Effect of Chronic Caffeine Consumption on Cardiac Tissue Metabolism in the Rabbit

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Summary: Previous studies on the ability of caffeine to enhance endurance and boost performance have focused on the energy substrates that are utilized by the skeletal muscle and the brain but nothing of such has been reported on cardiac tissue. This study was designed to investigate the effect of caffeine on cardiac tissue metabolism in the rabbit. The study was carried out on adult male New Zealand rabbits divided into 3 groups (n=5). Group I rabbits served as control and were given 0.5ml/Kg of normal saline while group II and III rabbits were administered with 2mg/Kg and 6mg/kg of caffeine respectively for 28 days. Blood samples were collected by retro orbital puncture for biochemical analysis. Animals were sacrificed by cervical dislocation and cardiac tissue biopsies were collected for biochemical and immunohistochemical analysis. Cardiac tissue glycogen concentration was determined by anthrone reagent method. Cardiac tissue CPT 1 activity and cAMP concentration were determined by immunohistochemistry and colorimetry techniques respectively, with assay kits obtained from Biovision Inc. The results showed that Caffeine at 2 and 6 mg/kg significantly inhibited MPO activity from 0.72±0.05 to 0.164±0.045 and 0.46±0.12 U/L respectively (p<0.05). Caffeine at 2mg/kg had no effect on serum nitric oxide but at 6mg/Kg, it significantly increased serum nitric oxide form 28.01±6.53 to 45.25±3.88μM of nitrite (p<0.05). Also, Caffeine at 2 and 6mg/kg increased cardiac tissue glycogen from 15.62±0.73 to 40.69±6.35 and 38.82±6.91mg/100g respectively and carnitine palmytol transferase 1 activity from 18.3 to 20 and 25.2% respectively. In conclusion, the study showed that caffeine consumption increased CPT 1 activity suggesting increased utilization of free fatty acids for energy metabolism and sparing of cardiac tissue glycogen by mechanism(s) which probably involved blockade of A1 adenosine receptors and cAMP signaling pathway.

INTRODUCTION

Caffeine remains the most commonly consumed stimulant in the world (Yang et al. 2009). It is present in many commercial beverages and medicines (Graham et al 2001; Chukwu et al 2006). In Nigeria, it is present in some common masticatory such as kola nut. Caffeine has been reported to boost performance and enhance endurance (Graham and Spriet 1991; Goldstein et al 2010). Central to caffeine's ergogenic ability is its capacity to alter substrate metabolism. Caffeine has been reported to alter skeletal muscle metabolism by increasing skeletal muscle lipolysis and oxidizing free fatty acids thereby sparing skeletal muscle glycogen usage (Jensen *et al*., 2007; Egawa *et al*., 2009). In the brain, caffeine increases glucose utilization in a number of regions such as the thalamus, hypothalamus, monoaminergic cell groupings and structures belonging to the extrapyramidal motor system (Nehlig *et al*., 1987, 1999). In the liver caffeine was reported to reduce the levels of hepatic lipid

content by activation of autophagy in in vitro and in vivo studies (Ray 2013). It has also been reported to alter glucose metabolism in the liver of dogs by increasing net hepatic glucose uptake (Pencek *et al.,* 2004). There is however, no available report on how caffeine alters cardiac tissue substrate metabolism. The cardiac muscle tissue is considered a metabolic omnivore as it uses lipids, glucose, lactate, ketones and amino acids as substrate to generate energy (Saddik *et al.,* 1991; Lopaschuk *et al.,* 1994; Stanley and Chandler 2002). The normal adult heart predominantly utilizes fatty acids to generate energy for its pumping activities. However, the heart has been noted to be flexible in its choice of substrate for energy metabolism, depending on substrate availability and metabolic effectors (Taegtmeyer *et al.,* 1994). The choice of substrate for energy metabolism by the cardiac muscle following caffeine administration is not clear. Thus, this study was designed to investigate

the effect of caffeine on cardiac tissue substrate metabolism in the rabbit.

