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Co-administration of caffeine and caffeic acid alters some key enzymes linked with reproductive function in male rats

S. F. Akomolafe[1] | A. J. Akinyemi[2] | G. Oboh[3] | S. I. Oyeleye[3,4] | O. B. Ajayi[1] | A. E. Omonisi[5] | F. L. Owolabi[1] | D. A. Atoyebi[1] | F. O. Ige[1] | V. A. Atoki[1]

[1]Department of Biochemistry, Ekiti State University, Ado Ekiti, Nigeria

[2]Department of Biochemistry, Afe Babalola University, Ado Ekiti, Nigeria

[3]Functional Foods and Nutraceuticals Unit, Department of Biochemistry, Federal University of Technology, Akure, Nigeria

[4]Department of Biomedical Technology, Federal University of Technology, Akure, Nigeria

[5]Department of Anatomic Pathology, Ekiti State University, Ado Ekiti, Nigeria

Correspondence
Seun F. Akomolafe, Department of Biochemistry, Ekiti State University, Ado Ekiti, Nigeria.
Email: purposefulseun@yahoo.co.uk

Summary

This study assessed the effects of caffeine combined with caffeic acid on some biomarkers of male reproductive function using normal albino Wistar rats. Rats were divided into four groups (n = 6) and treated for seven successive days; group 1 represents the control rats; group 2 rats were treated with 50 mg/kg body weight (BW) of caffeine only; group 3 rats were treated with 50 mg/kg BW of caffeic acid, while the rats in group 4 were cotreated with an equal combination of caffeine and caffeic acid. The results revealed significant increase in reproductive hormone, testicular and epididymal nitric oxide levels of the rats. Moreover, decreased oxidative stress in the testes and epididymides of the treated rats was evidenced by significant increase in total and nonprotein thiol levels, catalase and superoxide dismutase activities. Similarly, decreased testicular cholesterol level with concomitant elevation in testicular steroidogenic enzyme activities, glycogen and zinc levels were observed in the treated rats. No morphological changes were observed as revealed by the photomicrographs from light microscopy in treated rats. Nevertheless, the combination therapy exhibited additive/synergistic effect on these biochemical indices than when they were administered singly. This study suggests the combination therapy of caffeine and caffeic acid at the dose tested for improving male reproductive function.

KEYWORDS

caffeic acid, caffeine, fertility, hormones, steroidogenic enzymes

1 | INTRODUCTION

Caffeine (1, 3, 7-trimethylxanthine) is an alkaloid found in the leaves, seeds or fruits of at least 63 plant species worldwide and is part of a group of compounds known as methylxanthines. The most widely known sources of caffeine are coffee, cocoa beans, kola nuts and tea leaves (Barone & Roberts, 1996). Approximately 85% the world's population today uses substantial amounts of caffeine on a regular basis (Harris & Dawson-Hughes, 1994), and high caffeine containing energy drinks are widely consumed by adolescents (Reissig, Strain, & Griffiths, 2009). Caffeine intake has been associated with increased risk of spontaneous abortions (Dlugosz & Bracken, 1992; Nawrot et al., 2003), and some studies have reported a decrease in female fertility with increasing caffeine consumption (Bolumar, Olsen, Rebagliato, & Bisanti, 1997; Jensen et al., 1998), although both of these associations remain controversial. Previous studies on caffeine intake and semen quality have been contradictory with some revealing the beneficial effect while others reported the detrimental effect (Andersen, Jensen, & Carlsen, 2000; Jensen et al., 1998; Jørgensen, Carlsen, & Nermoen, 2002; Sobreiro, Lucon, & Pasqualotto, 2005).

Due to the contradiction surrounding caffeine intake on male fertility and the growing concern about a decrease in male reproductive health, recent research focuses on combining caffeine intake with established nutraceuticals with potent fertility enhancer in order to suppress the possible detrimental effect(s) that may associate with its consumption (Dorostghoal, Majd, & Nooraei, 2012).

Caffeic acid (3,4-dihydroxycinnamic acid) is a phenolic compound widely distributed in medicinal plants/foods such as fruits, vegetables, wine, coffee, olive oil among others (Nam, 2009). Caffeic acid and its derivative caffeic acid phenethyl ester (CAPE) are one of the most active compounds found in argan oil and many kinds of plants (Huang and Ferraro, 1992). Report has shown that caffeic acid has a stronger antioxidative effect compared to other phenolic acids such as *p*-coumaric and ferulic acids, in preventing low-density lipoprotein oxidation (Maurya & Devasagayam, 2010). Caffeic acid and CAPE have been reported to improve the reduction in the sperm characteristics in lambda cyhalothrin-induced oxidative damage of the testes (Abdallah, Fetoui, Zribi, Fakhfakh, & Keskes, 2012). Several studies have established fertility enhancing properties of caffeic acid and its derivative; however, their combined effect on male reproductive function remains unexplored. Hence, this study sought to investigate the effects of caffeine, caffeic acid and their combination (caffeine + caffeic acid) on some biomarkers of male reproductive function using normal albino Wistar rats.

