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Improvement in kidney damage resulting from stanozolol intramuscular injections in rats by Natural Cocoa Powder (NCP) administration

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A B S T R A C T

Background: Stanozolol, an anabolic steroid, has been shown to induce kidney damage through mechanisms involving oxidative stress and inflammation. Natural Cocoa Powder (NCP), known for its high antioxidant content, may offer protective effects against such damage.
Aim: This study aimed to evaluate the potential of Natural Cocoa Powder (NCP) in ameliorating kidney damage caused by stanozolol intramuscular injections in rats.
Methods: Thirty rats were randomly assigned into five groups (G1–G5) and fed with standard rat chow. G1 and G3 received 2 mg/kg stanozolol intramuscularly twice weekly, G2 and G4 received 5 mg/kg stanozolol twice weekly, while G5 served as the control with no stanozolol. G1, G2, and G5 had 24 h access to water, while G3 and G4 had water replaced with 2 % NCP from 6 am to 6 pm daily. After 8 weeks, kidneys were perfusion-fixed and analyzed histomorphometrically for proximal/distal tubules, Bowman's space, glomerular tuft, and renal corpuscle composition. Before and after treatments, renal function was measured by serum creatinine and blood urea nitrogen, whilst systemic inflammation was monitored by erythrocyte sedimentation rate and serum C-reactive protein tests.
Results and Discussion: Stanozolol administration significantly increased kidney damage in a dose-dependent manner, with marked rises in the volume densities of the proximal convoluted tubule (PCT), distal convoluted tubule (DCT), glomerular tuft, and renal corpuscle. At higher doses, PCT and DCT volume densities increased by 143 % and 223 %, respectively, compared to controls. Natural cocoa powder (NCP) mitigated these effects, reducing PCT, DCT, and glomerular tuft volume densities by 92 %, 77 %, and 86 %, respectively. Stanozolol also elevated serum markers of kidney dysfunction and inflammation, such as blood urea nitrogen (BUN), serum creatinine

Abbreviations: AAS, Anabolic Androgenic Steroids; BUN, Blood Urea Nitrogen; CRP, C-Reactive Protein; DCT, Distal Convoluted Tubules; ESR, Erythrocyte Sedimentation Rate; GFR, Glomerular Filtrate Rate; H&E, Haematoxylin and Eosin; NCP, Natural Cocoa Powder; NO, Nitric Oxide; PAS, Periodic Acid Schiff; PCT, Proximal Convoluted Tubule; ROS, Reactive Oxygen Species.

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(SCr), and C-reactive protein (CRP). While NCP significantly lowered BUN and SCr levels, it did not fully normalize CRP, which remained elevated in stanozolol-treated rats.
Conclusion: Increased volume density of kidney components indicated hypertrophy, which was associated with elevated serum creatinine, blood urea nitrogen (BUN), and C-reactive protein (CRP) levels, reflecting impaired renal function and heightened systemic inflammation. The reduction in these markers with NCP ingestion suggests its potential to mitigate stanozolol-induced renal damage. It is proposed that the antioxidant properties of NCP may have reduced inflammation by counteracting oxidative stress, thereby contributing to the observed improvement in kidney function and structure.

Introduction

Stanozolol is classified among the anabolic androgenic steroids (AAS). AAS are steroidal androgens (natural or synthetic) that are structurally related to and have similar effects as testosterone [6,20]. The discovery in the 1930s of the testicle extract testosterone, a more powerful androgen than androsterone, initiated the widespread scientific inquiry into the effects of AAS on strength and muscle building [26]. AAS are commonly abused as performance-enhancing substances among both professional athletes and recreational bodybuilders [22,31]. This has been reported to be approximately 10.0 %, 3.9 % and 20.0 % in the United States [9], Sweden [17] and Iran, [22] respectively.

AASs are desirable in some medical conditions such as chronic infections, extensive surgery, corticosteroid-induced myopathy, decubitus ulcers, burns, or severe trauma, which require reversal of catabolic processes or protein-sparing effects [6] and for treating C1-inhibitor deficient hereditary angioedema [31]. Despite the health benefits, stanozolol often result in glomerulopathy, marked proteinuria and hypertension following a short period of use, particularly when abused [4,21]. Chronic AAS use is associated with deleterious alterations in body tissues and organs of the renal, cardiovascular, nervous and endocrine systems [5,13]. In rats, intramuscular injection of 10 mg/kg body weight of Stanozolol once a week for a 12-week period negatively affected urinary pH and kidney morphology leading to a worse renal status [5].

The kidney is vulnerable to AAS insults because the drug may be metabolized into potentially toxic byproducts and highly subject to glomerular filtration [19,25]. Relevantly, nephrologists estimate the proportion of AAS users that become long-term abusers and are more likely to develop clinically significant kidney disease. Kanayama et al. [14] reported that approximately 30 % of AAS users develop dependence and therefore would be at a higher risk of developing the medical consequences of protracted AAS abuse. Indeed, AAS have been implicated in the kidney oxidative stress, inflammation and fibrosis [30], and Pellegrino et al. [24] posited that kidney oxidative stress results from accumulation of free radicals such as reactive oxygen species (ROS).

