Effect of caffeine therapeutic dose on rat organs: A biochemical and histological study

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ABSTRACT

This study was designed to investigate the effect of caffeine on gonads function related with biochemical assess in male rats. Caffeine is a chemical compound that may interfere with many organs' functions in the body, it may cause endocrine dysfunction in diagnosis of many clinical cases. Twenty-four adult male rats were randomly allotted to constituently control and treated groups having 6 rats in each one, all rats gavage daily for 40 days as follows: control group (GI) received 0.5 ml of distilled water. The treated groups were distributed as follow GII received (25mg caffeine/kg), GIII (50 mg caffeine/kg) and GIV (100mg caffeine/kg). Blood samples were collected from inferior vena cava of the heart of sacrificed animals and divided into two tubes; one contains EDTA for hematological analysis and the second tube was centrifuged at 3000rpm for 15min., the serum collected in an Eppendorf tube and stored at -20C^{O} for further laboratory investigation. Rats organs collected for histopathological study. Results reveal that the caffeine significantly increase serum testosterone, a sperm abnormality, RBCs, Hb, PLT. While, caffeine significantly decrease in sperm count, sperm motility and serum ALT, urea, WBCs, in all treated groups when compared to non-treated rats. Histopathological study showed different degree of suppression of spermatogenesis in testes, atrophy of glomeruli, vacuolation of hepatocytes and myocardial muscle cells in a high dose manner.

Keywords: Caffeine, Testosterone, Sperm, CBC.

INTRODUCTION

Coffee and other caffeine-containing beverages are widely consumed on a daily basis (1). Caffeine is now being added to food products such as potato chips, chocolates and bottled water which confirms it is growing popularity (2). It is a naturally occurring substance found in coffee beans, tea leaves, kola nuts and cocoa beans. Recently there has been an increase in energy drink consumption leading to caffeine abuse, with aggressive marketing and poor awareness on the consequences of high caffeine use (3).

It is therefore important to define the possible risks and benefits associated with caffeine intake, in order to be able to better inform both health professionals and the public. In fact, coffee is a complex chemical mixture reported to contain more than a thousand different chemicals including carbohydrates, lipids, nitrogenous compounds, vitamins, minerals, alkaloids and phenolic compounds (4).

Caffeine (1, 3, 7-trimethylxanthine) is a purine alkaloid that occurs naturally in coffee beans (4). Caffeine appears to exert most of its biological effects through the antagonism of the A1 and A2 subtypes of the adenosine receptors (5,6). Caffeine is rapidly and almost completely absorbed in the stomach and small intestines and distributed to all tissues, including the brain. Caffeine metabolism occurs primarily in the liver, which are ultimately excreted in the urine. The structure of caffeine is similar to adenosine in the body.

The caffeine binds to adenosine cell membrane receptors found in the heart, brain, smooth muscles, adiposities, and skeletal muscles. Caffeine can simultaneously affect a wide number of tissues in the body (7); it stimulates the CNS and increases the release of epinephrine. Caffeine has been shown to increase heart rate, metabolic rate, respiratory center outputs, fat oxidation and diuresis. It also decreases perception of pain and fatigue (8).

Caffeine usage may cause anxiety, heart palpitations, trembling, nervousness and facial flushing. These adverse effects are usually dose related. More side effects were reported when subjects consumed greater than 6 to 9 mg/kg body weight (9). Lethal half dose of caffeine is 150 to 200 mg/kg body weight (roughly 100 cups of coffee). Acute caffeine toxicity can cause hematemesis, hyperventilation, hyperglycemia, hypokalemia, metabolic acidosis and cardiac arrhythmia (10). This study aimed to determine the effect of therapeutic doses of caffeine on biochemical parameters and study the histopathological changes on some organs of rats.

MATERIALS AND METHODS

Experimental animals:

Twenty four adult male rats (232 ± 2.5 gm body weight) were housed (6 rats/cage) under optimum identical conditions (12/12 light, dark cycle, 22 ± 2 C°) where in these are allowed free access to pelleted rat chow and tap water, 24 adult male rats were randomly allotted to constituently control and treated groups having 6 rats in each group, according to guide for laboratory animals (11). All rats were gavage daily for 40 days as follows: control group (GI) received 0.5 ml of distilled water. Also, treated groups were distributed as follow GII received (25mg caffeine/kg), GIII (50 mg caffeine/kg) and GIV (100mg caffeine/kg). At the end study, blood samples were collected from inferior vena cava of the heart of sacrificed animals and divided into two tubes; one contains EDTA for hematological analysis and the second tube was centrifuged at 3000 rpm for 15min. The serum was collected in an Eppendorf tube and stored at -20C^{O} for further laboratory investigation. Rats' organs were collected for histopathological study.

