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STIGMASTEROL ATTENUATES OXIDATIVE STRESS IN SOME ORGANS OF *Trypanosoma congolense* INFECTED RATS

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Abstract

Stigmasterol has shown positive effects against *Trypanosoma congolense,* which induced organ pathological changes in rats. Therefore, to unravel a possible mechanism of this action, the effect of stigmasterol on oxidative stress in the liver, kidney, spleen, and heart of *T. congolense* infected rats was investigated. Rats were infected with *T. congolense* and on day 11 post-infection, the infected rats were treated with 100 and 200 mg/kg body weight (BW) of stigmasterol for 14 days. At the end of the experiment, the levels of reduced glutathione, malondialdehyde as well as the activities of catalase and superoxide dismutase were analyzed in the homogenates of liver, kidney, spleen, and heart obtained from the stigmasterol-treated infected rats and compared to non-infected and infected non-treated controls. The data showed evidence of induction of oxidative stress in all the organs caused by the infection. Treatments with the 100 and 200 mg/kg BW of stigmasterol attenuated the *T. congolense* induced oxidative stress in all the organs as indicated by significant ($P < 0.05$) amelioration of the levels of most of the markers. A similar trend was observed with diminazene aceturate, used as a standard drug. It is concluded that stigmasterol could attenuate *T. congolense* induced oxidative stress which might be due to a direct free radical scavenging effect or a consequence of the reported parasite clearance.

INTRODUCTION

African animal trypanosomiasis is caused by various species of *Trypanosoma,* with the most pathogenic species in animals being *Trypanosoma congolense* due to its devastating effects on cattle, while *Trypanosoma brucei brucei* and *Trypanosoma vivax* also contribute a substantial loss [1]. On the other hand, human African trypanosomiasis is exclusively confined to Africa because the two pathogenic species; *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense,* are mainly tsetse transmitted [2]. The first-stage clinical manifestations of African trypanosomiasis include fever, headache, lymphadenopathy, pruritis and splenomegaly [3]. Moreover, a prominent pathogenic manifestation of the disease in both humans and animals is anaemia [3, 4], which could be used to start a treatment in animals raised in endemic areas [5]. Trypanosomiasis-induced anaemia results from multiple factors that act individually or synergistically. These include direct erythrocyte destruction by the parasites, undulating pyrexia, parasite-derived enzymes and lipid peroxidation caused by oxidative stress [3]. Indeed, oxidative tissue damage on other organs such as the liver, kidney, brain, and heart has been described in trypanosomiasis with a concomitant reduction in endogenous defence mechanisms in the infected animals, and this largely contributes to the pathological features of the disease [3, 4, 6, 7]. Therefore, the use of antioxidants to tackle oxidative stress is a rational anti-disease approach to trypanosomiasis [7, 8].

Stigmasterol is a naturally occurring plant steroid and is a member of a larger group of secondary metabolites called phytosterols [9, 11]. The phytosterols have been reported to occur widely in plants and several bioactivities and important medicinal properties including anticancer, lipid lowering, anti-allergic and anti-inflammatory properties have been attributed to them [10]. Stigmasterol and its derivatives have been ascribed to several pharmacological activities and important health benefits [10] including antioxidative properties, antidiabetic among others [11]. More relevant to this article, stigmasterol reported to possess antitrypanosomal activity against *T. b. brucei in vitro* [12], and using animal models, we also recently reported that stigmasterol retards the proliferation and organ pathogenesis of *T. congolense* infection in addition to the inhibition of trypanosomal sialidase [13]. However, it is unknown whether the beneficial effects of stigmasterol on the *T. congolense* organ pathogenesis were mediated via an anti-oxidative dependent mechanism.

In view of the foregoing, this study investigated the protective effects of stigmasterol on *T. congolense*-induced oxidative stress in the liver, kidney, spleen, and heart of rats.

MATERIALS AND METHODS

Chemicals and Reagents

Dimethylsulphoxide (DMSO) and stigmasterol were procured from Sigma Chemical Company (St Louis, MO, USA), through Bristol Scientific Company Limited, Lagos, Nigeria. Diminazene aceturate was obtained from Eagle Chemical Company Ltd, Ikeja, Nigeria. Thiobarbituric acid (TBA) was purchased from Kem Light Laboratory limited, Mumbai, India.

