Research article

Changes in antioxidants status, atherogenic index and cardiovascular variables after prolonged doses of D-ribose-L-cysteine in male Wistar rats

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A B S T R A C T

D-ribose-L-cysteine (DRLC) acts as a rate limiting substrate for the synthesis of glutathione (GSH). GSH deficiency has been linked to oxidative stress, hypertension and cardiovascular diseases. There are limited findings on the effects of DRLC in the physiologic state. This study was therefore designed to investigate cardiovascular effects of different doses of DRLC in normal Wistar rats.

Fifteen male Wistar rats were assigned into 3 groups (n = 5). Group 1 was administered orally with 10 mg/kg distilled water (Control). Groups 2 and 3 were administered orally with DRLC 125 mg/kg and 250 mg/kg respectively daily for 8 weeks, respectively. Animals were weighed; blood pressure and heart rate measured using rat tail cuff method. They were euthanized, blood collected and organs harvested. Serum C-reactive protein (CRP) was determined through ELISA. Gamma glutamyl transferase (GGT), heart GSH, glutathione peroxidase (GPx), total thiol and lipid profile and were assessed through spectrophotometry. Data were expressed as mean ± SEM and compared by ANOVA at $P < 0.05$.

DRLC 250 significantly increased total thiol, GSH and GPx in heart tissues but decreased GGT, atherogenic index and CRP in normal male Wistar rats compared to DRLC 125 and control.

DRLC supplementation in normal male Wistar rats may sustain cardio functions and decrease atherogenicity.

1. Introduction

L-cysteine acts as a rate-limiting substrate for the overall biosynthesis of glutathione (GSH) [1] and several cysteine analogues have been developed to enhance its delivery into cells. D-ribose-L-cysteine (DRLC) is a cysteine analogue and pro-drug developed for boosting GSH synthesis [2]. It is a dietary supplement developed for delivering cysteine into cells thereby increasing cellular GSH levels [3]. Though, GSH is ubiquitously found in the body and it provides antioxidant functions, it can be easily depleted due to oxidative stress. Imbalance between antioxidants and oxidants in favour of oxidants which may affect physiological functions and redox signalling is termed oxidative stress [4]. Oxidative stress depletes GSH stores and it may result into hypertension and cardiovascular diseases [1, 5, 6]. GSH depletion is associated with inactivation and sequestration of nitric oxide (NO) (mediated by reactive oxygen species (ROS) which leads to diminished NO availability [1, 5]. Furthermore, some exogenous antioxidants supplementations may not reverse hypertension in GSH depletions [5]. This illustrates GSH roles as an important component of the antioxidant system.

In physiologically healthy mice, DRLC administered as a single dose (2 g/kg) increased GSH levels in organs such as heart, muscle tissue, liver, kidney and lungs without producing adverse effects [3]. In pathological states, studies have reported different activities of DRLC at varying doses. This includes anti-atherosclerotic effects of oral DRLC administration in lipoprotein (a) and apolipoprotein (e) deficient mice models [7, 8]. DRLC was also reported to attenuate memory deficit induced by lipopolysaccharide in mice models [9]. In Sprague-Dawley rats, DRLC administration attenuated reproductive hormones and sperm parameters in aluminium-induced testicular damage [10], Streptozotocin-induced diabetic rats [11] and also as an oral hypoglycaemic agent in pregnant-diabetic rats [12]. Other studies also showed DRLC enhanced wound healing [13] and halted the progression of intervertebral disc degeneration in rabbits [14]. These studies were either in genetically modified or diseased animals as there are limited data on the effect of prolonged DRLC administration in normal animals. Also, despite varying doses used in literature, there are inconsistencies on the systemic effect of DRLC in diseased models. GSH availability through DRLC supplementation may play important roles in regulating

cardiovascular functions as decreases in GSH level are linked with cardiovascular disturbances. This study was therefore designed to investigate the effect of DRLC on lipid profile, cardiovascular variables and some antioxidants enzymes in normal male Wistar rats using two doses.