Studied parameters:

The animal in this study were weighted after the adaptation period (at 0 time) and at the end of the experiment (at 40 days time). The blood samples were collected from inferior vena cava of the heart of sacrificed animals in ETDE tube for hematological parameters were measured by hematology analyzer (Genex Inc., Florida USA) included: total Erythrocytes count, Hemoglobin concentration, Total Leukocytes count and platelets. The second tube of blood was centrifuged at 3000rpm for 15min. and serum collected in Eppendorf tube for biochemical and hormonal analysis as follows, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activity were measured by U.V assay according to International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) without pyridoxal phosphate activation (12). The urea level was measured by ultra violet assay and the creatinine level was measured by UV assay (Modified Jaffe method) through the reagents used in automatic analyzer (Mindray) ACCENT-200 and ACCENT-200 II GEN (13). Serum testosterone was measured using AFIAS-6 (automated fluorescent immunoassay system) (14). The testes weight were measured immediately after collection from sacrificed animals. Measurement of sperm count, sperm motility and sperm normality was done as described by Evans and Maxwell. Caffeine sample used in this study was procured from Iraqi Sama Alfayhaa Pharmaceutical Industries, Himedia company, Iraq. Doses of caffeine prepared and used for this study were 25mg\kg, 50 mg\kg and 100mg\kg body weight of rats.

Histopathological study:

At the end of experiment, all animals were sacrificed and testes, kidney, liver and heart organs were collected, then preserved in 10% buffered formalin for histopathological examination. Slides

were stained with hematoxylin and eosin stain for examination according to Drury (15).

Statistical analysis:

Data were expressed as mean ± standard deviation (SD). In addition to used ANOVA analysis in experiment, least significant difference (LSD) was used to test the differences among means for ANOVA indicated a significant ($P<0.05$), using computerized SPSS version 11 (13).

RESULTS

Effect of caffeine on body weight of rats:

Table (1) showed no significant differences in body weight of rats before (0 time) and after treatment with caffeine among groups in comparison with control.

Table (1): The effect of caffeine on body weight of rats. N=6 (M ± SD)

Group	0 Time /gm	End time /gm
G1 Control	230.50 ± 22.53 a	294.75 ± 53.77 a
G2 25mg/kg	234.25 ± 18.76 a	299.75 ± 41.73 a
G3 50mg/kg	232.50 ± 16.58 a	297.50 ± 8.10 a
G4 100mg/kg	234.25 ± 20.66 a	298.50 ± 40.44 a
LSD	24.7	50.0

Values expressed in the small letters mean significant differences (P<0.05) level

Effect of caffeine on blood parameters:

Table (2) showed a significant ($P<0.05$) increase in RBCs count and PLT count in caffeine treated group 50 mg/kg and 100 mg/kg in comparison to the control group and 25 mg/kg caffeine treated group. On the other hand, there is a significant ($P<0.05$) increase in Hb concentration in high dose group when compared to other study groups, while the WBCs count are significantly ($P<0.05$) decreased in all caffeine treated group in comparison with control group.

Table (2): The effect of caffeine on blood parameters. N=6 (M ± SD)

Group	RBC $*10^6$cell/mm^3	HB g/dl	WBC $*10^3$cell/mm^3	PLT
G1 Control	5.87 ± 0.51 b	12.60 ± 0.59 bc	10.53 ± 0.75 a	431.50 ± 48.55 b
G2 25mg/kg	6.27 ± 0.14 b	13.11 ± 0.45 b	7.51 ± 0.53 b	438.71 ± 28.71 b
G3 50mg/kg	6.58 ± 0.24 a	13.66 ± 0.38 b	6.96 ± 0.71 b	561.83 ± 49.55 a
G4 100mg/kg	6.96 ± 0.52 a	14.68 ± 0.90 a	5.71 ± 0.49 bc	552.66 ± 56.71 a
LSD	0.68	1.01	1.25	114.5

Values expressed in the small letters mean significant differences (P<0.05) level

Effect of caffeine on serum AST and ALT level:

Table (3) showed no significant differences in serum AST level between caffeine treated groups when compared to control group while there is a significant ($P<0.05$) decrease in serum ALT level in caffeine treated groups in comparison to control group.

Effect of caffeine on serum urea and creatinine level:

Table (4) showed a significant ($P<0.05$) decrease in serum urea levels in all caffeine treated groups in comparison to control group. On the other hands, there is a significant ($P<0.05$) increase in serum creatinine level in G4 and G3 group when compared to G2 and control group.