The present study was provoked in part by investigators who experimentally prevented or minimized effects of ROS and free radicals in inflammatory processes by use of high antioxidant-containing substances [35]. Chemicals and medicinal plants with high antioxidant levels such as selenium and *Sonchus asper*, have been explored for their potential in attenuating the nephrotoxic effects of ROS [15,35]. *Theobroma cacao* (cocoa) is among plants known to contain very high variety and concentrations of antioxidants [8,10,16]. Natural cocoa abounds in polyphenols which include catechins, epicatechin and proceyanidins, and is known to have anti-inflammatory activity [23]. The high levels of flavanols in natural cocoa powder (NCP) and their demonstrated ability to scavenge free radicals have been cited for extenuation of inflammation and minimization of tissue damage in experimental diseases [2,8,32].

Despite cocoa's recognized antioxidant properties, the potential of NCP for nephroprotection in AAS-induced kidney damage has not been previously evaluated. The present study aims to evaluate the potential of prandial Natural Cocoa Powder (NCP) in ameliorating kidney damage caused by stanozolol intramuscular injections in rats. This is in alignment with the World Health Organization's Sustainable Development Goal 3 (SDG 3) to ensure healthy lives and promote well-being for all at all ages.

Materials and methods

Acquisition and acclimatization of rats

Ethical approval for the study was obtained from the University of Ghana College of Health Sciences Ethical and Protocol Review Committee (CHS-Et/ M.8- P20.9/ 2017–2018). Thirty (30) male Sprague Dawley rats 14–16 weeks old, weighing between 140 g and 220 g were acquired from the Animal Experimentation Unit of the School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle-Bu Campus. Only male rats were used because they have no lunar hormonal cycle to confound the experiment. The rats were kept in the Animal Experimentation Unit throughout the study with an average temperature of 28±2 °C, a relative humidity of 80±2 % and a 12 h light/dark cycle. The animals were fed with standardized rat chow and water for a week to acclimatize. After acclimatization, the animals were weighed and randomly put into 5 groups (G1-G5) with 6 animals in each group. Each rat was kept in a rat cage of dimension 20.3 cm × 28.7 cm × 17.3 cm. Animals were weighed weekly.

Preparation and administration of 2 % (w/v) Natural Cocoa Powder (NCP)

Unsweetened natural cocoa powder (GoodFood®, KEL Kakawa Company Ltd. Accra, Ghana) with batch number KK1801A was

used. Aqueous NCP was prepared and administered daily as described by Sokpor et al. [32]. Eight grams (8.0 g) of NCP was dissolved in 400 ml of freshly boiling-hot tap water to make 2 % (w/v) NCP. The solution was allowed to cool to room temperature before being made available to the rats. The concentration used has proved efficacious in previous studies in our laboratory and the detailed composition of NCP is already published [2,8,32].

Stanozolol administration and treatment of rats

A pilot study was done to confirm that 2.0 and 5.0 mg/kg body weight stanozolol [34] would cause kidney injury observable at the light microscope level within 8 weeks. Groups G1 and G3 received 2 mg/kg stanozolol (Sigma-Aldrich, Germany) intramuscular injection twice weekly at three-day intervals. Groups G2 and G4 received 5 mg/kg stanozolol intramuscular injection twice weekly at three-day intervals. Stanozolol was injected into the gluteus maximus muscle of rats. In addition, G1 and G2 received 500 ml of clean tap water for 24 h *ad libitum*. Groups G3 and G4 were given unrestricted access to freshly prepared 400 ml of 2 % (w/v) NCP (in a graduated drinking bottle) for 12 h from 6 am to 6 pm, and then followed by 500 ml of clean tap water for 12 h (6 pm to 6 am). The normal/control group (G5) was given 500 ml of clean tap water for 24 h. All groups were given standard rat chow. The treatment was done for 8 weeks for all the five groups. In clinical settings, stanozolol is often prescribed in doses ranging from 2 to 4 mg daily for therapeutic use. However, in the context of performance enhancement, users may exceed these doses, with reported abuses reaching upwards of 20 mg per day [18]. Thus, the selected doses were intended to evaluate both lower therapeutic levels and higher doses that reflect potential misuse in athletes.

Blood sampling and biochemical assays

Blood samples were collected from rats before commencement (baseline) and at termination of treatments (8 weeks) for biochemical analysis. Blood samples were taken by cardiac puncture after anaesthetizing the animals by diethyl ether inhalation. About 1.5 ml of blood was collected from each animal into plain gel separation blood sample bottles, kept in an ice chest and transported to the Medical Biochemistry Department of the University of Ghana Medical School for analysis. The samples were left to stand for 30 min at room temperature to facilitate clotting and serum separation. Samples were centrifuged at 3000 rpm for 5 min, and the serum collected into labelled Eppendorf tubes. A fully automated Vitros 5.1/Fs Chemistry Analyzer (serial number: 34,000,615) was used to measure the serum levels of selected biochemical markers of renal function – creatinine, blood urea nitrogen (BUN) and inflammation, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)

Kidney harvest and tissue processing

Following week 8 blood sampling, the animals were sacrificed. Rats were anaesthetized via chloroform inhalation for perfusion fixation with 10 % neutral buffered formalin (pH = 7.24–7.28) after flushing the system with approximately 250 ml of 0.9 % saline. Effective fixation was affirmed when all muscles ceased twitching and rat became stiff. The anterior abdominal wall was incised, and the kidneys harvested. The kidneys were weighed using a chemical balance and their volumes determined via water displacement. They were then fixed by immersion in 50 ml of 10 % buffered formalin for a week before tissue processing for light microscopy. The left kidney was cut into two longitudinal slices 0.20–0.25 mm thick. The right kidney was cut transversely into approximately five slices 0.20–0.25 mm thick. The 1st, 3rd and 5th slices of the right kidney were picked for tissue processing. A total of five kidney slices were obtained for each rat, which were routinely processed and embedded in paraffin wax (melting point 56 °C). Tissue blocks were trimmed and sectioned at 5 μm using a rotary microtome (Leica RM 2125, Germany). Three sections 20 μm apart were picked from each tissue block. One section of each kidney block was picked for Haematoxylin and Eosin (H&E) staining. Two sections per block were picked for Periodic Acid Schiff (PAS) staining. A total of 15 microtomy sections were obtained for each rat for stereological studies.