Experimental Animals and Trypanosome Parasites

Twenty-five healthy Wistar rats in the weight range of 170 - 220 g were procured from the animal house of Pharmacology Department, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, (ABUZ) Nigeria. The protocols for animal care met the rules and regulations of the experimental animal ethics committee of ABUZ and the guidelines of the Good Laboratory Practice (GLP) regulations. The animals were fed with commercial rat chow (ECWA Feeds, Jos, Nigeria) and water *ad libitum*. The *T. congolense* (savannah strain) was obtained from the National Institute of Trypanosomiasis and Onchocerciasis Research (NITOR), Kaduna, Nigeria. Obtained parasites from the blood of a benefactor rat at topmost parasitemia and diluted in physiologically cold saline were used for the infection of experimental animals.

Animal Grouping and Treatment

Approximately 1×10^4 *T. congolense* per 100 g body weight (BW) was used via intraperitoneal injection to infect four groups of five rats each, whereas rats of one matched group were uninfected. When parasitemia approximately reached 10^6 trypanosomes/ml blood which was on day 11 post-infection (pi), two groups of infected rats were orally treated with 100 (ITSG100) and 200 mg/kg BW (ITSG200) of stigmasterol dissolved in 5% DMSO while another infected group was treated with 3.5 mg/kg b.w. of diminazene aceturate (ITDA). The remaining group of infected rats (IC) and the uninfected group of rats were treated with 5% DMSO (NC). The treatments lasted for two weeks.

Collection of Organs and Tissue Processing

Animals were humanely euthanized by chloroform anesthesia and the organs (liver, kidney, spleen, and heart) were collected from each animal, washed in normal saline (0.9% NaCl) to get rid of adhering tissues and wiped with filter paper. Organs fragments were homogenized in normal saline (1:10 w/v), centrifuged (800 xg; 10 min; 4°C) and a crude homogenate was used for lipid peroxidation assay. The supernatant was further centrifuged (10,000 xg; 20 min; 4°C) and the resultant supernatant was then collected in a micro tube and stored at −20 °C until further analysis of the antioxidative parameters.

Biomarkers Analysis

Thiobarbituric acid reactive substances, expressed as malondialdehyde (MDA) equivalents, were measured to determine the extent of lipid peroxidation using the method as described by Fraga et al. [14]. Reduced glutathione (GSH) level was determined using the dithiobisnitrobenzoic acid (DTNB) method described by Ellman [15]. Superoxide dismutase (SOD) activity was measured based on its ability to inhibit the autooxidation of epinephrine as described by Misra and Fridovich [16] while catalase activity was determined using the hydrogen peroxide method as described by Aebi [17].

Statistical Analysis

All data were shown as mean ± standard deviation of five animals. Statistical software package (SPSS for Windows, version 20, IBM Corporation, NY, USA) using Tukey's-HSD multiple range post-hoc test was used to analyze the data. Values were considered significantly different at $P < 0.05$.

RESULTS

The most common biomarker used in assessing the extent of free radical generation, oxidative stress and lipid peroxidation is the accumulation of malondialdehyde (MDA) in tissues. Alongside MDA accumulation, measurement of the levels of reduced glutathione as well as activities of catalase and SOD gives valuable information on tissue oxidative status. Tables 1-4 show the levels of these markers in the liver, kidney, spleen, and heart in all rat groups. The level of MDA is significantly ($P < 0.05$) elevated in all the organs of the infected groups compared to the non-infected group. Treatment with 100 and 200 mg/kg BW stigmasterol as well as diminazene aceturate significantly reduced MDA levels in all organs (except in the liver of ITSG200 group) related to the infected control. Conversely, liver GSH level was reduced by the infection although this was not significant (Table 1) and treatment of infected animals with stigmasterol resulted in a significant ($P < 0.05$) increase in GSH compared to the non-treated control. Liver SOD activity was elevated by the infection and all treatments did not ameliorate the increase while catalase activity was neither affected by infection nor the treatments.

In the other organs, the GSH level in the kidney and spleen was significantly reduced, whereas only the catalase activity in the kidney was significantly elevated by the infection. Interestingly, all the treatments ameliorated these changes (Tables 2 and 3). No significant change in SOD activity was induced by the infection or treatments in the kidney or spleen. Heart SOD activity was significantly reduced ($P < 0.05$) by the infection and only the treatment with 200 mg/kg stigmasterol and the standard drug significantly reversed the decrease (Table 4).