Table (3): The effect of caffeine on serum AST and ALT level. N=6 (M ± SD)

Group	AST U/L	ALT U/L
G1 Control	106.5 ± 2.51 a	15.0 ± 2.58 a
G2 25mg/kg	95.75 ± 6.80 a	7.25 ± 1.50 c
G3 50mg/kg	103.2 ± 9.03 a	8.50 ± 1.29 c
G4 100mg/kg	106.0 ± 6.37 a	11.5 ± 3.00 b
LSD	8.32	2.77

Values expressed in the small letters mean significant differences (P<0.05) level

Table (4): The effect of caffeine on serum urea and creatinine level. N=6 (M ± SD)

Group	Urea mg/dL	Creatinine mg/dL
G1 Control	31.7 ± 3.53 a	0.45 ± 0.07 c
G2 25mg/kg	19.8 ± 6.27 b	0.44 ± 0.05 c
G3 50mg/kg	21.0 ± 3.74 b	0.49 ± 0.04 b
G4 100mg/kg	26.5 ± 5.80 b	0.61 ± 0.09 a
LSD	6.27	0.08

Values expressed in the small letters mean significant differences (P<0.05) level

Effect of caffeine on serum testosterone and testes weight:

Table (5) showed a significant (P<0.05) increase in serum testosterone level in all caffeine treated groups in comparison to control group while there is no significant differences in the weight of testes between caffeine treated groups and control group.

Effect of caffeine on sperm characteristics:

Table (6) showed a significant (P<0.05) decrease in sperm count, sperm motility and sperm normality in 100mg/kg and 50mg/kg caffeine group in comparison to control and 25mg/kg caffeine treated group. On the other hands, the table also showed a significant (P<0.05) increase in sperm abnormality in 100mg/kg and 50mg/kg caffeine group in comparison to control and 25mg/kg caffeine treated group.

Table (5): The effect of caffeine on testes weight and serum testosterone level. N=6 (M ± SD)

Group	Testes weight/ gm	Testosterone/ ng/ml
G1 Control	1.22 ± 0.20 a	0.66 ± 0.04 d
G2 25mg/kg	1.22 ± 0.22 a	1.06 ± 0.00 c
G3 50mg/kg	1.25 ± 0.26 a	3.49 ± 0.20 b
G4 100mg/kg	1.32 ± 0.09 a	5.43 ± 0.17 a
LSD	0.24	0.16

Values expressed in the small letters mean significant differences (P<0.05) level

Table (6): The effect of caffeine on sperm properties. N=6 (M ± SD)

Group	Sperm count $*10^6/\mu L$	Sperm motility %	Sperm normal %	Sperm abnormal %
G1 Control	55.49 ± 2.64 a	95.0 ± 0.81 a	90.0 ± 0.00 a	10.0 ± 0.00 c
G2 25mg/kg	54.46 ± 1.80 a	93.75 ± 1.25 a	90.0 ± 0.00 a	10.0 ± 0.00 c
G3 50mg/kg	48.44 ± 2.20 b	62.5 ± 2.88 b	87.5 ± 2.88 b	12.5 ± 2.88 b
G4 100mg/kg	47.83 ± 1.87 b	30.0 ± 0.00 c	50.0 ± 0.00 c	50.0 ± 0.00 a
LSD	2.70	2.04	1.81	1.81

Values expressed in the small letters mean significant differences (P<0.05) level

Histopathological study

Testes: Testes of control rats showed normal seminiferous tubules and normal spermatogenesis, the treated groups showed different degree of suppression of spermatogenesis which increase with high dose of caffeine administered as shown in figure (1).

Kidney: Kidney histopathological study of control group showed normal glomeruli and normal cortical tubules and the caffeine groups showed a little effect on kidney tissue presented as vacuolation of glomeruli as shown in figure (2).

Liver: There is no pathological changes in healthy control liver tissue of control groups showed a normal lobular hepatic architecture with central vein, while the treated groups showed mild changes in liver presenting as bile duct proliferation, central vein dilatation and vacuolation of hepatocytes that observed in high dose caffeine group as shown in figure (3).

Heart: Histopathological study of control group showed normal myocardial muscle cells. Caffeine treated groups showed vacuolation of myocytes, congestion of blood vessels and degeneration of cardiac muscle cells that noted in high dose caffeine group as shown in figure (4).