Light microscopy

Microscope fields containing the renal corpuscles and tubules were captured (using × 40 objective lens) onto a computer (HP Compaq dx2300 Microtower) with the aid of a digital eyepiece (Lenovo Q350 USB PC Camera) connected to a Leica Galen III (catalogue no. 317,506, serial no.1207XU) light microscope. Six micrographs were systematically sampled [32] from each of the 15 microtomy sections obtained from each rat. Therefore, a total number of 90 micrographs were obtained per animal for histomorphometric assessment.

Volume density estimation for renal corpuscles, PCTs and DCTs

The volume density of the renal corpuscle (including glomerular tufts and Bowman's space) were determined using point counting with Cavalieri principle [11]. Using Adobe Photoshop CS6 Extended (version 13.0.1) software, a stereological grid consisting of uniformly spaced points, 1 cm × 1 cm was superimposed over each micrograph of the renal corpuscle. The number of test points that hit glomeruli and Bowman's space were counted for each renal corpuscle. The volume densities of the Bowman's space and glomeruli (VG) were calculated using the equation below:

$$Vv = \left(a \Big/ p \sum P\right) \times t \Big/ M^2$$

Where V indicates volume, ΣP is the sum of all test points encountered, (a/p) is the area per point of the stereological grid, t is the thickness of the section and M is the linear magnification [11]. The same equation was used to calculate the volume densities for PCTs and DCTs.

Data analysis

Graph Pad Prism Version 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Levene's test for homogeneity was done to determine normality of all data. One-way analysis of variance (ANOVA) and Tukey's posthoc *t*-test were used to determine the differences in mean values between groups. Statistically significant differences had $p < 0.05$.

To enhance evaluation of stanozolol dose-dependency on severity of renal damage and degree of amelioration by prandial NCP for each histological variable, comparison of lower and higher doses studied was obtained by subtracting the value for G5 (control) rats from corresponding Group value (G1, G2, G3, or G4), and expressing the difference as a percentage of the control value. Similarly, the measured biochemical markers were further analyzed by first determining the 'before' and 'after' treatment differences. Thereafter, the relative (before & after) change with respect to control (G5) rats was determined by the equation: $\left[\frac{Gn-G5}{G5}\right]x$ 100. Where G means group; and n = 1,2,3, or 4.

Results

Volume densities of renal corpuscle, PCT and DCT

Representative of systematically random samples of morphology of renal corpuscles, PCTs and DCTs in the different rat groups (G1-G5) are shown in Figs. 1 and 2. Quantitative data on the kidney histovolumetric variables assessed is presented in Table 1. Tukey's post hoc indicated significant differences in the histomorphometry between the groups (Table 1). PCT volume densities of G1 and G2 rats were significantly higher in comparison with G5 ($p < 0.0001$). G4 also had a significant increase in PCT volume at 95 % confidence level ($p < 0.001$). However, no statistically significant difference was observed in G3 when compared to G5. Similar trends were observed for DCT, glomerular tuft and renal corpuscle volume densities (Table 1). ANOVA between the groups did not reveal any significant difference in the volume density of Bowman's space ($p = 0.6580$).

White arrows indicate profiles of DCTs, block white crosses show profiles of PCT. G1 rats were given 2 mg/kg stanozolol intramuscular injection twice weekly and drinking tap water. G2 rats were given 5 mg/kg stanozolol intramuscular injection twice weekly and drinking tap water. G3 rats were given 2 mg/kg stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G4 rats were given 5 mg/kg stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G5 rats were Normal/Control and were neither given stanozolol injections nor NCP and had drinking tap water. All treatments lasted 8 weeks.

White double headed arrows indicate glomerular tufts, White single headed arrows show PCTs, and asterisks represent renal (Bowman's)