Table 1. Effect of stigmasterol on oxidative stress markers in the liver of *T. congolense* infected rats

	Normal control (NC)	Infected control (IC)	ITSG100	ITSG200	ITDA
MDA (nmol/mg protein)	595.20 ± 199.50^{a}	1858.00 ± 507.80^{b}	1658.30 ± 4.70^{ab}	1755 ± 33.10^{b}	1630 ± 23.70^{ab}
GSH (µg/g)	25.83 ± 2.62^{ab}	18.68 ± 3.93^{b}	29.97 ± 5.57^{a}	28.78 ± 2.69^{a}	31.17 ± 6.72^{a}
SOD (U/mg protein)	14.85 ± 3.73^{a}	8.22 ± 2.70^{b}	8.19 ± 1.17^{b}	9.10 ± 0.29^{b}	5.80 ± 3.85^{b}
Catalase (U/mg protein)	68.60 ± 16.36^{a}	48.56 ± 7.83^{a}	61.70 ± 6.24^{a}	47.84 ± 9.48^{a}	56.39 ± 18.09^{a}

Values are expressed as mean ± SD of 5 rats. Different superscript letters (a and b) along a row represent a statistically significant difference (Tukey's multiple range post-hoc test, $P < 0.05$). NC is an uninfected (normal) control group while IC is an infected untreated control group. ITSG100 and ITSG200 are infected groups that were treated with 100 and 200 mg/kg BW of stigmasterol respectively while ITDA is an infected group treated with 3.5 mg/kg BW of diminazene aceturate.

Table 2. Effect of stigmasterol on oxidative stress markers in the kidney of *T. congolense* infected rats

	Normal control (NC)	Infected control (IC)	ITSG100	ITSG200	ITDA
MDA (nmol/mg protein)	1720 ± 123.90^{a}	3189 ± 875.10^{b}	2055 ± 743.90^{ab}	2051 ± 519.90^{ab}	1835 ± 481.00^{a}
GSH (µg/g)	25.98 ± 2.00^{a}	20.06 ± 3.69^{b}	27.17 ± 4.08^{a}	27.03 ± 4.14^{ab}	27.62 ± 4.33^{a}
SOD (U/mg protein)	10.60 ± 10.69^{a}	8.85 ± 1.98^{a}	10.30 ± 2.06^{a}	10.10 ± 2.57^{a}	9.89 ± 1.58^{a}
Catalase (U/mg protein)	57.10 ± 8.28^{a}	119 ± 32.70^{b}	66.00 ± 31.80^{a}	70.20 ± 27.60^{ab}	46.50 ± 15.30^{a}

Values are expressed as mean ± SD of 5 rats. Different superscript letters (a and b) along a row represent a statistically significant difference (Tukey's multiple range post-hoc test, $P < 0.05$). NC is an uninfected (normal) control group while IC is an infected untreated control group. ITSG100 and ITSG200 are infected groups that were treated with 100 and 200 mg/kg BW of stigmasterol respectively while ITDA is an infected group treated with 3.5 mg/kg BW of diminazene aceturate.

Table 3. Effect of stigmasterol on oxidative stress markers in the spleen of *T. congolense* infected rats

	Normal control (NC)	Infected control (IC)	ITSG100	ITSG200	ITDA
MDA (nmol/mg protein)	1128 ± 443.60[a]	2990 ± 987.30[b]	1678 ± 501.50[a]	1473 ± 518.80[a]	1217 ± 238.90[a]
GSH (µg/g)	26.51 ± 2.13[a]	18.71 ± 3.30[b]	29.14 ± 5.46[a]	26.75 ± 0.95[a]	28.13 ± 2.67[a]
SOD (U/mg protein)	8.68 ± 1.26[ab]	6.83 ± 1.85[a]	9.49 ± 1.33[ab]	9.72 ± 1.50[ab]	10.70 ± 1.95[b]
Catalase (U/mg protein)	101.60 ± 40.76[ab]	128.30 ± 27.34[ab]	152.80 ± 64.35[a]	93.98 ± 23.91[ab]	75.62 ± 20.07[b]

Values are expressed as mean ± SD of 5 rats. Different superscript letters (a and b) along a row represent a statistically significant difference (Tukey's multiple range post-hoc test, $P < 0.05$). NC is an uninfected (normal) control group while IC is an infected untreated control group. ITSG100 and ITSG200 are infected groups that were treated with 100 and 200 mg/kg BW of stigmasterol respectively while ITDA is an infected group treated with 3.5 mg/kg BW of diminazene aceturate.