MATERIALS AND METHODS

Experimental animals

Adult male New Zealand rabbits with body weights ranging from 1.3 - 2.0 Kg were purchased from the University of Ibadan Veterinary Animal House and acclimatised for a period of 2 weeks in the Central Animal House of the University of Ibadan before the experiment was carried out. The animals were housed 1 per cage and were given regular rabbit laboratory chow and water *ad libitum*. Ethical regulations were observed all through experimental periods in accordance with national and institutional guidelines for the protection of the animal's welfare (PHS, 1996). Protocol for animal use was in consonant with the criteria outlined in the Guide for the care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health.

Experimental design

Adult male New Zealand rabbits were divided into 3 groups (n=5). Group I rabbits served as control and were given 0.5 ml/Kg of normal saline while groups II and III rabbits were administered with 2 mg/Kg and 6 mg/kg of caffeine respectively. Caffeine or normal saline administration in the rabbit was carried out daily through oral gavage for twenty eight (28) days. At the end of 28 days, blood samples were collected by retro orbital puncture for biochemical analysis and then animals were sacrificed by cervical dislocation followed with collection of cardiac tissue biopsies for biochemical and immunohistochemical analysis.

Plasma and Serum Preparation

About 3 mL of blood was collected from the retro-orbital venous plexus of the animals into EDTA sample bottles and plain sample bottles before animals were sacrificed by cervical dislocation. Blood samples were centrifuged at 4000 rpm for 15 minutes to obtain plasma and serum respectively.

Isolation of Post-Mitochondrial Fraction of the rabbit heart

Biopsies of rabbit heart was harvested on dry ice, rinsed in saline, and homogenized in aqueous potassium buffer (0.1 M, pH 7.4) and the homogenate centrifuged at 10,000 rpm (4ºC) for 10 min to obtain the supernatant fraction.

Determination of Cardiac cAMP concentration, Blood glucose level and Cardiac glycogen content.

Direct competitive immunoassay principle was used in the determination of cardiac cAMP concentration in post-mitochondrial fractions of the heart. The post-mitochondrial fractions were diluted with 0.1 M HCL and cAMP assay kit (Biovision Inc.) was used to determine the respective cAMP concentrations with the aid of a microplate reader at optical density of 450 nm. Blood glucose was determined with one touch

glucometer using glucose oxidase method. The glucometer was checked against standard glucose solution at regular interval to ensure accuracy. Glycogen content of cardiac muscle tissue was determined by the anthrone reagent method as earlier described by Ishehunwa et al. (2013).

Biochemical Analysis

The serum myeloperoxidase activity was measured spectrophotometrically by a peroxidase-coupled assay system involving O-dianisidine and hydrogen peroxide as previously described by Xia and Zweier (1997). Production of Nitric Oxide was evaluated by measuring the level of nitrite (an indicator of NO) in the serum with the aid of Griess reagent system and assay kit was purchased from Promeg. Serum total cholesterol and triglyceride concentrations were determined by spectrophotometric methods using commercially available Randox Kits.

Immunohistochemistry of Cardiac Carnitine Palmitol Transferase I activity

The whole heart was fixed in 10% buffered formalin. The cardiac tissue was processed and embedded in paraffin wax. Altogether, 5–6 mm thick sections were made. Tissue sections were deparaffinized and hydrated using xylene followed by passage through ethanol of decreasing concentration (100–80%). The manufacturer's protocol for the localization of antigens in tissue sections was followed. Ready-to-use IHC kit for the study was purchased from Biovision Inc. and CPT I antibodies from Bioss USA. The immunoreactive positive expression of CPT I intensive regions were viewed starting from low magnification on each slide then with 100× magnifications using a photo microscope (Olympus) and a digital camera (Toupcam VR, Touptek Photonics, Zhejiang, China). The immune-positive reactions were quantified with Image J software.

Statistical Analysis

All values are expressed as mean±SE. The test of significance between two groups was estimated by Student's t-test. The test of significance amongst all groups was estimated by One way Analysis of Variance (ANOVA) with Tukey's posthoc test using Graph pad prism 5.0 with p-values <0.05 considered statistically significant.