2. Materials and methods

2.1. Animals

Fifteen male Wistar rats (11 weeks old) weighing 180–200g were purchased from the Central Animal House, University of Ibadan. The animals were housed in sterile sanitary beds and maintained under standard environmental conditions. They were fed with standard rats' chow and provided water *ad-libitum* throughout the study period. Ethical approval (UI-ACUREC/18/0128) was obtained from the Animal Care and Use Research Ethics Committee of the University of Ibadan, Nigeria. Animals were divided into three groups of five (5) animals each. Control group was orally administered with 10 mg/kg of distilled water daily for eight weeks. DRLC 125 group was orally administered with 125 mg/kg of DRLC daily, for eight weeks. DRLC 250 group was orally administered with 250 mg/kg of DRLC daily, for eight weeks. Distilled water and DRLC were administered through an oral cannula. At the end of the study (week 8), the animals were anaesthetised by infusion of ketamine (60 mg/kg) and xylazine (10 mg/kg) and blood was collected. The blood was centrifuged at 1372 x g for 15 min, at – 4 °C using a cold centrifuge (Centurion Scientific Ltd., West Sussex, United Kingdom). The resultant supernatant serum samples were pipetted into separate plain bottles. Heart and liver tissues of the rats were excised and a uniform portion (100 g) was homogenized in 10% w/v ice-cold 0.1 M phosphate buffer (pH 7.4). Biochemical markers were assayed in the serum and heart homogenates of the rats through diagnostic kits.

2.2. Chemicals

Assay kits used for lipid profile (total cholesterol, triglyceride, and lipoproteins), glutathione peroxidase and gamma glutamyl transferase were obtained from Fortress Diagnostic (Antrim, UK). Lipid peroxidation (malondialdehyde assay kits) and Nitric oxide were obtained from Oxford Biomedical Research, Inc. (USA). Ellman reagent [5,5-dithiobis- (2-nitrobenzoate) DTNB] and thiobarbituric acid (TBA) were from (Sigma–Aldrich, St. Louis, USA). C-reactive protein was from (Elabscience Biotechnology Inc, USA) and D-Ribose-L-Cysteine was obtained from Max International, Salt Lake City, Utah, USA.

2.3. Body weight and blood pressure measurements

Body weights were taken weekly and measured using a digital weighing scale. Heart rate, systolic and diastolic pressures were measured with the CODA non-invasive tail-cuff method at the end of week 8. Through a tail cuff sphygmomanometer and a photoelectric sensor, blood pressure was measured in conscious restrained rats with a photoelectric sensor and a tail-cuff sphygmomanometer, using the Kent RTBP2000 series Rat Tail System (Kent Scientific Corporation 457 Bantam Road. Litchfield, CT) [15]. The rats were allowed to move into a pre-heated restrainer with their tails exposed for 30 min prior to the measurements. Tail cuff was closely fitted to the base of the tail and behind the cuff was a pulse sensor. The cuff was automatically deflated and inflated between 90 seconds to 30 minutes. Then through a PowerLab/400 (software Chart for Windows®) system, the pulse signal and pressure in the occlusion cuff were recorded and monitored. Pulse signals initiation after inflation peak was correlated with the pressures in the occlusion cuff which was used to obtain the readings. Blood pressure and heart rate values were obtained from an average of 5 consistent readings.

2.4. Determination of glutathione

GSH was measured on the principle of oxidation of reduced GSH by 5,5,-dithiobis-2-nitrobenzoic acid (DTNB) (aromatic disulphide compound), to form glutathione oxidized form and 5,thio-2-nitrobenzoic acid [16]. At absorbance of 412nm, yellow colour formed as a result of DTNB reduction was measured.

2.5. Glutathione peroxidase (GSH-Px) assessment

Measurement of GSH-Px was determined by a previously described method [17]. GSH-Px in the presence of hydrogen peroxide (H_2O_2) facilitates the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). GSSG is reduced to GSH through nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase. GSH-Px activity was then calculated by reading absorbance reduction at 340nm during NADPH to NADP oxidation.

2.6. Determination of catalase (CAT) activity

CAT was assessed through a previously described method [18]. H_2O_2, by catalase, is broken down to oxygen and water and it is presented in the ultraviolent spectrum as reduced absorbance. At 240 nm, H_2O_2 reaches maximum absorbance. This absorbance reduction is directly proportional to CAT enzymes activities.

2.7. Determination of Malondialdehyde (MDA) level

Determination of Malondialdehyde (MDA) level principle was based on thiobarbituric acid (TBA) and MDA reaction at 95 °C [19]. TBA and MDA react at 532 nm absorbance to form pink pigment. For 15 min, the reaction was performed at pH 2–3 and sample mixed (2.5 vol of 10% (w/v) trichloroacetic acid) to precipitate protein. Through centrifugation, precipitate was pelleted and supernatant reacted with TBA (0.67%) for 15 min in boiling water-bath. Absorbance was read after 15 min at 532 nm. Values were compared with standard solutions (1,1,3,3-tetramethoxypropane) and results expressed in μM.