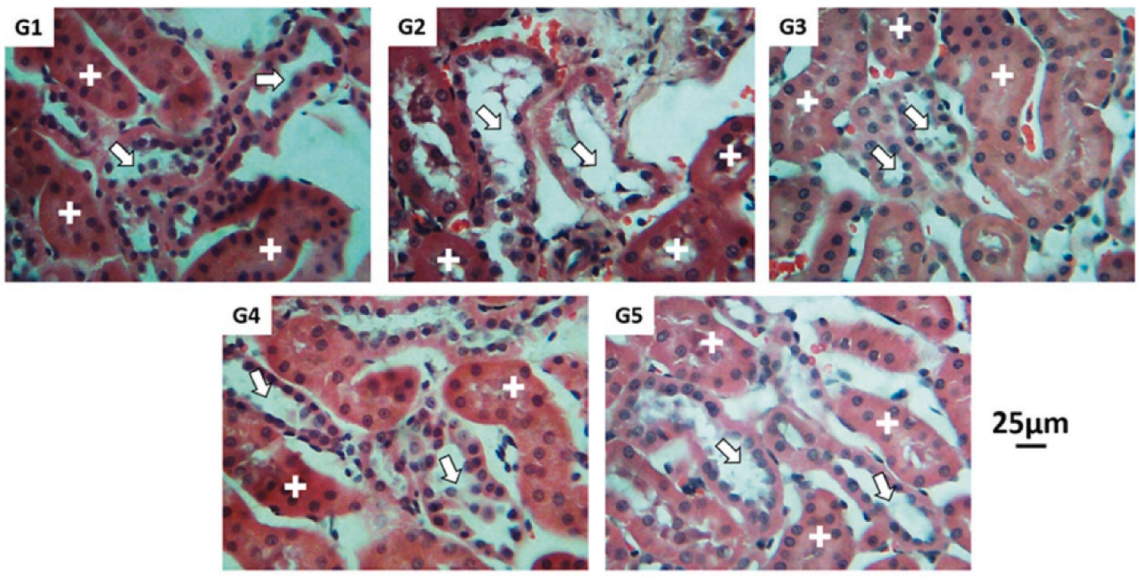

Fig. 1. Photomicrographs of representative sections of kidney tissue from the experimental groups. H & E stain.

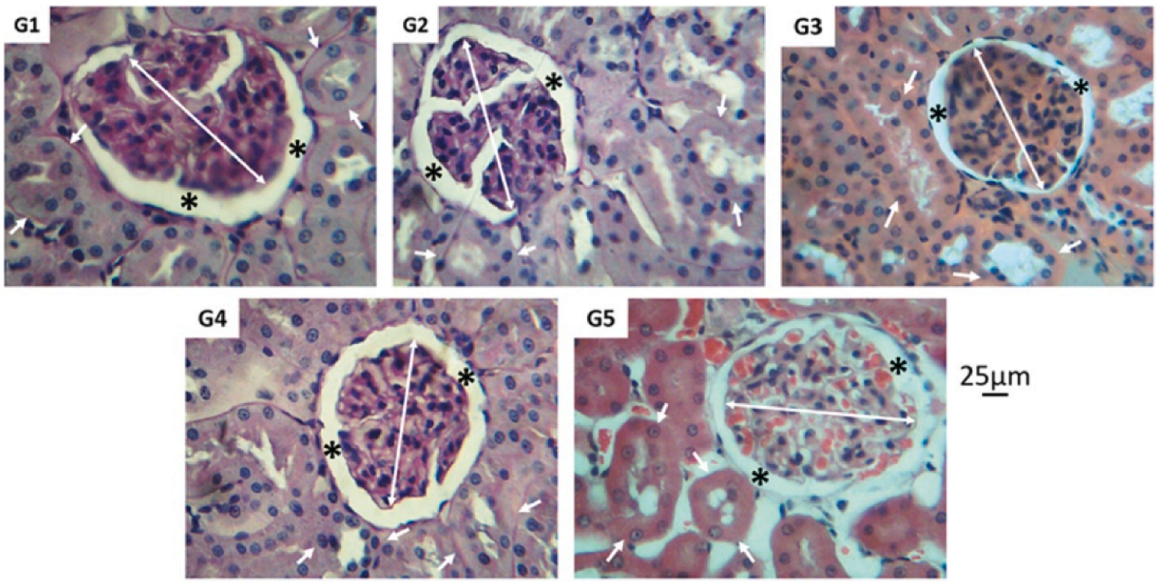

Fig. 2. Photomicrographs showing representative PAS-stained sections of kidney from experimental groups.

Table 1
Summary of mean volume densities of renal variables assessed by morphometry and statistical comparisons.

Morphometric Variable	G1[a]	G2[b]	G3	G4[c]	G5	P value
PCT (x 10^2 µm^3)	11.5 (SD 0.6) ***	14.8 (SD 0.6) ***	7.5 (SD 0.1)	9.2 (SD 0.4) **	6.1 (SD 0.3)	<0.0001
DCT (x 10^2 µm^3)	3.46 (SD 0.5) *	4.2 (SD 0.3) **	1.7 (SD 0.4)	3.2 (SD 0.3) *	1.3 (SD 0.1)	0.0011
Bowman's Space (x 10^2 µm^3)	1.7 (SD 0.4)	1.9 (SD 0.6)	1.2 (SD 0.3)	1.5 (0.3	1.1 (SD 0.3)	0.6580
Glomerular Tuft (x 10^2 µm^3)	4.4 (SD 0.2) **	5.6 (SD 0.2) ***	2.7 (SD 0.2)	3.6 (SD 0.2) *	2.3 (SD 0.2)	<0.0001
Renal Corpuscle (x 10^2 µm^3)	5.8 (SD 1.0) *	7.3 (SD 0.6) **	3.8 (SD 0.2)	5.1 (SD 0.1	2.4 (SD 0.7)	0.0041