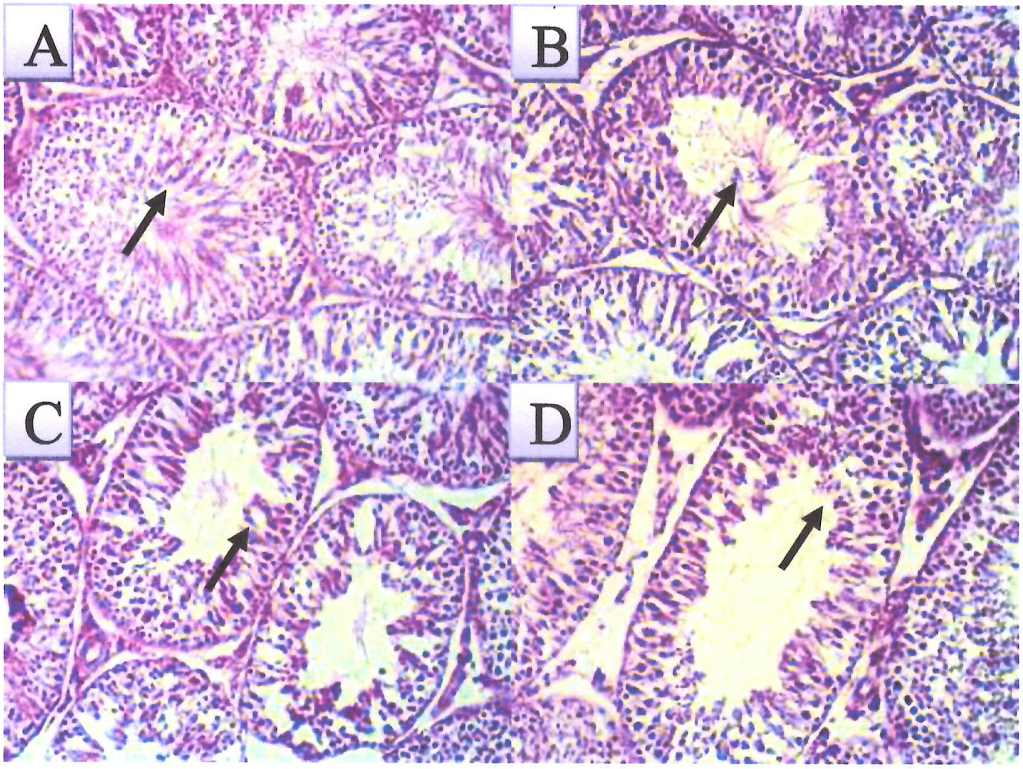

Figure (1): Testes of rat (A) control group showed normal spermatogenesis in seminiferous tubules. (B) 25mg/kg caffeine showed little effect on spermatogenesis. (C) 50mg/kg caffeine showed moderate suppression of spermatogenesis and vacuolation of primary spermatocytes. (D) 100mg/kg caffeine showed marked suppression of spermatogenesis, vacuolation of spermatogonia and primary spermatocytes. H&E stain 200X