2.8. Determination of Nitric oxide (NO) and C-reactive protein (CRP)

Level of nitrite was calculated being the main product of NO oxidation in aqueous solution and this was used to determine plasma NO. Sulphanilic acid is converted by reaction with nitrite acid solution quantitatively to diazonium salt. This forms azo dye through coupling of *N*-(1-naphthyl) ethylenediamine and can be quantified at 548nm absorbance spectrophotometrically. C-reactive protein (CRP) was determined using ELISA and the procedure followed was in accordance with the manufacturer's instructions.

2.9. Gamma glutamyl transferase (GGT)

The test principle is based on substrate L-γ-glutamyl-3-carboxy-4-nitroanide. In the presence of glycylglycine, it is converted to 5-amino-2-nitrobenzoate by γ-GT and absorbance is measured at 405nm. The increase in absorbance is directly proportional to GGT activity. The test procedure was followed according to manufacturer's instruction.

2.10. Total thiol

The total thiol (total sulfhydryl groups) content was determined using a previously described method [20]. About 50μL of the sample was mixed with 0.6 ml of Tris–EDTA buffer, 40 μL of 10 mM DTNB in methanol. The final volume was made up to 1 ml by adding MeOH. The reaction mixture was incubated at room temperature for 20 min and the absorbance was measured at 412 nm. The content of total thiols was calculated using molar extinction coefficient of 13,600/M/cm.

2.11. Lipid profile and atherogenic index (AI) estimation

High density lipoprotein (HDL), triglycerides (TG) and total cholesterol (TC) were measured with commercially available kits in accordance with manufacturer's instructions. Low density lipoprotein cholesterol (LDL-c) was calculated using Friedwald's equation. LDL = TC – HDL – (TG/5) [21]. Very low density lipoprotein (VLDL) was calculated using this formula: VLDL = TG/5 and expressed as mg/dl and AI estimated as log (TG/HDL) [22].

2.12. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Statistically significant differences among groups were calculated using one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test for multiple comparisons. Statistical significance was defined as p < 0.05.

3. Results

3.1. DRLC mediated changes in antioxidants status

Administration of DRLC at both doses (125 and 250 mg/kg) significantly increased heart GSH and total thiol in normal male Wistar rats compared to control (Table 1). Serum GSH was only significantly elevated in the DRLC 250 group (P < 0.01) but not the DRLC 125 (p < 0.32) compared to the control, respectively. Heart GPx was only significantly elevated in the DRLC 125 group (P < 0.02) but not the DRLC 250 (p < 0.21) compared to the control, respectively (Table 1). Liver GGT was significantly decreased in DRLC 125 (4.2 ± 1.0) and DRLC 250 (1.6 ± 0.4) compared to control (10.9 ± 0.6). DRLC decreased MDA only in DRLC 250 (0.8 ± 0.1) compared to control (4.9 ± 0.8). DRLC also increased total thiol in DRLC 250 (5.4 ± 0.3) compared to control (4.1 ± 0.4).

3.2. Effects of DRLC on CRP, atherogenic index, and cardiovascular variables (AI) in normal Wistar rats

Figure 1A and B show the effects of DRLC treatment on AI and CRP respectively. There was reduction in the CRP of DRLC 250 (123.9 ± 5.3) compared to control (173.6 ± 6.1) P < 0.0001. DRLC 250 was also significantly decreased when compared to DRLC 125 (163.8 ± 5.2) P < 0.0009. AI of the DRLC 250 (0.07 ± 0.05) at P < 0.02 and DRLC 125 (0.12 ± 0.03) at P < 0.04 were both significantly decreased compared to the control (0.25 ± 0.04). However, there were no significant differences in body weight, NO, SP, DP and heart rate of both the DRLC 125 and 250 compared to the control (Table 2).