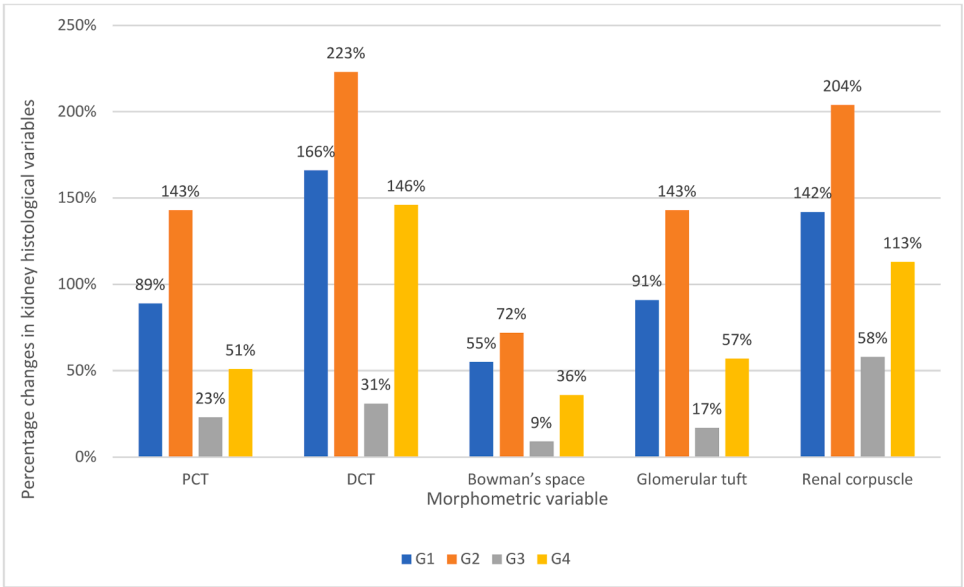

Fig. 3. Dose-dependence of stanozolol-induced kidney damage indicated by percentage changes in kidney histological variables assessed in rat treatment groups compared with values for control (G5) animals presented in Table 1.

space. Tissue limited externally by the renal space (asterisks) and glomerular tuft (double headed arrow) constitute the renal corpuscle. G1 rats were given 2 mg/kg stanozolol intramuscular injection twice weekly and drinking tap water. G2 rats were given 5mg/kg stanozolol intramuscular injection twice weekly and drinking tap water. G3 rats were given 2 mg/kg stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G4 rats were given 5 mg/kg stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G5 rats were Normal/Control and were neither given stanozolol injections nor NCP and had drinking tap water. All treatments lasted 8 weeks.

P value represents significant level for the one-way ANOVA (followed by Tukey's posthoc test) for between groups comparison with *=$p < 0.05$; **=$p < 0.001$ and ***=$p < 0.0001$. Superscript a= G1 v G5; b= G2 v G5; c= G4 v G5. PCT is proximal convoluted tubule; DCT is distal convoluted tubule; and SD stands for standard deviation. *G1 rats were given 2 mg/kg stanozolol intramuscular injection twice weekly and drinking tap water. G2 rats were given 5mg/kg stanozolol intramuscular injection twice weekly and drinking tap water. G3 rats were given 2 mg/kg stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G4 rats were given 5 mg/kg stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G5 rats were Normal/Control and were neither given stanozolol injections nor NCP and had drinking tap water. All treatments lasted 8 weeks.*

Fig. 3 shows synopsis of relative changes in assessed histomorphological variables with respect to higher and lower dose stanozolol with and without prandial NCP, when each treatment group's values were compared with corresponding values for control (G5) animals. For all 5 histological variables studied, lower dose stanozolol produced less increases in volume density than higher dose for rats in groups that did not drink NCP (G1 and G2) and those that did (G3 and G4) (Fig. 3).

Higher dose stanozolol without NCP (G2) minus lower dose stanozolol without NCP (G1) produced respective percentage increase of 55, 57, 17, 52, and 62 in PCT, DCT, Bowman's space, glomerular tuft, and renal space. Similar arithmetic indicated volume density increase of 28 % (PCT), 11 % (DCT), 27 % (Bowman's space), 40 % (glomerular tuft), and 55 % (renal corpuscle) for higher dose stanozolol with NCP ingestion relative to lower dose with NCP (G4-G3). Thus, apart from Bowman's space higher dose stanozolol produced smaller percentage increases in volume composition when rats drank cocoa than when they did not.

For lower dose stanozolol (G1 minus G3), NCP consumption decreased volumetric composition as follows; 66 % (PCT), 135 % (DCT), 46 % (Bowman's space), 74 % (glomerular tuft), and 84 % (renal corpuscle). At higher dose stanozolol (G2 minus G4) NCP ingestion decreased PCT (92 %), DCT (77 %), Bowman's space (36 %), glomerular tuft (86 %), and renal corpuscle (91 %) compared with that produced by the same dose without NCP intake. Thus, drug damage was reduced by NCP ingestion to a greater percentage for PCT, glomerular tuft, and renal corpuscle but not for DCT and Bowman's space.

PCT-proximal convoluted tubule, and DCT stands for distal convoluted tubule. NCP-natural cocoa powder. *G1-rats given 2 mg/kg (lower dose) stanozolol intramuscular injection twice weekly and drinking tap water. G2- rats given 5mg/kg (higher dose) stanozolol intramuscular injection twice weekly and drinking tap water. G3- rats given 2 mg/kg (lower dose) stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G4-rats given 5 mg/kg (higher dose) stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h per day. G5- rats Normal/Control and were neither given stanozolol injections nor NCP and had drinking tap water. All treatments lasted 8 weeks.*

Taken together the above results show the following: (1) Renal damage associated with stanozolol administration was evidenced by significant increases in volume density of PCT, DCT, glomerular tuft, and renal corpuscle but not Bowman's space. However, NCP ingestion resulted in significant reduction in percentage increases in volume composition of studied kidney components. (2) Higher stanozolol dose caused greater increases in kidney volume components with and without NCP consumption than lower drug dose, suggesting expected accentuation of damage by more stanozolol molecules at higher dosage. However, the percentage reduction in stanozolol-induced elevation of kidney component volumes associated with NCP ingestion was greater at higher stanozolol dose than at lower dose. Given the fixed NCP concentration (2 %) and volume available to rats, the apparently improved amelioration at higher drug dose suggests ideal modulating effect.