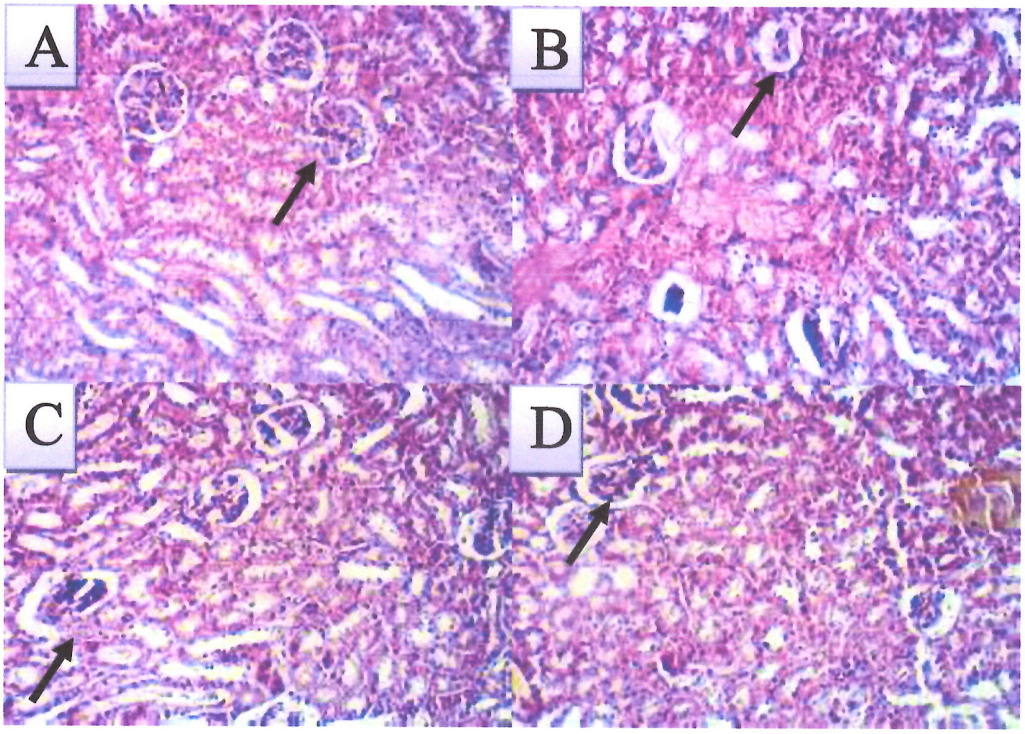

Figure (2): Kidney of rat (A) control group showed normal glomeruli and cortical tubules. (B) 25mg/kg caffeine showed atrophy of glomeruli in some field. (C) 50mg/kg caffeine showed a slight vacuolation of glomeruli. (D) 100mg/kg caffeine showed vacuolation of glomeruli. H&E stain 200X.

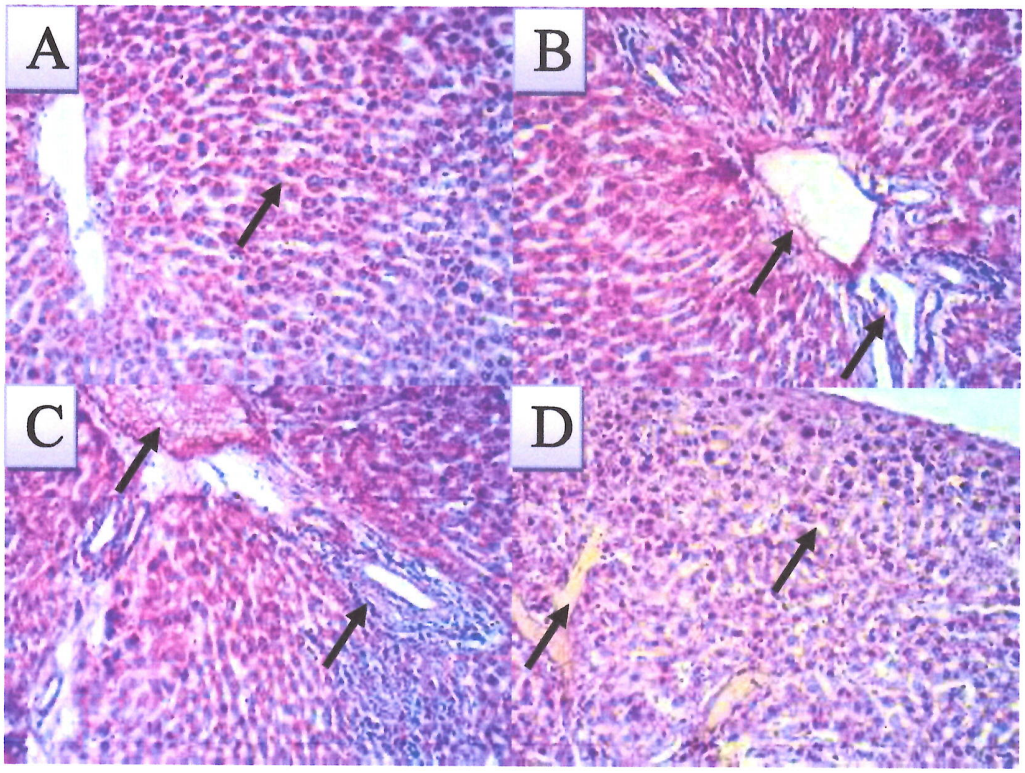

Figure (3): Liver of rat (A) control group showed normal hepatocytes architecture. (B) 25mg/kg caffeine showed dilation of central vein and bile duct proliferation. (C) 50mg/kg caffeine showed slight vacuolation of hepatocytes, bile duct proliferation and aggregation of inflammatory cells. (D) 100mg/kg caffeine showed marked vacuolation of hepatocytes and congestion of portal vein. H&E stain 200X.