2 | MATERIALS AND METHODS

2.1 | Source of chemicals

Caffeine, caffeic acid, nicotinamide adenine dinucleotide (NAD), dehydroepiandrosterone (DHEA), testosterone, bovine serum albumin (BSA), sodium pyrophosphate were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used for the experiments were of analytical grade, while the water was glass distilled. All the kits used for the bioassay were sourced from Randox Laboratories Ltd. (Crumlin, Northern Ireland, United Kingdom).

2.2 | Animal experiment

All animal procedures were approved, and prior permission from Ekiti State University Animal Ethical Committee was obtained as per the prescribed guidelines. The bioethical allowance reference number was AFO022SAKO02. Twenty-four male albino Wistar rats weighing between 122 and 137 g were purchased from College of Medicine, Ekiti State University, and were housed in the Ekiti State University Central Animal Unit. The rats were allowed to adapt to the new environment for two weeks before the experiment. They were kept under standard condition (inverted 12-hr light/dark cycles), constant temperature (22 ± 2°C) and humidity (70% ± 4%) with access to standard feed and water ad libitum. The animals were handled according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals. Ethical care was maintained throughout the experimental period.

After two weeks of acclimatisation, the rats were divided into four groups (1, 2, 3 and 4, respectively) using completely randomised design with six rats in each group. Group 1 (positive control) received water by gavage throughout the experiment; Group 2 received 50 mg/kg body weight (BW) of caffeine orally; Group 3 received 50 mg/kg BW of caffeic acid, while Group 4 received an equal combination of caffeine and caffeic acid (50 mg/kg caffeine + 50 mg/kg caffeic acid) orally. All the animals were treated for seven successive days. Daily feed intake was monitored, and BW was taken both at the beginning and at the end of the experiment. The choice of doses at 50 mg/kg BW for both caffeine and caffeic acid was according to the study of Kantamala, Vongsakul, and Satayavivad (1990). For the group that received both compounds, rats were orally administered 50 mg/kg of caffeine before 50 mg/kg of caffeic acid.

2.2.1 | Necropsy

All the animals were sacrificed 24 h after the last treatment under ketamine and xylazine anaesthesia. Testis and epididymis were removed and cleared of adhering tissues, rinsed in ice-cold 1.15% potassium chloride and dried with blotting paper. The weights of the organs were recorded in gram (g) and expressed as g/100 g BW. The blood sample was collected by cardiac puncture into plain sample tube and allowed to stand for about an hour at room temperature, after which they were centrifuged at 3,000 *g* for 10 min using a bench top centrifuge (MSE, England). Sera obtained from the respective sample tubes were used for hormonal assays. The right testes was cut into two pieces; the first section was fixed in Bouin's fixative for 6 hr, then transferred to formalin, sectioned and stained routinely with haematoxylin and eosin and then viewed under a light microscope (Olympus/3H-Tokyo, Japan) for histopathologic examination. Left testes and caudal epididymis were processed for biochemical analysis.

2.2.2 | Steroidogenic enzyme assay

The left testes and epididymis of each rat were homogenised in 50 mM Tris–HCl buffer (pH 7.4). The resulting homogenate was centrifuged at 10,000 *g* for 15 min at 4°C, and the supernatant was subsequently collected for estimation of antioxidant status. For the determination of testicular steroidogenic enzymes (3β- and 17β-hydroxysteroid dehydrogenase [HSD]) activities, testicular tissue (50 mg/ml) was homogenised in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA at 4°C and centrifuged at 10,000 *g* for 30 min. Protein concentration was determined by the method of Lowry, Rosenbrough, Farr, and Randall (1951).

2.2.3 | 3β-HSD determination

3β-Hydroxysteroid dehydrogenase was measured by mixing 250 μl of the supernatant with 250 μl of 100 μM sodium pyrophosphate buffer, pH 8.9, 10 μl ethanol containing 30 μg dihydroxyl epiandrosterone (DHEA) (Sigma) and 240 μl of 25 mg% BSA (Bangalore Genei, India). Enzymatic activity was measured after addition of 50 μl of 0.5 μM NAD to the mixture in a spectrophotometer at 340 nm against a blank (without NAD) (Talalay, 1962). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

2.2.4 | 17β-HSD determination

17β-Hydroxysteroid dehydrogenase was measured by mixing 250 μl of the supernatant with 250 μl of 440 μM sodium pyrophosphate buffer

(pH 10.2), 10 μl ethanol containing 0.3 μM testosterone (Sigma) and 240 μl of 25 mg% BSA. Enzyme activity was measured after addition of 50 μl of 0.5 μM NAD to the mixture in a spectrophotometer at 340 nm against a blank (without NAD) (Jarabak et al., 1962). One unit of enzyme activity was the amount causing a change in absorbance of 0.001/min at 340 nm.

2.2.5 | Biochemical assay

Estimation of testicular cholesterol

Testicular total cholesterol was determined according to the method of Zlatkis, Zak, and Boyle (1953) with slight modification. Briefly, testicular tissue (50 mg/ml) was homogenised in ether:alcohol mixture (1:3), centrifuged at 10,000 × g for 10 min, 0.1 ml supernatant was mixed with 4.9 ml ferric chloride solution (50 mg $FeCl_3 \cdot 6H_2O$, dissolved in 100 ml Acetic acid) in a screw capped centrifuge tube and allowed to stand for 15 min. The mixer was centrifuged at 10,000 × g for 10 min, and 1.5 ml of conc. H_2SO_4 was added to 2.5 ml of the clear supernatant and incubated for about 30 min at room temperature for colour development. A stock concentration of 0.25 μg/μl (250 mg/ml) of standard cholesterol was prepared. The colour intensity of unknown and standard was read against blank at 560 nm in a spectrophotometer. The result was expressed in mg/g tissue.