Serum markers of kidney function and inflammation

Baseline levels for each of the serum markers for kidney (BUN, SCr) and inflammation (CRP and ESR) were not significantly different among the five groups of rats (supplementary data 1). After various treatments however, unpaired *t*-tests of all four biochemical variables yielded significantly higher values in G1 and G2 rats that did not drink NCP in comparison with untreated control rodents (G5). Rats that drank NCP (G3 and G4) had after-treatment values of serum markers that were not significantly different from control (G5) values, except for higher dose stanozolol treated (G4) rats whose CRP of 4.50 mg/l (SE 0.22) was statistically higher (P =0.024) than the 3.5 mg/l (SE 0.22) for control (G5) rats. The serum C-RP after-treatment value for lower-dose Stanozolol without NCP (G1) was 7.5 mg/l (SE 0.22); higher-dose Stanozolol without NCP (G2) was 9.83 mg/l (SE 0.65); and lower-dose Stanozolol with NCP (G3) was 4.16 mg/l (SE 0.31). Unpaired *t*-test comparisons with the control group (G5), yielded probabilities of 0.001 (G1), 0.0002 (G2), and 0.994 (G3). Similarly, for serum BUN comparison with control value gave a significant increase in the mean concentration of G1 [8.42 mmol/L (SE 0.23); $p = 0.0017$] and G2 [7.93 mmol/L (SE 0.23); $p = 0.0009$] using unpaired *t*-test. There was no statistically significant difference in mean BUN concentrations for G3 [5.95mmol/L (SE= 0.32); $p = 1.199$] and G4 [6.06 (SE 0.34); $p = 1.039$] when compared with G5[5.90 mmol/l (SE 0.34)].

SCr significantly increased at the end of treatments for G1 [93.30 μmol/L (SE 2.26); $p = 0.0009$] and G2 [103.00 μmol/L (SE 3.70); $p = 0.0004$] in unpaired *t*-test comparison with G5. There was no statistically significant difference in mean SCr concentrations for G3 [75.17 μmol/L (SE= 2.89); $p = 0.0764$] and G4 [70.83 μmol/L (SE 3.49); $p = 0.1205$] when compared with G5 [70.00 μmol/l; SE 3.7]. ESR after the treatments, significantly increased for G1 [1.31 (SE 0.01) mm/h; $p = 0.0006$] and G2 [1.53 (SE= 0.01) mm/h; $p = 0.0001$] using unpaired *t*-test comparison with G5. There was no statistically significant difference in mean ESR for G3 [1.015 mm/h

(SE 0.04); $p = 0.2420$] and G4 [1.05 mm/h (SE 0.04); $p = 0.1920$] when compared with G5 [0.97 mm/h (SE 0.04)]

Table 3 shows changes in the measured biochemical markers before and after treatment with lower and higher dose stanozolol with and without prandial NCP (Dataset A). Lower dose stanozolol produced less percentage increase in serum markers for kidney function (BUN & SCr) and systemic inflammation (ESR & CRP) than higher dose for animals (G1 and G2) that did not drink NCP (Table 3, Dataset A). Although differences were not significant, Table 3 Dataset A indicated that the trend for animals that drank NCP (G3 and G4) was greater percentage change in kidney function markers for lower stanozolol than higher dose.

The relative (before & after) change with respect to control (G5) rats determined by the equation: $\left[\frac{Gn-G5}{G5}\right]x\ 100$ is presented as Dataset B in Table 3. Strikingly, inflammatory markers showed 2 to 5-fold greater percentage increases than renal function markers (Table 3, Dataset B). Table 3 further confirms that for animals that drank NCP (G3 and G4), percentage increases were greater for lower dose stanozolol in serum BUN and creatinine but smaller for ESR and CRP. Table 3, Dataset B shows that in all 4 serum markers studied, lower dose stanozolol produced smaller percentage increases in serum markers for kidney function and systemic inflammation than higher dose for animals that did not drink NCP (G1 and G2).

To more clearly evaluate how changes in serum biochemical markers were impacted by lower/higher dose stanozolol, corresponding percentage changes in Table 3 Dataset B were obtained by simple subtraction. For rats that did not drink NCP, values for higher dose (G2) minus values for lower dose (G1) yielded the respective increases of 140 % (BUN), 113 % (SCr), 1900 % (ESR), and 488 % (CRP). Higher stanozolol dose-related changes (G4 minus G3) in rats that drank NCP were BUN (negative 24 %), SCr negative 26 %), ESR (100 %), and CRP (206 %). Thus, lower dose stanozolol without NCP ingestion elicited greater percentage increases in biochemical variables than higher dose stanozolol with NCP ingestion. Arithmetic determination of impact of NCP ingestion was obtained by subtracting smaller percentage changes recorded for stanozolol treatment with prandial cocoa (Table 3, columns 4 and 5) from corresponding greater values obtained without cocoa intake (Table 3, columns 2 and 3). For lower dose stanozolol, serum markers without NCP (G1) minus values with NCP (G3) gave decreases of 696 % (BUN), 311 % (SCr), 3370 % (ESR), and 1012 % (CRP). Higher dose stanozolol without NCP (G2) minus values with NCP (G4) yielded decreases in serum markers of 866 % (BUN, 450 % (SCr), 5170 % (ESR), and 1294 % (CRP). Thus, NCP apparently exerted greater modulating effect evidenced by greater percentage decreases in biochemical markers at higher than lower dose stanozolol.

