on by an abnormally unfavorable pregnancy environment, which results in a decreased placenta weight (Furukawa *et al.,* 2011). At later stages, placenta growth may be hindered by cytotoxic effects, metabolic abnormalities, or alterations in hormonal and inflammatory pathways, leading to significant decreases in placenta weight, diameter, and thickness. The substantial reduction in placenta weight, diameter, and thickness observed in the TLD-treated groups in this study raises the possibility that TLD may interfere with angiogenesis, change the proliferation of trophoblast cells, or have direct cytotoxic effects on placenta cells, which would result in decreased growth and size.

Histological result this study revealed the presence of fetal blood vessel filled with fetal blood in the control groups compared to TLD-treated groups which shows presences of dilated fetal blood vessel filled with fetal blood and dilated vacoulation. There is presence of maternal sinusoids filled with maternal blood in the control groups compared to TLD-treated groups which shows presences of maternal sinusoids that are dilated and congested with maternal blood. The results of this study is in contrast with those of Magdy *et al.* (2023), who reported that during the fetal developmental phase, ribavirin administration resulted in deteriorated necrotic areas, congestion, and glycogen cyst degeneration in the basal zone. The spongiotrophoblast also displayed pyknotic. Degenerated large cells with pyknotic nuclei are also present. Comparing the treated groups to the control, the labyrinth zone displayed structural disorientation, dilated maternal sinusoids, necrotic change, and blood vessels lined with a deteriorated endothelial layer. The study reveals that, antiretroviral treatments such as TLD can disrupt the structure and function of the placenta, which may result in the development of trophoblastic cells and the circulation of blood between the fetus and the mother. This may result in vacoulation, dilatation and congested foeto-maternal vascular bed, which may impact fetal development and nutrition exchange as well as the circulations of the mother and fetus.

This study revealed that, daily administration of TLD caused an increase with insignificant difference in the concentration of malondialdehyde in the placenta, causes a decrease with insignificantly difference in the glutathione peroxidase, superoxide dismutase and catalase in the TLD-treated groups compared to the control except in the concentration of reduced glutathione which was significantly reduced in the treated groups compared to the control. The findings of this study contradict with those of

Magdy *et al.* (2023), who found that ribavirin use significantly raised MDA levels and significantly decreased SOD, CAT, and GSH levels in the placenta when compared to the control. The metabolic processing of the medication combination may be the cause of the study's negligible rise in malondialdehyde (MDA) brought on by enhanced lipid peroxidation. The slight decrease in enzymes such as CAT, SOD, and GPx indicates a disruption in antioxidant defence. A notable decrease in reduced glutathione (GSH), however, indicates that the medications may either consume GSH or prevent its regeneration, which would impact the activity of antioxidant enzymes.

5. CONCLUSION

Tenofovir Disoproxil Fumurate/Lamivudine/Dolutegravir (TLD) administration during pregnancy causes significant decrease in the body weight of the treated group, as does the placenta weight, thickness, and diameter. Also, the concentration of reduced glutathione decreased, glutathione peroxidase, superoxide dismutase, and catalase decreased with insignificantly differences, and the concentration of malondialdehyde increased with insignificantly differences, but there was no significant difference in the number of placenta compared to the control group. Additionally, TLD treatment revealed vacoulation, dilatation and congested foeto-maternal vascular bed. Due to the negative effects on placenta morphology, TLD, a known therapeutic agent in the management of HIV patients, is not completely safe during pregnancy as these effects could lead to infertility in females during the reproductive period. While its prescription might be necessitated by medical exigency, the risks should be weighed against the benefits. Adjuvant therapies that may help to reduce the effects of oxidation stress, improve foetal-maternal wellbeing and growth should include to females of child-bearing age who are on TLD and wants to get pregnant. Concerns over long-term TLD usage by HIV-positive people were thus highlighted by this study, particularly for women of reproductive age who wish to get pregnant. Further research should be done to determine which specific components in this formulation that may pose greater risk, as TLD is a combination therapy.

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