Estimation of testicular glycogen

Testicular glycogen was estimated using the method of Nicholas, Robert, and Joseph (1956) with a slight modification. Briefly, 1 ml of 5% Trichloroacetic acid (TCA) was added to 50 mg testicular tissue, homogenised and centrifuged at 3,000 rpm for 15 min. In a centrifuge tube, 1 ml supernatant and 5 ml 95% ethanol were mixed and allowed to stand in water bath at 37–40°C for 3 hr. After completion of precipitation, tubes were centrifuged at 3,000 rpm for 15 min. The supernatant was gently decanted from packed glycogen, and the tubes were allowed to drain in an inverted position for 10 min. The packed glycogen was dissolved by adding 2 ml of distilled water, this was considered as sample, and the blank was prepared by 2 ml water. The standard was prepared by 2 ml solution containing 0.1 mg glucose. Ten millilitre of anthrone reagent (0.05% anthrone, 1% thiourea and 66% H_2SO_4 v/v) was added into each tube with vigorous but consistent blowing and good mixing. All the tubes were kept in cold water and then immersed in boiling water bath for 15 min. The tubes were removed again to cold water bath and cooled to room temperature. The reading of unknown (sample) and standard was taken against blank at 620 nm in spectrophotometer. The value of glycogen content was expressed in mg/g tissue.

Estimation of testicular and epididymal zinc content

Testicular and epididymal zinc was determined according to the method of Mas, Romeu, Alemany, and Arola (1985). Briefly, testicular (0.5 g/5 ml) tissue was digested with concentrated nitric acid at 100°C for 2 hr in boiling water bath and after 24 hr, the clear digest was diluted to 10 ml with deionised water. The zinc level was estimated by atomic absorption (Model no. A Analyst-200, PerkinElmer Make) and was expressed in mg/g of wet tissue.

2.2.6 | Hormonal assay

Serum LH and FSH assays

The serum levels of luteinising hormone (LH) and follicle stimulating hormone (FSH) were measured using available radioimmunoassay kits. They were determined by a double-antibody RIA using 125I-labelled radioligand as described by Taya et al. (1985). Intra-assay and interassay coefficients of variation were 5.4% and 6.9% for LH, and 4.8% and 11.4% for FSH, respectively. The sensitivity of LH and FSH assays was 1.9 pg per tube and 9.8 pg/tube, respectively.

Serum testosterone assay

Serum-free testosterone was determined using an enzyme immunoassay (EIA) kit (Immunometrics, UK Ltd). This method is based on the competition of serum testosterone and alkaline phosphatase-labelled testosterone in binding to a limited amount of fluorescein-labelled polyclonal antitestosterone antibody (Biswas et al., 2001). The reaction was terminated by the addition of EIA stop buffer (glycine buffer pH 10.4 containing sodium hydroxide and a chelating agent), and the optical density was measured at 550 nm. The testosterone concentration of the test sample is interpolated from a calibration curve using testosterone EIA standards.

Measurement of nitric oxide

Nitric oxide (NO) content in testes and epididymis supernatant was estimated in a medium containing 400 ml of 2% vanadium chloride (VCl_3) in 5% HCl, 200 ml of 0.1% *N*-(l-naphthyl)ethylenediaminedihydrochloride, 200 ml of 2% sulphanilamide (in 5% HCl). After incubating at 37°C for 60 min, nitrite levels, which corresponds to an estimative of levels of NO, were determined spectrophotometrically at 540 nm, based on the reduction of nitrate to nitrite by VCl_3 (Miranda, Espay, & Wink, 2001). Testes and epididymis nitrite and nitrate levels were expressed as nanomole of NO/milligram of protein.

2.2.7 | Estimation of antioxidant status

Determination of catalase activity

Catalase (CAT) activity using hydrogen peroxide as substrate according to the method of Clairborne (1995). Briefly, the assay mixture contained 2 ml of H_2O_2 solution and 2.5 ml of phosphate buffer in test tubes. 0.5 ml of properly diluted sample was rapidly added to the reaction mixture by a gentle swirling, and the reaction proceeded at room temperature. One millilitre portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid reagent at 60 s intervals for three minute. The H_2O_2 contents of the withdrawn solutions were subsequently determined.

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of autoxidation of epinephrine at pH 10.2 at 30°C according to Misra (1989). Briefly, an aliquot of 0.2 ml of the diluted microsome (tissue homogenate) was added to 2.5 ml of 0.05 M carbonate buffer pH 10.2 to equilibrate in a cuvette and the reaction

started by the addition 0.3 ml of 0.3M of adrenaline. The reference cuvette contained 2.5 ml of carbonate buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of distilled water. The increase in absorbance at 480 nm was monitored every 30 s for 150 s. The activity of SOD of the samples was subsequently calculated.