3.3. DRLC decreased LDL, TG and VLDL in serum

TC and HDL of the DRLC 125 and 250 were not significantly different when related to the control. TG of the DRLC 250 (16.5 ± 1.7) was
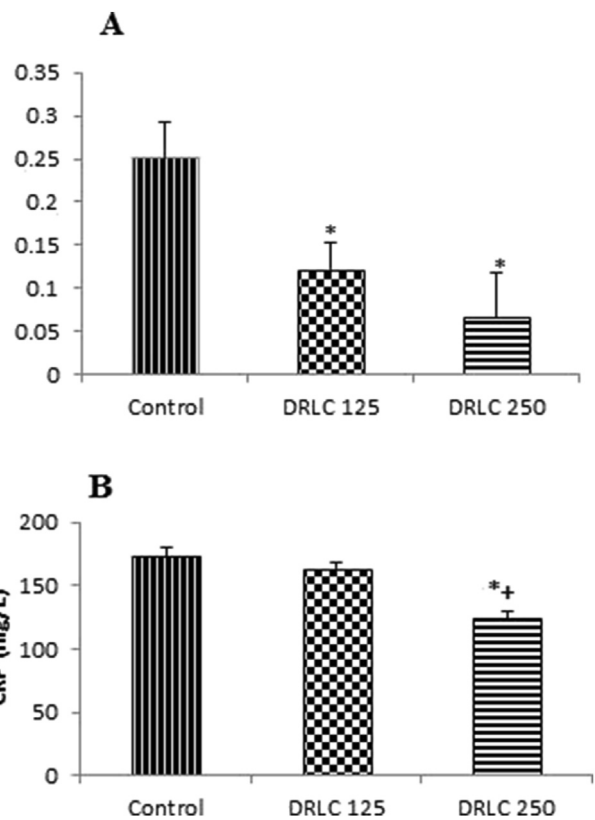

Figure 1. (A): Atherogenic index and **(B):** C-reactive protein in Control, DRLC (D-ribose-L-cysteine) 125 and DRLC (D-ribose-L-cysteine) 250 of Wistar rats after 8 weeks of DRLC treatment. n = 5. Values are expressed as Mean ± SEM, * significantly different from control (p < 0.05). + Significantly different from DRLC 125 (p < 0.05).

significantly decreased compared to the control (26.5 ± 0.7) at P < 0.0008 but the control was not significantly different from the DRLC 125 (24.1 ± 1.1). VLDL and LDL of DRLC 250 (3.3 ± 0.3; 102.3 ± 3.4, respectively) at P < 0.0008 and P < 0.03 were significantly decreased compared to the control (5.1 ± 0.1, 109.6 ± 0.9, respectively). The control was not significantly different from the DRLC 125 (4.8 ± 0.2; 105.5 ± 3.6, respectively) Figure 2.

4. Discussion

Antioxidants supplementations are now widely used because they are believed to prevent oxidative stress in cells. Oxidative stress is linked to the pathogenesis of various non-communicable diseases such as diabetes mellitus, cardiovascular diseases, dyslipidaemia and

Table 1. Comparison of serum reduced glutathione (GSH), serum Catalase (CAT), serum malondialdehyde (MDA), Heart GSH, Heart glutathione peroxidase (Heart GSH-Px), serum Total thiol and Liver Gamma Glutamyl Transferase (Liver GGT) after 8 weeks of DRLC administration.

Variables	Control	DRLC 125	DRLC 250
Serum GSH (mM)	2.2 ± 0.1	2.7 ± 0.2	3.3 ± 0.2*
Serum CAT (µmol/min/mL)	17.5 ± 0.3	20.6 ± 2.0	20.8 ± 1.9
Serum MDA (µM)	4.9 ± 0.8	2.9 ± 0.9	0.8 ± 0.1*
Heart GSH (mM)	5.8 ± 0.7	6.8 ± 0.8*	15.5 ± 1.5*+
Heart GPx (u/L)	1.9 ± 0.2	3.3 ± 0.3*	2.7 ± 0.4
Serum Total thiol (nmol/mg)	4.4 ± 0.9	8.8 ± 1.1*	14.2 ± 0.8*+
Liver GGT (u/L)	10.9 ± 0.6	4.2 ± 1.0*	1.6 ± 0.4*

Values are expressed as Mean ± SEM, * significantly different from control (p < 0.05).
+ Significantly different from DRLC 125 (p < 0.05).

Table 2. Comparison of Body weight, Nitric Oxide (NO), Systolic Pressure (SP), Diastolic Pressure (DP) and Heart rate after 8 weeks of DRLC administration.