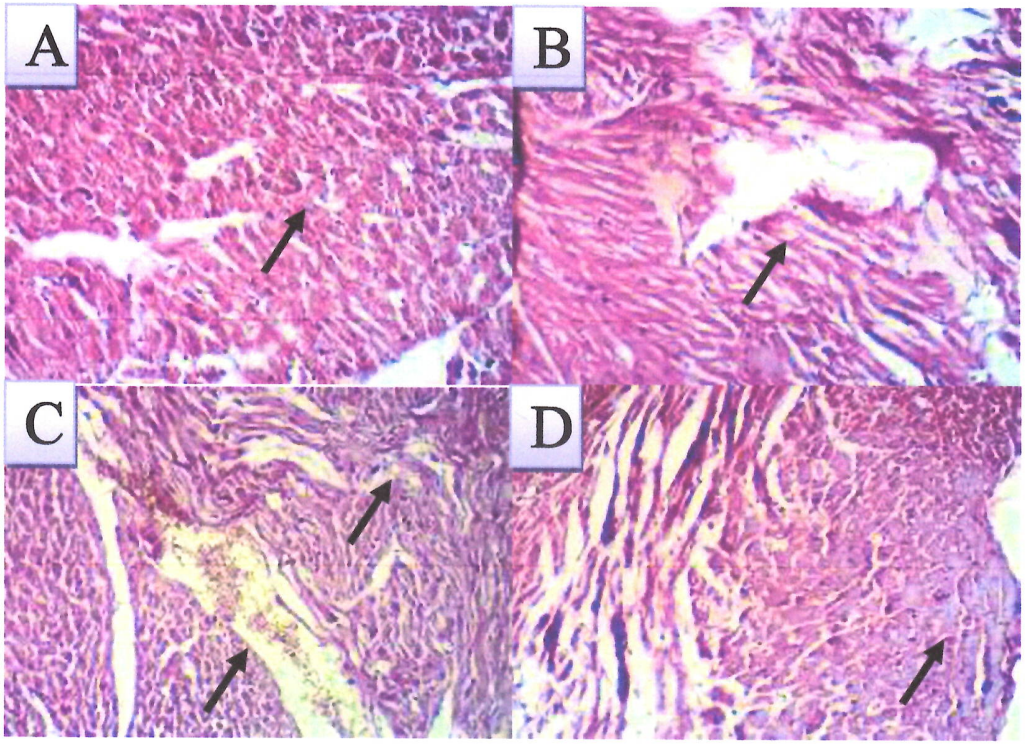

Figure (4): Heart of rat (A) control group showed normal myocardial muscle cells. (B) 25mg/kg caffeine showed vacuolation of some myocardial muscle cells. (C) 50mg/kg caffeine showed vacuolation of myocardial muscle cells and congestion of blood vessels. (D) 100mg/kg caffeine showed degeneration of myocardial muscle cells somewhat grayish in color. H&E stain 200X.

DISCUSSION

This study revealed the effect of caffeine therapeutic doses on male rat testes, kidneys, livers and hearts. Results showed no significant differences in body weight between treated and non-treated rats, similar findings were reported previously by others (16-18), while Shirali *et.al.* reported an increase in body weight of rat treated with caffeine (19). This may be related to the synergism effect of caffeine and carnitine.

The current study showed that the blood parameters monitored an increase in RBCs, Hb and PLT level, on the other hand, a decrease in WBCs level. These results are in consistent with (20), giving the reasons that may be related to the effect of raised testosterone which increases the body mass which associated with increase oxygen demands that lead to increase level of RBC that are provided by erythropoietin hormone, while another group of researchers had another findings (16, 21,22). The differences may be related to higher therapeutic dose of caffeine used by others.

Caffeine is metabolized in liver cells. Results showed no changes in AST and ALT levels in caffeine groups. This may be linked to use of therapeutic doses of caffeine that are overcome by hepatocyte with no severe changes. this finding was confirmed by histopathological examination of liver tissue, which included bile duct proliferation, a slight vacuolation of hepatocytes (figure 3), since the effect of therapeutic dose was the goal of this study. These findings were in agreement with (23,24) and disagreed with other findings (20,25,26). The differences may be related to higher dose administration of caffeine and its long duration.

Results revealed a decrease in urea level and increase in creatinine level, which may be due to diuretic effect produced by caffeine on kidney cells, while increase activity of muscle cells stimulated by caffeine lead to increase creatinine concentration and not due to kidney damage, although higher doses administration may result in kidney damage. These findings were confirmed by histopathological evaluation, which showed only a slight atrophy of some glomeruli of kidney (figure 2). This result wasn't in line with (16) who reported that caffeine administration caused increase urea and creatinine levels due to higher doses of caffeine used.