Total thiol determination

Total thiol content was determined according to the method previously described by Ellman (1959). Briefly, the reaction mixture consisted 40 µl of testicular or epididymal homogenate, 10 µl of 10 mM 5^{I}, 5^{I} – dithiobis-(2-nitrobenzoic acid) (DTNB) and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 200 µl. The mixture was incubated for 30 min at ambient temperature and then read the absorbance at 412 nm. A standard curve was plotted for each measurement using cysteine as a standard and the results expressed as mol/mg protein.

Determination of nonprotein thiols (NPSH)

Nonprotein thiol (NPSH) levels were determined by the method of Ellman (1959). Briefly, an aliquot of testicular or epididymal homogenate was mixed (1:1) with 10% trichloroacetic acid. Subsequent to precipitation of protein, the resulting solution was centrifuged at 10,000 *g* for 5 min at 4°C and the free SH groups were determined in the supernatant. The reaction mixture consisting 50 µl of sample, 450 µl phosphate buffer and 1.5 ml of 0.1 mM of 5,5-dithiobis 2-nitro benzoic acid was incubated for 10 min at 37°C. The absorbance was measured at 412 nm. NPSH levels were expressed as mol/mg of protein.

2.3 | Statistical analysis

The data reported herein are the means of six replicates ($n = 6$). Means separation was carried using Fisher's protected least significant difference test at ($p < .05$). One way analysis of variance was performed to evaluate relationship between control and treated groups. All statistical analysis were performed using JMP Release 10.0 statistical package (SAS Institute, Inc., Cary, NC, USA, 2010).

3 | RESULTS

3.1 | Body weight, absolute and relative organ weights

The body weight, absolute and relative organ weights of control and treated rats are presented in Table 1. Following the treatment of the rats with caffeine, caffeic acid and their combination, the BW of all the groups significantly ($p < .05$) increased during the period of seven days when compared with the control rats. Nevertheless, combination of caffeine and caffeic acid (caffeine + caffeic acid) synergistically increase the BW gain ($p < .05$) when compared with caffeine or caffeic acid treated group. Also, a significant ($p < .05$) increase in the testes and epididymides weights was observed in all the treatment groups when compared to the control.

3.2 | Reproductive hormones and nitric oxide levels

Treatment with caffeine, caffeic acid and their combination significantly ($p < .05$) increased the testosterone, LH and FSH levels when compared with the normal group (Figure 1a). However, as shown in Figure 1a, co-administration of both caffeine and caffeic acid resulted in synergistic increase in the reproductive hormones when compared with either caffeine or caffeic acid alone. NO level in the testes and epididymis was clearly elevated in the treated groups when compared with the control (untreated) group (Figure 1b). However, the combination (caffeine + caffeic acid) caused a synergistic effect on the NO level both in the testes and epididymis.

3.3 | Testicular and epididymal antioxidant status

The antioxidant status of the testes and epididymis in normal and treated rats is presented in Figure 2a–e. The levels of malondialdehyde (MDA), a maker of lipid peroxidation, in both testis and epididymis decreased significantly ($p < .05$) in the treated rats with

TABLE 1 Body weight (BW), absolute and relative organ weights of rats treated with caffeine, caffeic acid and their combination (in g)

Treatment groups	Control	Caffeine	Caffeic acid	Combination
Initial BW	137.60 ± 8.93	134.60 ± 10.24	127.40 ± 5.63	128.80 ± 7.28
Final BW	150.23 ± 9.95	151.09 ± 9.83	142.68 ± 7.30	149.44 ± 8.81
Weight gain	12.63	16.49[a]	15.28[a]	20.64[a]
Weight gain %	8.41	10.91	10.71	13.81[a]
Absolute testis weight	0.97 ± 0.14	2.14 ± 0.09[a]	2.10 ± 0.21[a]	2.13 ± 0.34[a]
Absolute epididymis weight	0.32 ± 0.06	0.61 ± 0.10[a]	0.52 ± 0.08[a]	0.59 ± 0.12[a]
Relative testis weight	0.66 ± 0.14	0.79 ± 0.06[a]	0.72 ± 0.03[a]	0.79 ± 0.05[a]
Relative epididymis weight	0.18 ± 0.02	0.21 ± 0.04[a]	0.24 ± 0.03[a]	0.22 ± 0.05[a]

Data are expressed as means ± *SD*.

[a]Significantly different from control ($p \leq .05$; $n = 6$ animals per group). ANOVA–Duncan's test.