Variables	Control	DRLC 125	DRLC 250
Body weight (g)	260.2 ± 4.1	259.4 ± 4.7	254.3 ± 9.8
NO (µM)	1.7 ± 0.2	2.7 ± 0.2	3.1 ± 0.3
Systolic pressure (SP) (mmHg)	130.2 ± 3.2	136.2 ± 2.4	134.4 ± 2.9
Diastolic pressure (DP) (mmHg)	99.0 ± 7.8	92.6 ± 3.4	96.8 ± 2.4
Heart rate (bpm)	272.8 ± 23.7	285.2 ± 13.3	301.8 ± 10.3

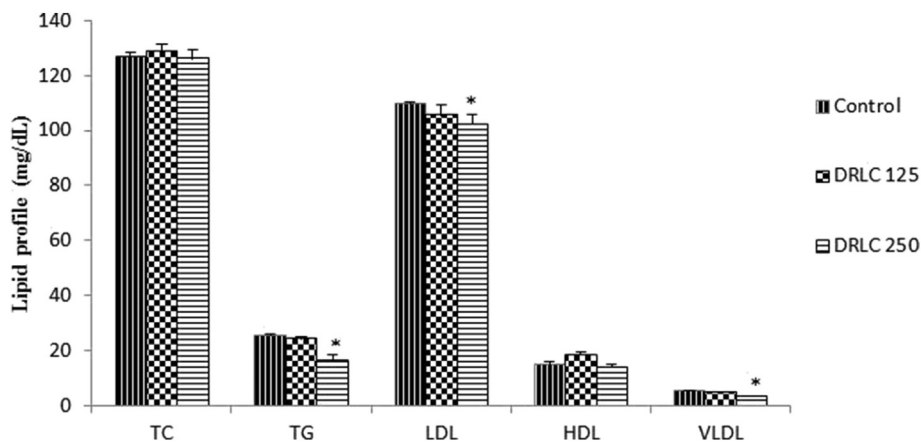Values are expressed as Mean ± SEM.

Figure 2. Lipid profile in Control, DRLC (D-ribose-L-cysteine) 125 and DRLC (D-ribose-L-cysteine) 250. TC = Total Cholesterol; TG = triglycerides; LDL = Low density lipoprotein; HDL = High density lipoprotein; VLDL = Very low density lipoprotein cholesterol in Wistar rats after 8 weeks of DRLC treatment. n = 5. Values are expressed as Mean ± SEM, * significantly different from control (p < 0.05).

neurodegenerative diseases as well as cancer [4, 23, 24, 25, 26]. GSH plays a vital role in regenerating other antioxidants (such as ascorbate, tocopherol, alpha lipoic acid and co-enzymes Q) and serves as a cofactor for GSH dependent enzymes (such as glutaredoxin or protein disulphide isomerase) [27, 28] subsequently increasing the body's protective enzymes against Reactive Oxygen Species (ROS) [29]. GSH supplementation through DRLC caused marked elevations in GSH level at both doses in cardiac tissue but was only elevated at DRLC 250 mg/kg in serum. This shows that even at reduced dosage, there was abundance of GSH in tissues. Usually, hepatocytes supply GSH that is found in plasma and plasma GSH is kept at low concentration because of metabolism of GSH by many other cells [30]. This makes glutathione to be generally more predominant in animal cells (0.5–10 mmol/L) than in plasma (2–20 µmol/L) [6, 30]. Furthermore, half-life of GSH in tissues varies. Previous finding demonstrated that 16 h after single DRLC administration at 2 g/kg in mice, there was increased and maintained elevation in heart GSH with increases equally found in other tissues as well [3]. This may also be the reason for the abundance of GSH in heart tissue in this study. GSH abundance in heart tissues reflects the protection DRLC administration can afford thus providing adequate protection against oxidative stress. Although, GSH pro-drug such as N-acetylcysteine (NAC) also increases GSH level, there are associated side effects. Higher dose of NAC (1.0 g/kg bw) was reported to be effective in increasing GSH levels but with adverse effects resulting from impulsive release of NAC in a toxic manner [3]. However, DRLC is released in a much slower and controlled manner that is facilitated through intracellular GSH synthesis and L-cysteine uptake by the liver [3, 7].