Several clinical and animal studies had mentioned that caffeine use caused serum testosterone elevation in adult human and adult animals (27,28). This result was in line with the results obtained by the current study and is also in agreement with Park *et.al.* and Joshua *et.al.* (29,30). This elevation of

testosterone may be related to increase activity of leydig cells by caffeine administration. However, this result was not in line with (31) due to immature rat used in the study. On the other hands, no effect of caffeine on rat testes weight were observed in this study as reported also by others (29,32-34). Results also indicated a decrease in sperm count, motility and sperm normality may be related to the effect of caffeine on spermatogenesis and sperm germinal cells this result is in line with (30), this result is supported by histopathology of testes (figure 1), that revealed a different degree of suppression of spermatogenesis in rat treated by caffeine depending on dose manner, this result is in agreement with (35).

Caffeine causes stimulation of nervous system that leads to increase activity of body tissues (8). In the current study heart tissue showed a vacuolation of cardiac muscle cells (figure 4), but with high doses cardiac muscles degenerated as reported by Happonen *et.al.* (36). This result may be linked to the effect of caffeine by rising level of cortisol and epinephrine which affect heart tissue cells (37,38).

CONCLUSION

The current study determined that high doses caffeine may have a deleterious effect on some organs and biochemical parameters and further studies also are required to determine the relationship between caffeine and endocrine organs.

REFERENCES

1. Nawrot P.; Jordan S.; Eastwood J.; Rotstein J.; Hugenholtz A. and Feeley M. (2003). Effects of caffeine on human health. *Food Add. Contam.* 20(1):1-30.
2. Temple JL.(2009). Caffeine use in children: what we know, what we have to learn and why we should worry. *Neurosci. Biobehav. Rev.* 33:793-806.
3. Bagwath PL. (2011). Energy drinks and the neurophysiological impact of caffeine. *Frontier. Neuroasci.* 5(116):1-8.
4. Spiller MA. (1998). The Chemical Components of Coffee. *Caffeine*. pp.: 97-161.
5. Evans SM. and Griffiths RR.(1999). Caffeine withdrawal: A parametric analysis of caffeine dosing conditions. *J. Pharm. Exp. Ther.* 289:285-294.
6. Battram DS.; Shearer J.; Robinson D. *et al.* (2004). Caffeine ingestion does not imp the re-synthesis of proglicogen and macroglicogen after prolonged exercise and carbohydrate supplementation in humans. *J. Appl. Physiol.* 96:943-950.
7. Neuwinger HD. (2000). African Traditional Medicine, a Dictionary of Plants Use and Application, Med pharm GmbH Publishers, Stuttgart, Germany.
8. Hursel R. and Westerterp-Planteng MS. (2013). Catechin-and caffeine-rich teas for control of body weight in humans. *Amer. J. Clini. Nutr.* 98(6):1682S-1693S.
9. Kendall KL.; Moon JR.; Fairman CM.; Spradley BD.; Tai CY.; Falcone PH. *et al.*(2014). Ingesting a pre-workout supplement containing caffeine, creatine, β-alanine, amino acids, and B vitamins for 28 days is both safe and efficacious in recreationally active men. *Nutr. Res.* 34(5):442-449.
10. Guide for the care and use of laboratory animals. (2011). 8th ed. Washington: National Academic Press.
11. Keisler BD. and Armsey TD. (2006). Caffeine as an ergogenic aid. *Curr. Sports. Med. Rep.* 5(4):215-219.
12. Khadhair AA. and Majeed SK. (2017). Study the toxicity of digoxin on liver and their transaminase enzymes level in rat. *Kufa. J. Vet. Med. Sci.* 8(2):73-80.
13. Khadhair AA. and Majeed SK. (2018). Toxopathological and biochemical study of multiple doses digoxin in sprague dawley rats. *Bas. J. Vet. Res.* 17(1):136-146.
14. Wilson JD.; George FW. and Griffin JE. (1981). The hormonal control of sexual development. *Science.* 211:1278-1284.
15. Drury RA. (1967). Carlton's Histological Technique. 4th ed. Oxford University Press., Toronto. P:140.
16. Emmanuel A.; Majesty D.; Benjamin A.; Peter A. and Princess U. (2017). Effect of Caffeine on Some Selected Biochemical Parameters Using Rat Model. *Adv. Bio.* Pp.:1-8.
17. Inoue H.; Kobayashi-Hattori K.; Horiuchi Y.; Oishi Y.; Arai S. and Takita T. (2006). Regulation of the body fat percentage in developmental-stage rats by methylxanthine derivatives in a high-fat diet. *Biosci. Bio. Biochem.* 70(5):1134-1139.
18. Zheng G.; Sayam K.; Okubo T.; Juneja LR. and Oguni I. (2004). Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine in mice. *In Vivo.* 18(1): 55–62.
19. Shirali S.; Hosseini SA.; Ashtary-Larky D.; Daneghian M. and Mirlohi MS. (2016). Effect of caffeine co-ingested with carnitine on weight, body-fat percent, serum leptin and lipid profile changes in male teen soccer players: A randomized clinical trial. *Int. J. Pediatr.* 4(10):3685-3698.
20. Camerone A.; Sweet E.; Bottino A.; Bittar C.; Veiga C. and Cameron LC. (2007). Effect of caffeine supplementation on haematological and biochemical variables in elite soccer players under physical stress conditions. *Brit. J. Sports. Med.* 41(8):523-530.
21. Duru MK.; Agomuo EN. and Amadi B. A.(2012). Biochemical studies on 'Udu' an antimalarial concoction used in Umunchi village, Isiala Mbano L.G.A of Imo State, Nigeria. *Con. J. Pharm. Toxicol. Res.* 5(2): 28-34.
22. Adebayo AH.; Abolaji AO.; Opata TK. and Adegbenro IK. (2010). Effects of ethanolic leaf extract of *Chrysophyllum albidum* G. on