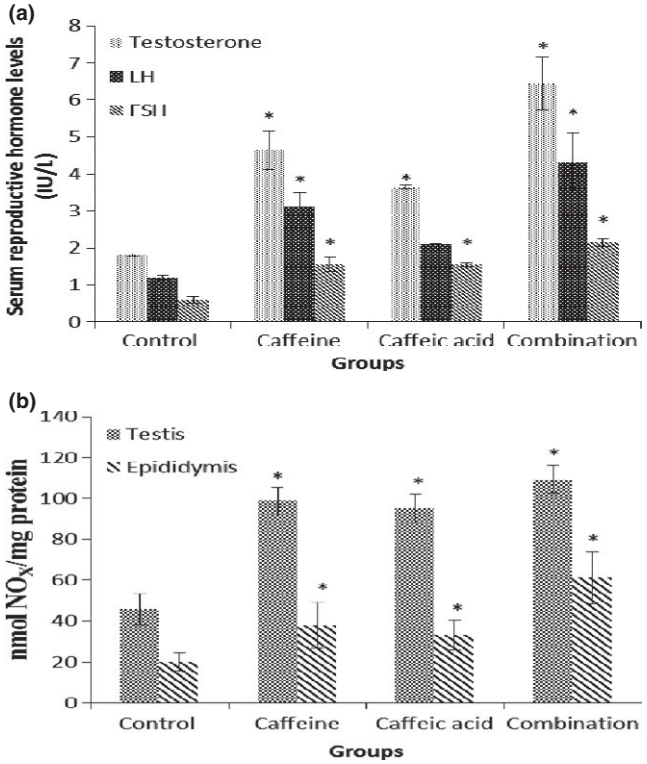

FIGURE 1 (a) Effect of caffeine, caffeic acid and their combination on the level of serum testosterone, luteinising hormone (LH) and follicle stimulating hormone (FSH) of rats. Data are presented as mean ± *SD* ($n = 6$). *Mean values are significantly ($p < .05$) different compared to control group. (b) Effect of caffeine, caffeic acid and their combination on rat's testicular and epididymal nitric oxide (NO) level. Data are presented as mean ± *SD* ($n = 6$). *Mean values are significantly ($p < .05$) different compared to control group

concomitant elevation in the CAT, SOD activities as well as total thiol and NPSHs levels. Nevertheless, the combination has a more pronounced effect on the antioxidant status than when they are administered singly.

3.4 | Results of testicular biochemical parameters and steroidogenic enzyme activities

Results of the testicular biochemical parameters in treated group showed significant ($p < .05$) increase in testicular glycogen content (Figure 3a), zinc (Zn) content (Figure 3b), and with a significant decrease in cholesterol content (Figure 3c). Also, the activity of the steroidogenic enzymes (3β- and 17β-HSD) increased significantly ($p < .05$) (Figure 3d) when compared with the control rats. However, the combination (caffeine + caffeic acid) caused a synergistic effect on the activities of the two enzymes.

3.5 | Histopathology results

The photomicrographs from the light microscopy showed well-formed seminiferous tubules containing normal cells of the spermatogenic series, and there is no loss of cellular details (Figure 4a–d).

4 | DISCUSSION

Caffeine and caffeic acid are dietary compounds which are widely present in many fruits, vegetables and coffee. Their consumption is large because they are present in most popular coffee and tea worldwide (Boyer & Liu, 2004). In the present study, we investigated the effects of caffeine, an alkaloid combined with caffeic acid, a phenolic acid with fertility enhancing property on some biomarkers of male reproductive function using normal albino Wistar rats. We observed a significant rise in the testes and epididymides weights after treatment with caffeine, caffeic acid and their combination. This increase may be due to increase availability of androgen (Izatus et al., 2013). Testicular size and weight are the best fundamental assessment of spermatogenesis. Increases in testicular weight are mostly related to the number of spermatozoa present in the tissue (Parisa, Leyla, Hamed, & Jasem, 2014).

In males, LH acts upon the Leydig cells of the testis and is accountable for the production of testosterone, an androgen that exerts both endocrine activity and intratesticular activity on spermatogenesis (Crosnoe, Grober, Ohl, & Kim, 2013). Testosterone is a male hormone that has a noteworthy impact on spermatogenesis (Lee, Coughlin, & Bellinger, 2001). FSH regulates the growth of seminiferous tubules and maintenance of spermatogenesis in males. FSH is also essential for sperm production. It supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation. Diminished secretion of LH or FSH can cause a failure of gonadal function (hypogonadism). This condition is typically evidenced in males as failure in production of normal numbers of spermatozoa.

The marked increase in LH and FSH levels with concomitant elevated testosterone level observed in the present study indicates a favourable effect of caffeine, caffeic acid and their combination on male reproductive function in vivo thereby contributing to the normal testicular and epididymal functions (Lampiao, 2013).

The chronic inhibition of NO can affect sperm function, and hence, availability of normal NO level is a vital mediator in the male reproductive tracts (Chamness et al., 1995). Also, NO can act as a free radical scavenger, halting and even inhibiting production of superoxide anions (Clancy, Leszczynska-Piziak, & Abramson, 1992; Gibson & Lilley, 1997) which cause lipid peroxidation, a process which leads to functional impairment of spermatozoa (Agarwal, Hamada, & Esteves, 2012).

The result of the present study demonstrated that the combination of caffeine and caffeic acid resulted in a synergistic increased of NO level in the testis and epididymis as there was significant ($p < .05$) when compared with rats treated with caffeine or caffeic acid alone. The increase in testis and epididymis NO levels suggests the possible availability of NO which in turn may improve male reproductive function.