For each Biochemical marker, Data set A represents the difference between mean values at baseline and end of treatment (8 weeks) for each group of rats; Data set B represents the relative (percentage) change in value compared to the control (group 5) obtained as $\left[\frac{Gn-G5}{G5}\right]x100$ *. Where n is 1,2,3, or 4 for data in Table 1.*

G1 rats were given 2 mg/kg (lower dose) stanozolol intramuscular injection twice weekly and drinking tap water. G2 rats were given 5mg/kg (higher dose) stanozolol intramuscular injection twice weekly and drinking tap water. G3 rats were given 2 mg/kg (lower dose) stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h and water12 h per day. G4 rats were given 5 mg/kg (higher dose) stanozolol intramuscular injection twice weekly and drinking 2 % NCP 12 h and water 12 h per day. G5 rats were Normal/Control and were neither given stanozolol injections nor NCP and had drinking tap water 24 h. All treatments lasted 8 weeks.

In a nutshell, assays of biochemical markers in this study revealed the following. (1) Percentage increases caused by stanozolol administration in biochemical markers for kidney function and systemic inflammation were greater than percentage increases in histovolumetric composition. Also, percentage increases caused by stanozolol administration in biochemical markers for systemic inflammation was 2- to 5-fold greater than for markers of kidney function. (2) Percentage reduction in stanozolol-induced increases in some renal function and all biochemical markers was greater at higher drug dose than at lower dose when rats ingested NCP.

Discussion

This study sought to determine whether stanozolol-induced kidney damage would be mitigated by NCP ingestion owing to antioxidant and anti-inflammatory properties of component dietary nutrients. It also assessed how lower dose (2 mg/kg) and higher dose (5 mg/kg) impacted variables studied.

The volumetric component increases resulting from stanozolol administration may be ascribed mainly to hypertrophy. It is postulated that stanozolol caused increased intraglomerular pressure to induce glomerular and tubular hypertrophy [39]. In an excellent and extensive review of the pathophysiology of acute kidney injury, Basile and co-workers [7] proffered that vascular arteriole vasoconstriction in response to tubuloglomerular feedback may account for decreased glomerular filtration rate (GFR) in

Table 3
Changes in serum biochemical markers for renal function and systemic inflammation in rat groups.

Biochemical Marker		G1	G2	G3	G4	G5
Blood Urea Nitrogen or BUN (mmol/L)	Data set A	2.59	3.01	0.50	0.43	0.30
	Data set B	763 %	903 %	67 %	43 %	——-
Serum Creatinine or SCr (μmol/L)	Data set A	32.47	40.33	10.67	8.83	7.00
	Data set B	363 %	476 %	52 %	26 %	———
Erythrocyte Sedimenta-tion Rate or ESR (mm/h)	Data set A	0.397	0.587	0.06	0.07	0.01
	Data set B	3870 %	5770 %	500 %	600 %	———
C–Reactive Protein or CRP (mg/ml)	Data set A	4.33	6.50	0.99	1.67	0.33
	Data set B	1212 %	1700 %	200 %	406 %	——-

acute tubular injury. It is suggested that when the kidney injury became chronic as in this study, the glomerulus and convoluted tubules dilated to compensate and improve GFR. Noteworthily, massive muscle breakdown occurring in AAS-using weightlifters may lead to rhabdomyolysis and myoglobin-induced renal failure, and Basile et al. [7] include myoglobin from rhabdomyolysis as an endogenous compound that may cause nephrotoxicity. It is also conceivable that kidney injury induced by stanozolol may have instigated chronic inflammatory processes both in the glomeruli and tubuli, resulting in elevated synthesis and reduced degradation of extracellular matrix, with excessive tubulointerstitial collagen accumulation [29]. The latter authors postulate that consequential glomerular sclerosis, tubulointerstitial fibrosis, and tubular atrophy cause a further loss of functioning renal mass, thereby closing a vicious circle of disease progression by increasing intraglomerular pressure and hypertrophy of the remaining glomeruli. Other factors including cellular swelling and oedema have been implicated in the renal injury resulting from perturbed intrarenal blood flow, but these could not be determined at the light microscope level of the present work.

By far the most remarkable finding of this study is the huge magnitude of increases in systemic inflammatory markers arising from stanozolol administration, and equally noteworthy is the significant reductions achieved by prandial NCP. Our discussion is based on the interesting link between antioxidants and anti-inflammatory compounds in kidney injury that has been comprehensively reviewed [7].

According to Basile et al. [7], reactive oxygen species (ROS) may influence the effects of vasoconstrictors and vasodilators and lead to an increase in renal vascular resistance. This may have elicited adaptive glomerular and tubular hypertrophy in the present study. Furthermore, whereas ROS may also influence haemodynamics and inflammation, the cellular defence mechanisms in response to injury may particularly augment renal vasoconstrictor responses in the renal medulla. Inflammatory mediators are synthesized and released by both tubular epithelial cells and activated leukocytes. Tubular epithelial cells produce TNF-α, IL-1, IL-6, IL-8, TGF-β, MCP-1, ENA78, RANTES and fractalkines, while leukocytes may produce IL-1, IL8, MCP-1, reactive oxygen species and eicosanoids. These factors act in concert to promote inflammation in a positive feedback loop promoting further kidney injury. In this scenario ROS may block the normal homeostatic mechanism maintaining medullary perfusion and these responses may be normalized with the use of antioxidants. It is posited that this may explain the massive decreases in the inflammatory markers (ESR and C-RP) in the rats treated with stanozolol and which also drank NCP. The operative mechanism is akin to administration of the non-selective adenosine receptor antagonist theophylline which preserved renal blood flow when administered before ischemic vasoconstriction. Notably, the methylxanthine theobromine and flavanols in NCP have vasodilatory activity [12,28]. We therefore concur that cytokines [33,38] that can interrupt the amplification of this inflammatory cascade during acute kidney injury may have therapeutic implications, as demonstrated by the anti-inflammatory activity [33] of NCP in present work.