biochemical and haematological parameters of albino Wister rats. *Afr. J. Biol.* 9(14):2145-2150.
23. Tucker KL. (2003). Dietary Intake and Bone Status with Aging. *Current. Pharm. Des.* 9(31):1-18.
24. Kynast-Gales SA. and Massey LK. (1994). Effect of caffeine on circadian excretion of urinary calcium and magnesium. *J. Amer. Coll. Nutri.* 13(5):467-472.
25. Mathew A.(2001). Liver enzymes (elevated) in Principles of Health Choice. Media Inc. available at: http://www.liverenzyme.com/
26. Manne V. and Saab S. (2015). Coffee as modulator of liver injury: fact and fiction. *Clin. Liver Dis.* 6(6): 139-141.
27. Sarobo C.; Lacorte LM.; Martins M. *et al.* (2012). Chronic caffeine intake increases androgenic stimuli, epithelial cell proliferation and hyperplasia in rat ventral prostate. *Int. J. Exper. Pathol.* 93(6):429-437.
28. Ramlau-Hansen CH.; Thulstrup AM.; Bonde JP.; Olsen J. and Bech BH.(2008). Semen quality according to prenatal coffee and present caffeine exposure: two decades of follow-up of a pregnancy cohort. *Human Reprod.* 23(12):2799-2805.
29. Park M.; Choi Y.; Choi H.; Yim JY. and Roh J. (2015). High doses of caffeine during the peripubertal period in the rat impair the growth and function of the testes. *Int. J. Endocrinol.* 1-9.
30. Joshua O.; Sunday O.; John O. and Titilope O. (2017). Excessive Caffeine Intake Disrupts Testicular Architecture, Spermatogenesis and Hormonal Levels in Experimental Wistar Rats. *IOSR. J. Enviro. Sci. Toxicol. Food Technol.* 11(10):8-18.
31. Bae J.; CHoi H.; CHoi Y. and Roh J. (2017). Dose-and time-related effects of caffeine on the testis in immature male rats. *Exper. Animals.* 66(1):29-39.
32. Kwak Y.; Choi H. and Roh J. (2017). The effects of caffeine on the long bones and testes in immature and young adult rats. *Toxicol. Res.* 33(2):157-164.
33. Whitby KE.; Collins TF.; Welsh JJ.; Black TN.; O'Donnell M.; Gray GC. *et al.* (1986). Reproduction study of caffeine administration to male Osborne-Mendel rats. *Food Chem. Toxicol.* 24: 277–282.
34. Tinwell H.; Colombel S.; Blanck O. and Bars R. (2013). The screening of everyday life chemicals in validated assays targeting the pituitary-gonadal axis. *Regul. Toxicol. Pharmacol.* 66: 184-196.
35. Friedman L.; Weinberger MA.; Farber TM. *et al.* (1979). Testiscular atrophy and impaired spermatogenesis in rats fed high levels of themethyl xanthines caffeine, theobromine, or theophylline. *J. Enviro. Pathol. Toxicol.* 2(3):687-706.
36. Happonen P.; Voutilainen S. and Salonen JT. (2004). Coffee drinking is dose-dependently related to the risk of acute coronary events in middle-aged men. *J. Nutri.* 134(9):2381-2386.
37. Lochen ML. and Rasmussen K. (1996). Palpitations and lifestyle: impact of depression and self-rated health. The Nordland Health Study. *Scand. J. Social. Med.* 24(2):140-144.
38. Rosmarin PC. (1989). Coffee and coronary heart disease: a review. *Prog. Cardio. Dis.* 32(3):239-245.