The balance between antioxidant defence system and ROS production in the male reproductive system is required to maintain the regulation of normal sperm function/fertility (Aitken, 1999; Aybek, Aybek, Rota, Sxen, & Akbulut, 2008). Oxidative damage to spermatozoa in many cases of oligospermia usually coexists with a reduction in the antioxidant capacity, which can increase the deleterious effects of

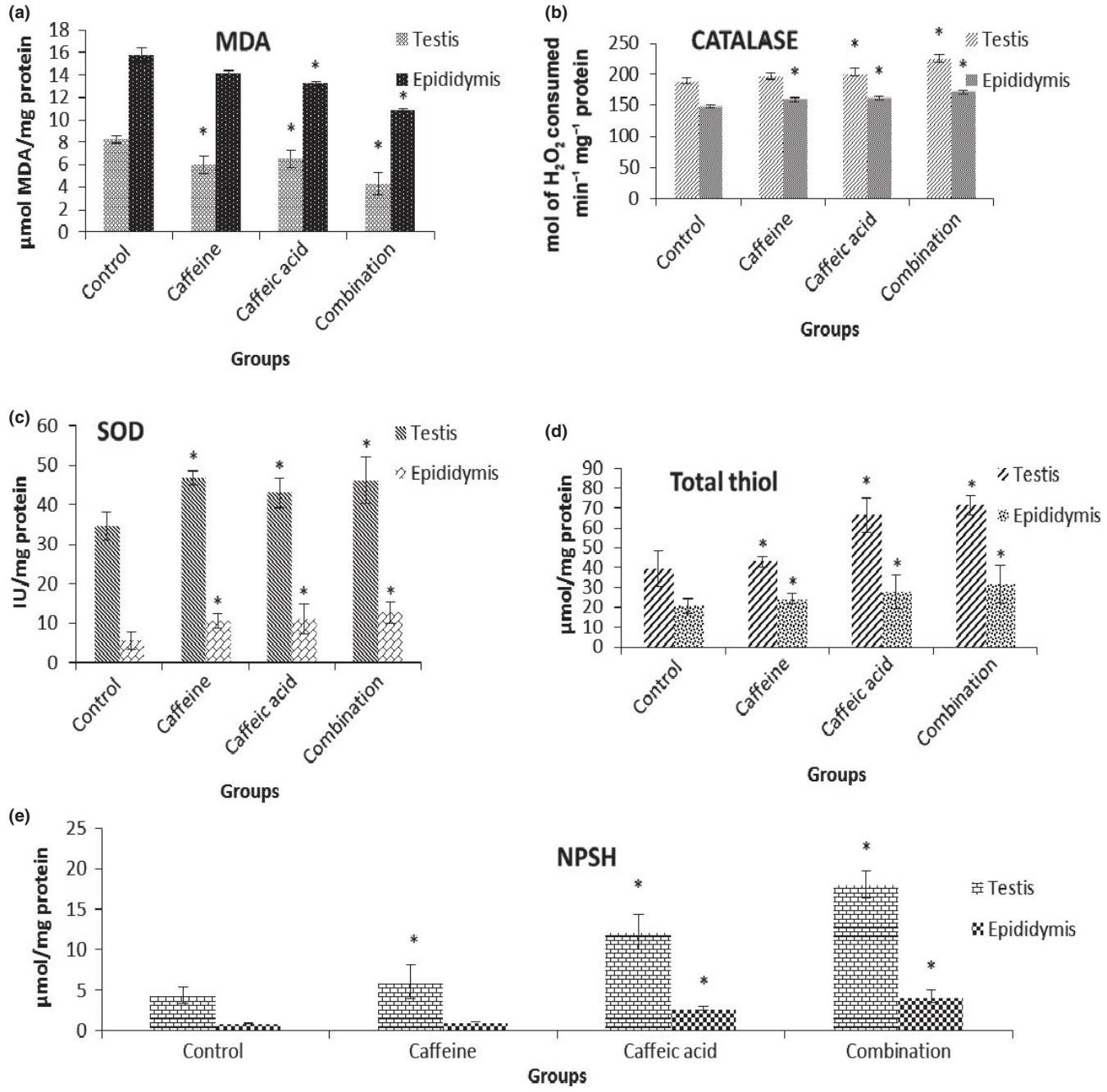

FIGURE 2 Effect of caffeine, caffeic acid and their combination on rat's testicular and epididymal: (a) malondialdehyde (MDA) level, (b) catalase activity, (c) superoxide dismutase (SOD) activity, (d) total thiol level and (e) non protein thiol (NPSH) level. Data are presented as mean ± *SD* ($n = 6$). *Mean values are significantly ($p < .05$) different compared to control group

the free radicals (Zini, de Lamirande, & Gagnon, 1993). Moreover, we observed a significant elevation in the testicular and epididymal CAT, SOD activities, total thiol and NPSH levels in the treated rats with a concomitant decrease in MDA production. The combination caused a synergistic increase in all the parameters when compared with either caffeine or caffeic acid alone (Figure 2a–e). These observations could result in the sufficiency of the testes and epididymis antioxidant status to effectively prevent induction of oxidative stress in the treated rats. Akinyemi et al. (2015) reported an imbalance between antioxidant defence system and ROS production in the testes and epididymis of hypertensive male rats and concluded that this might be an indication of oxidative stress which can cause male infertility. The increase in total thiol and NPSH levels observed in this study may be due to the protective role of caffeic acid on the testicular androgenesis and spermatogenesis (Huang, Roby, & Pace, 1995). Glycogen serves as main source of energy for sperm cells which is directly proportional to the steroid hormone level (Govardhan & Changamma, 2014). In the present study, increased level of testicular glycogen (Figure 3a) in the treated groups indicates the availability of nutrients for the development of spermatozoa. Indrani, Subhasish, and Nirmal (2014) reported