It is needful to discuss why systemic inflammatory markers registered far greater percentage increases than renal function markers in this study. It has recently been observed in respect of COVID-19 [20,38], that when tissues in the body are damaged either by viruses or other means (stanozolol in our study) the body's natural healing response involves releasing inflammatory molecules including cytokines into the blood stream. A concept of secondary effect of cytokines rather than the initial cause of acute injury amplifying and broadening the proinflammatory response is important in escalating what has become known as the inflammatory (or more commonly cytokine) storm [36]. Cytokine storms damage organs throughout the body including heart and liver [20]. In this scenario the immune system becomes nonadaptive, resulting in highly elevated inflammatory markers. It is conceivable that the systemic inflammation markers in our study being in response to possible multiple organ damage via a cytokine storm, elicited excessively higher percentage elevations than markers for the limited kidney damage. NCP consumption presumably prevented the cascading cytokine storm and so remarkably extenuated the elevations in inflammatory markers. It is notable too that NCP in our study apparently exerted ideal immunomodulatory effect by increasing its protective activity in response to higher dose of stanozolol. This could be achieved by enhancing the immune response at the onset of injury but suppressing its escalation later to curtail potential cytokine storm [36]. Thus, besides the advantage of being nutraceutical, NCP's modulatory activity in this study confirms it as efficacious in treating conditions in which an inflammatory storm is implicated, such as malaria [1,2].

The question of whether our findings of nephron-protective benefit of NCP ingestion against stanozolol damage can be extrapolated to humans demand addressing. We have previously shown that a concentration of 2 % weight per volume is optimal for consumption by the rats as well as for health benefit of rats [2,8,32] and humans [1]. Higher concentrations increased rate of sedimentation after a few hours and blocked the delivery tube making it impractical for the rats to suck/drink. For humans, higher concentrations have a disagreeable taste (too bitter) and increases nuisance of frequent copious urination and consequential occurrence of constipation (Addai, unpublished findings). Rats used in this study had an average weight of 175.2 g and voluntarily consumed an average volume of 50.85 ml NCP suspension daily. In humans (with an average weight of 6200 g) that would translate to 1799.5 ml NCP suspension daily. Therefore, in the present study each rat took a daily dose of 1.02 g NCP for two months to exhibit the observations we report. Given that one rat month is roughly equivalent to two and a half human years [27], this would translate to a daily dose of 35.99 g NCP for five years in humans. Evidence from dietary studies suggest that such dosage will be tolerated by the human body [3,37].

Limitation

While this study provides knowledge into the effects of stanozolol and the potential ameliorative properties of Natural Cocoa Powder (NCP) on kidney damage, several limitations should be noted. Even though the study duration was sufficient to observe acute renal changes but may not capture long-term effects of stanozolol or the sustained protective role of NCP. Future studies could extend the timeline to explore chronic impacts. And also while a 2 % NCP concentration was chosen based on previous research, no dose-response evaluation was performed, so testing different concentrations may reveal greater protective effects.

Conclusion

This study has demonstrated damage to kidneys by stanozolol administration in terms of increased volume density of PCT, DCT, glomerular tuft, and renal corpuscle. Excessively increased markers of systemic inflammation confirmed high inflammatory activity of stanozolol and significant reduction by NCP ingestion evidenced anti-inflammatory benefit. It is posited that amelioration of stanozolol-induced hypertrophy of kidney components by prandial NCP is probably attributable more to attenuation of inflammation than oxidation, albeit interplay between the two phenomena portend mutual potentiation. Though it was an animal study, the results are promising to have health benefits in humans, thus contributing to healthy lives and promoting well-being for all at all ages in line with SDG 3. The results indicate the importance of dietary interventions, like NCP, in mitigating drug-induced organ damage, which could inform broader health initiatives, including strategies to prevent kidney disease and manage inflammation-related conditions. Future studies could further explore these findings to contribute to evidence-based dietary recommendations and global health priorities related to chronic disease prevention and overall well-being.

Declarations

Ethics approval and consent to participate

Approval for the research was obtained from the University of Ghana College of Health Sciences Ethical and Protocol Review Committee (CHS-Et/ M.8- P20.9/ 2017–2018). Procedures involving the care and use of the animals were carried out in compliance with national and international guidelines for the use of animals in biomedical research.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

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Authors contribution

FKA and DO conceptualized and designed the study. Experimentation was conducted by DO. BAB and KA-O drafted the manuscript and coordinated input by co-authors in data analyses and interpretation, as well as critical review of final manuscript. All authors actively participated in compilation and analyses of results, reviewed the manuscript, and all gave approval for submission.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

No other co-author declares any potential competing interest except Frederick Kwaku Addai (FKA), who is a non-salaried Director of KEL Kakawa Company Ltd., the company that packages GoodFood® natural Cocoa Powder for health promotional consumption in Ghana (See Addai FK [1]. Natural cocoa as diet-mediated antimalarial prophylaxis. Med. Hypotheses 74:825–830). KEL made no financial contribution to this study which was a M.Phil. Thesis Project for which FKA was lead supervisor.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2024.e02468.

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