Thiols are compounds specific to sulfhydryl groups and play important roles in defense against free radicals. Decreased thiol contents have been linked with type 2 diabetes mellitus [31, 32], alcohol abusers [33], hypertension [34, 35] and oxidative stress [36] while increased thiol levels inversely correlates with these conditions [36]. In this study, thiol increases illustrates evidence of supplementation with DRLC and also

shows the protection this affords against reactive oxygen species and oxidative stress. Gamma glutamyl transferase (GGT) determination has been used to assess liver functions [37]. Aside this, GGT also serves the important role of metabolizing GSH extracellularly thereby resisting GSH intracellular degradation [36,37]. This implies that decrease in hepatic GGT illustrates that GSH degradation is tightly regulated and well controlled by DRLC supplementation. In addition, GGT activities illustrates there was no damage to liver cells after prolonged exposure which depicts a safety profile of DRLC. Furthermore, GSH degradation requires GGT as it is the only enzyme that initiates catabolism of GSH and GSH adducts (such as GSSG, glutathione S-conjugates, and glutathione complexes) in the extracellular space [38]. This agrees with evidences from previous studies that GSH maintains antioxidants homeostasis by regulating the release of DRLC in a pulsatile fashion for GSH synthesis [3].

These findings also showed that DRLC could decrease serum triglycerides in normal Wistar rats. Hypertriglyceridemia, an independent risk factor for coronary atherosclerosis, is well correlated with high LDL and linked to increased risk of cardiovascular diseases [39, 40]. There was reported decrease in TC and TG levels in Wistar rats fed 30 mg/kg DRLC for a month [11]. Although, LDL level was unchanged, VLDL and AI were not determined in that study, the result from this finding is in concordance with DRLC ability in decreasing dyslipidaemic indices. Decreases observed in TG, LDL and VLDL at 250 mg/kg in this study may be associated with the longer duration of exposure and increased dose administered to the animals. VLDL formation in liver involves a lipidation process through apolipoprotein B. In a previous study, decrease in cholesterol and LDL was reported to be associated with increased catabolism of LDL mechanistically resulting from catabolism of LDL receptors and apolipoprotein B [7]. Similarly, it could be inferred there are possibilities that high dose of DRLC prevented the lipidation of VLDL and TG through apolipoprotein B and LDL receptors in this study. Moreover, reduction in TG level shows potentiating ability of DRLC in decreasing CVD risks. This also supports the decrease observed in AI. Atherogenic

index (AI) increase correlates with lipoprotein size and it is an indicator of atherogenic lipoprotein status [41]. AI predicts cardiovascular risk factors and coronary events. Observed decrease in AI after long term administration in normal animals showed DRLC may decrease cardiovascular risk by decreasing VLDL and triglycerides in normal rats at both doses.

C-reactive protein is secreted in response to inflammation and it also serves as a predictor of cardiovascular diseases (CVD). As CRP elevation is associated with CVD, its reduction in serum suggests reduced risk of developing CVD [42]. The observed ability of DRLC to reduce CRP at 250 mg/kg in this study indicates that it could reduce the risk of developing CVD. In a previous study, CRP decrease was reported to be dose-response dependent as humans with higher intake of fruits and vegetables were 65% less likely to have higher plasma CRP and homocysteine concentrations [43]. Moreover, CRP proatherogenic action is well documented in literatures because of its specific binding to LDL and VLDL [44, 45, 46]. This may be linked to decrease observed in the TG, VLDL and AI in this study.

There were no changes in NO concentration and blood pressure after eight weeks of DRLC supplementation in normal Wistar rats at both doses. This may also be due in part to decreases observed in serum CRP and AI at both doses because elevated CRP and AI depict proatherogenic state [46]. CRP plays pivotal roles in atherogenesis and it inhibits endothelial NO synthase expression (which is responsible for NO production) in endothelial cells [42]. Though these were in normal rats fed with dietary supplements, it is not expected that they observe decreases in blood pressures but low circulating CRP and AI (which are predictive risk factors for incidence of cardiovascular diseases) suggest a protective role from DRLC. There are possibilities that in hypertensive states, DRLC may decrease blood pressures and other cardiovascular risk factors. As it has been shown that through increased GSH synthesis, DRLC decreased dyslipidaemic and atherogenic indices. Thus, changes observed in this study may be attributed to increases in cellular antioxidants.

5. Conclusion

Although, there are positives resulting from prolonged administration of DRLC 125 mg/kg supplementation, increased dose (250 mg/kg) may possess the ability to yield better responses without instigating adverse effects in healthy animals. Also, there are indications that supplementation with DRLC 250 mg/kg may enhance decrease in triglycerides, atherogenic index and C-reactive protein, thereby sustaining cardio functions in physiologic states.

Declarations

Author contribution statement

Abodunrin Adebayo Ojetola: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Temitope Gabriel Adedeji: Analyzed and interpreted the data; Wrote the paper.

Adesoji A. Fasanmade: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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