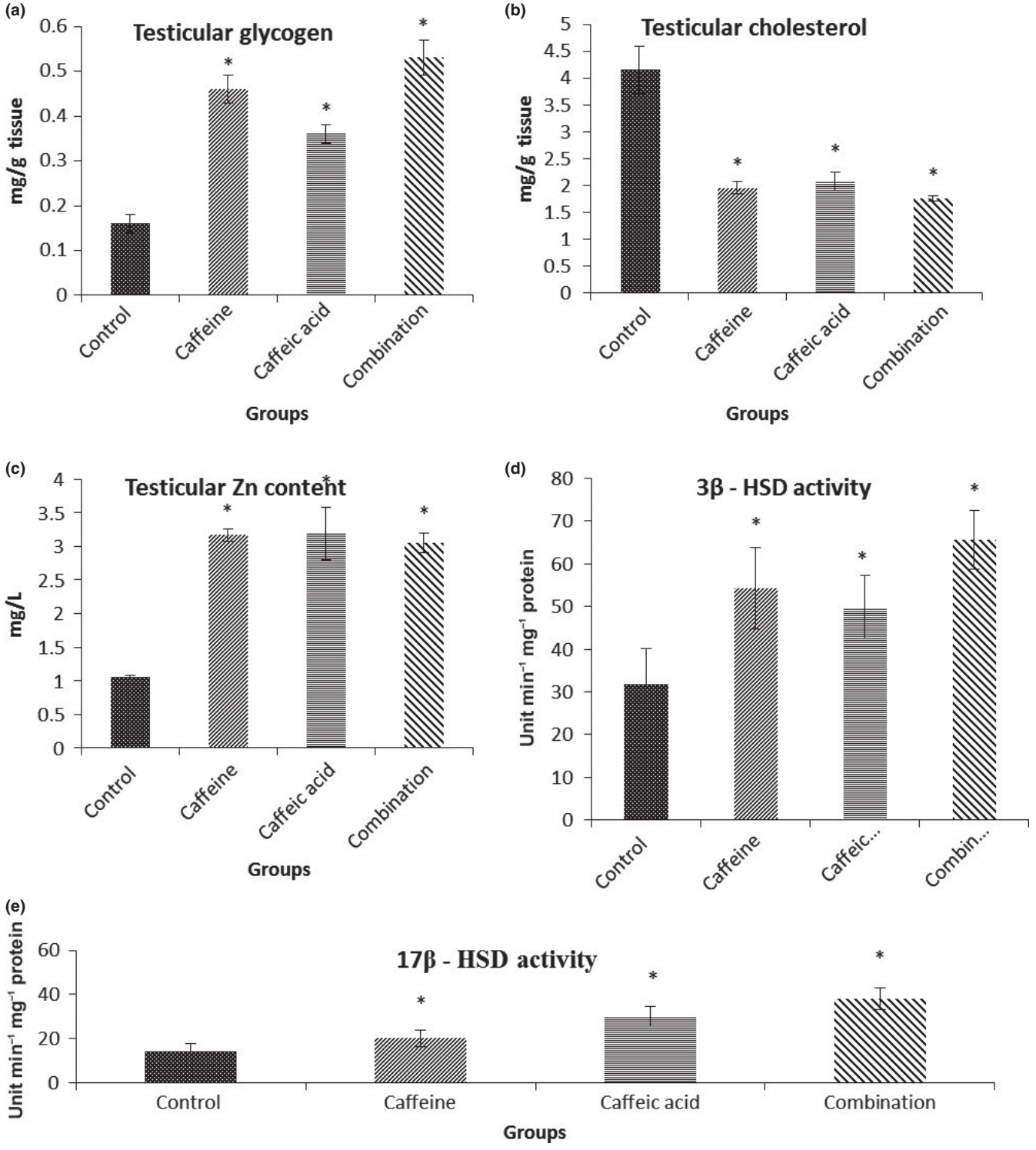

FIGURE 3 Effect of caffeine, caffeic acid and their combination on rat's testicular: (a) glycogen content, (b) cholesterol content, (c) zinc content, (d) 3β-hydroxysteroid dehydrogenase (HSD) activity and (e) 17β-HSD activity. Data are presented as mean ± *SD* ($n = 6$). *Mean values are significantly ($p < .05$) different compared to control group

that a decrease in glycogen and fructose contents in testis and seminal vesicles can decrease the number of mature and motile spermatozoa. In support of that, glycogen can serve as nutrient for the development of spermatozoa, which in turns may increase the number of mature and motile spermatozoa (Niknam, Ruzbehani, & Mahmoudi, 2014).