Table 4. Effect of stigmasterol on oxidative stress markers in the heart of *T. congolense* infected rats

	Normal control (NC)	Infected control (IC)	ITSG100	ITSG200	ITDA
MDA (nmol/mg protein)	2467 ± 340.00[a]	2919 ± 1185[b]	1470 ± 691.60[a]	1734 ± 681.70[ab]	1150 ± 576.80[a]
GSH (µg/g)	23.32 ± 3.63[a]	18.81 ± 3.29[a]	26.78 ± 6.06[a]	24.53 ± 5.79[a]	25.85 ± 4.80[a]
SOD (U/mg protein)	11.84 ± 1.07[a]	6.53 ± 1.49[b]	7.58 ± 1.87[b]	10.00 ± 2.02[ab]	9.14 ± 2.74[ab]
Catalase (U/mg protein)	73.96 ± 15.47[a]	80.95 ± 22.46[a]	74.19 ± 23.10[a]	72.04 ± 20.59[a]	57.95 ± 8.76[a]

Values are expressed as mean ± SD of 5 rats. Different superscript letters (a and b) along a row represent a statistically significant difference (Tukey's multiple range post-hoc test, $P < 0.05$). NC is an uninfected (normal) control group while IC is an infected untreated control group. ITSG100 and ITSG200 are infected groups that were treated with 100 and 200 mg/kg BW of stigmasterol respectively while ITDA is an infected group treated with 3.5 mg/kg BW of diminazene aceturate.

DISCUSSION

The involvement of free radicals in the pathology of parasitic infections including trypanosomes has been established [3, 18]. Free radical generation is a complex mechanism involving both the host and the parasites which are both armed with endogenous defense mechanisms [19]. However, the capacity of the host defense systems to prevent oxidative burst is often overwhelmed when excess free radicals are generated by the parasites [6] and this makes the antioxidant system a suitable therapeutic target [8]. The present study demonstrated that stigmasterol could protect the anti-oxidative system in the various organs of *T. congolense* infected animals.

African trypanosomes survive freely in the bloodstream of their host and release toxic hydrogen peroxide into the host because they lack the principal detoxification enzyme catalase, as well as other important antioxidant enzymes; glutathione reductase and thioredoxin reductase [19, 3]. Other trypanosome generated free radicals are also discharged into the bloodstream of the host. Consequently, oxidative organ damage is a hallmark of trypanosome infections. In this study, the trypanosomes were found to stimulate oxidative stress as established by the increased MDA levels with decreased GSH and antioxidant enzyme levels in the organs of infected animals. However, stigmasterol ameliorated, to a large extent, the above-mentioned symptoms of oxidative stress in the liver, kidney, and spleen of rats infected with the *T. congolense*. Interestingly, stigmasterol has been reported to possess anti-trypanosomal activities *in vitro* [12] and ameliorated *T. congolense* induced anemia, renal and hepatic damage as well as splenomegaly in rats [13] in addition to *in vitro* free radical scavenging activity [11]. Along with its numerous medical benefits, stigmasterol serves as a precursor in the production of steroids such as progesterone, androgens, estrogens, corticoids, and vitamin D_3 [20, 21]. Therefore, the observed ameliorative effects of hepatic and renal damages observed with stigmasterol treatment of *T. congolense*-infected rats [13] could be connected to the reduction of oxidative stress in the organs considering the role of oxidative stress in the induction of these pathological features during the disease [6, 7]. These are indeed vital observations because anti-disease strategies against trypanosome that target host pathologic and physiologic responses are especially desirable [3]. However, it is also possible that the observed effects were due to a reduced parasite load recorded in the stigmasterol treated *T. congolense* infected rats [13] because stigmasterol treatment appears to prevent increased MDA concentration (oxidative stress) in all the organs.

The effects of stigmasterol on the oxidative stress status of the organs (liver, kidney, and spleen) may also be extended to other organs such as the heart because the

oxidative stress biomarkers in the heart were also largely modulated by the stigmasterol

In conclusion, prevention of *T. congolense*-induced organ pathology by stigmasterol in infected rats could be associated with its antioxidative properties. It is not clear at this point if this is a direct primary antioxidant effect or a consequence of the parasite clearance ability of the compound. It will be interesting to investigate the antioxidative effects of stigmasterol during infection with other parasites, especially intracellular parasites, to find out if the antioxidative properties extend beyond bloodstream trypanosomes infection.

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CONFLICT OF INTEREST

There is no conflict of interest in this study. Also, all the authors have declared that they have no conflict of interest.

ETHICAL APPROVAL

Ethical approval for the study was obtained from the institutional ethics committee on animal use and care of Ahmadu Bello University (ABU), Zaria, with Approval No: ABUCAUC/2022/041.

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