Moreover, increased testicular zinc level (Figure 3b) in response to the treatment may have positive effects on serum testosterone concentration, as Zn controls the testosterone metabolism, semen volume (Altaher & Abdrabo, 2015), sperm maturation and maintenance of germinal epithelium (Alsalman, Almashhedy, & Hadwan, 2013). Chia, Ong,

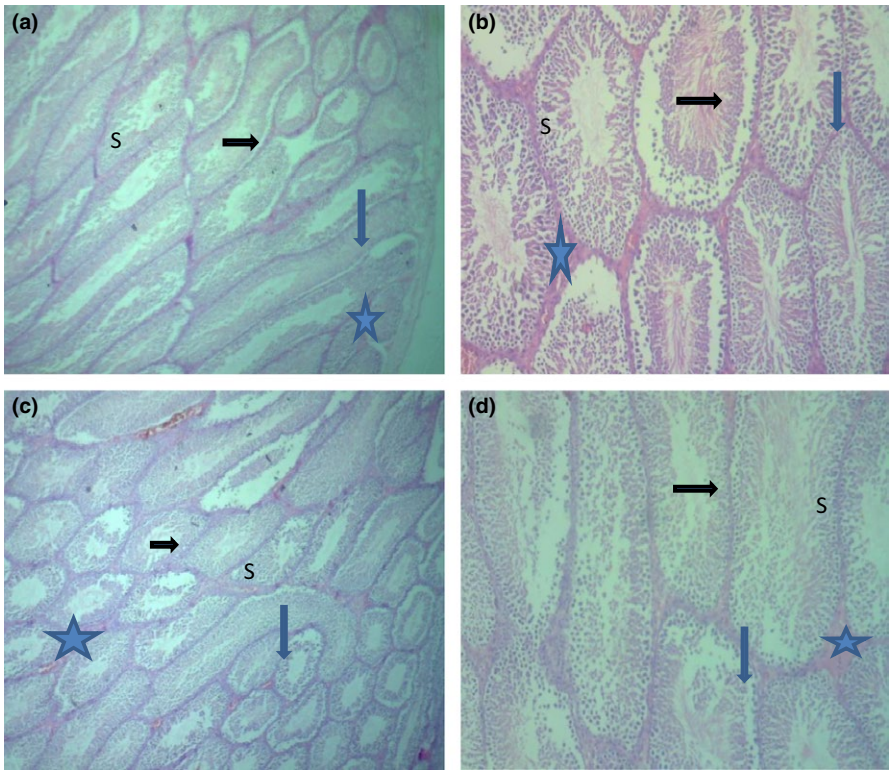

FIGURE 4 Representative photomicrographs of testes sections (viewed under light microscope at ×200 magnification) from control (a), caffeine (b), caffeic acid (c) and combination of caffeine and caffeic acid (d) treated groups under normal condition. The photomicrographs showed no histological abnormalities; the seminiferous tubules are well differentiated with all the developing stages of spermatogenesis present in all the groups. Leydig and Sertoli cells are present in all. Seminiferous tubule (S), lumen (black arrow), Sertoli cell (blue arrow), Leydig cell (star)

Chua, Ho, and Tay (2000) reported that low sperm count, atrophy of the seminiferous tubules, failure of spermatogenesis and abnormal sperm production are associated with decreased zinc content.

It is well known that cholesterol is an essential precursor for the production of testosterone (Traish & Kypreos, 2011) which is actually responsible for maintaining the normal activities of the male reproductive system (Ruige, Ouwens, & Kaufman, 2013). The decreased level of testicular cholesterol (Figure 3c) indicates that cholesterol was used for the production of testosterone (Biswas & Deb, 1965) which can be supported by the increased activities of two testicular steroidogenic key enzymes Δ5-3β- and 17β-HSD in all the treated groups (Figure 3d) in this study.

The histological sections of the current study showed many spermatogenic cells which indicated that spermatogenesis was positively facilitated by caffeine, caffeic acid and their combination. The lumen of control group was less densely filled as compared to that of treated groups which was fully filled with sperm flagella. Also, the histology results correlate well with the increased weight of testicles of the treated groups when compared with control which may due to increased number of spermatozoa (Parisa et al., 2014). A previous study reported that sertoli cells play a major role in the modulation of spermatogenesis and altering rates of sperm produced (Russell & Griswold, 1993). Thus, it is possible that the caffeic acid could have interacted with Sertoli cells and contributed positively to the spermatogenesis.

5 | CONCLUSION

In conclusion, the results obtained in the present study demonstrated that the combined effects of caffeine and caffeic acid effectively improved antioxidant status in the testes and epididymides, reproductive hormone levels, testicular biochemical parameters and activity of steroidogenic enzymes. However, the combined effect of the two compounds is more pronounced compared to when they are singly administered. Therefore, we can suggest that the combine therapy of these two dietary compounds as tested in this study could have great importance in modulating male hormonal values and oxidative stress.

CONFLICT OF INTEREST

ASF declares that she has no conflict of interest. AAJ declares that he has no conflict of interest. OG declares that he has no conflict of interest. OSI declares that she has no conflict of interest. AOB declares that she has no conflict of interest. OAE declares that he has no conflict of interest. OFL declares that she has no conflict of interest. ADA declares that he has no conflict of interest. IFO declares that she has no conflict of interest. AVA declares that he has no conflict of interest.

ETHICAL APPROVAL

All animal procedures have been approved and prior permission from Ekiti State University Animal Ethical Committee was obtained as per the prescribed guidelines. The bioethical allowance reference number was AFO022SAKO02.

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