RESULTS

Effect of Caffeine on Serum Myeloperoxidase Activity, Nitrite Concentration, Cholesterol, Triglyceride and Blood Glucose

Table 1 shows the effect of administration of caffeine on serum myeloperoxidase activity, nitrite concentration, total cholesterol level, plasma triglyceride and blood glucose concentrations. Caffeine produced a significant reduction in MPO activity in response to the two doses administered. The lower dose of caffeine produced a more significant reduction in MPO activity than the higher dose. Caffeine, however, caused an increase in

Table 1: Effect of 28 days administration of caffeine on serum myeloperoxidase activity, nitrite concentration, cholesterol, triglyceride and blood glucose concentrations

GROUP	MPO (U/L)	NOx (μM)	Blood glucose (mg/dl)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
Control	0.72±0.05	28.07±2.53	118.2±4.81	56.34±6.03	103.4±7.27
CAF (2mg/kg)	0.16±0.05***	31.94±2.52	121.2±5.70	120.20±29.89*	142.8±21.10**
CAF (6mg/kg)	0.46±0.07*	45.25±3.88*	191.4±4.82***	117.00±24.85*	144.5±11.52***

Figure 1: Effect of chronic administration of caffeine on cardiac tissue glycogen
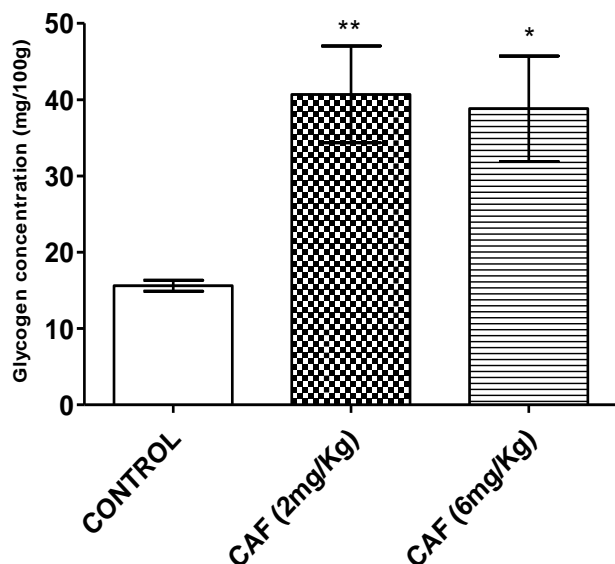

Figure 2: Effect of chronic administration of caffeine on cardiac tissue cAMP concentration

serum nitrite levels and this was significant only at a dose of 6 mg/kg. The two doses of caffeine also significantly increased the blood glucose levels in a dose-dependent manner. Administration of low and high doses of caffeine also significantly increased serum total cholesterol and triglyceride levels. There is no significant difference in the levels of total cholesterol and triglycerides in response to the two doses of caffeine.

Effect of Caffeine on Cardiac Tissue Glycogen
The effect of chronic administration of caffeine on cardiac glycogen tissue content is shown in figure 1. Caffeine increased cardiac glycogen content from 15.62±0.73 to 40.69±6.35 and 38.82±6.91 mg/100g of cardiac tissue at doses of 2 and 6 mg/kg respectively.

Effect of Chronic Administration of Caffeine on Cardiac Tissue cAMP Concentration
Figure 2 shows the effect of chronic administration of caffeine on cardiac tissue cAMP concentration in rabbits. Caffeine at doses of 2 and 6 mg/kg significantly increased cardiac tissue cAMP concentrations from 5.097±0.289 to 6.840±0.257 and 6.358±0.139 pmol/well respectively when compared to control.
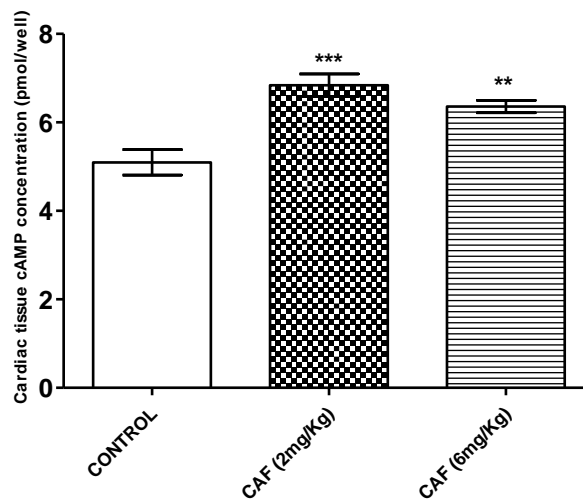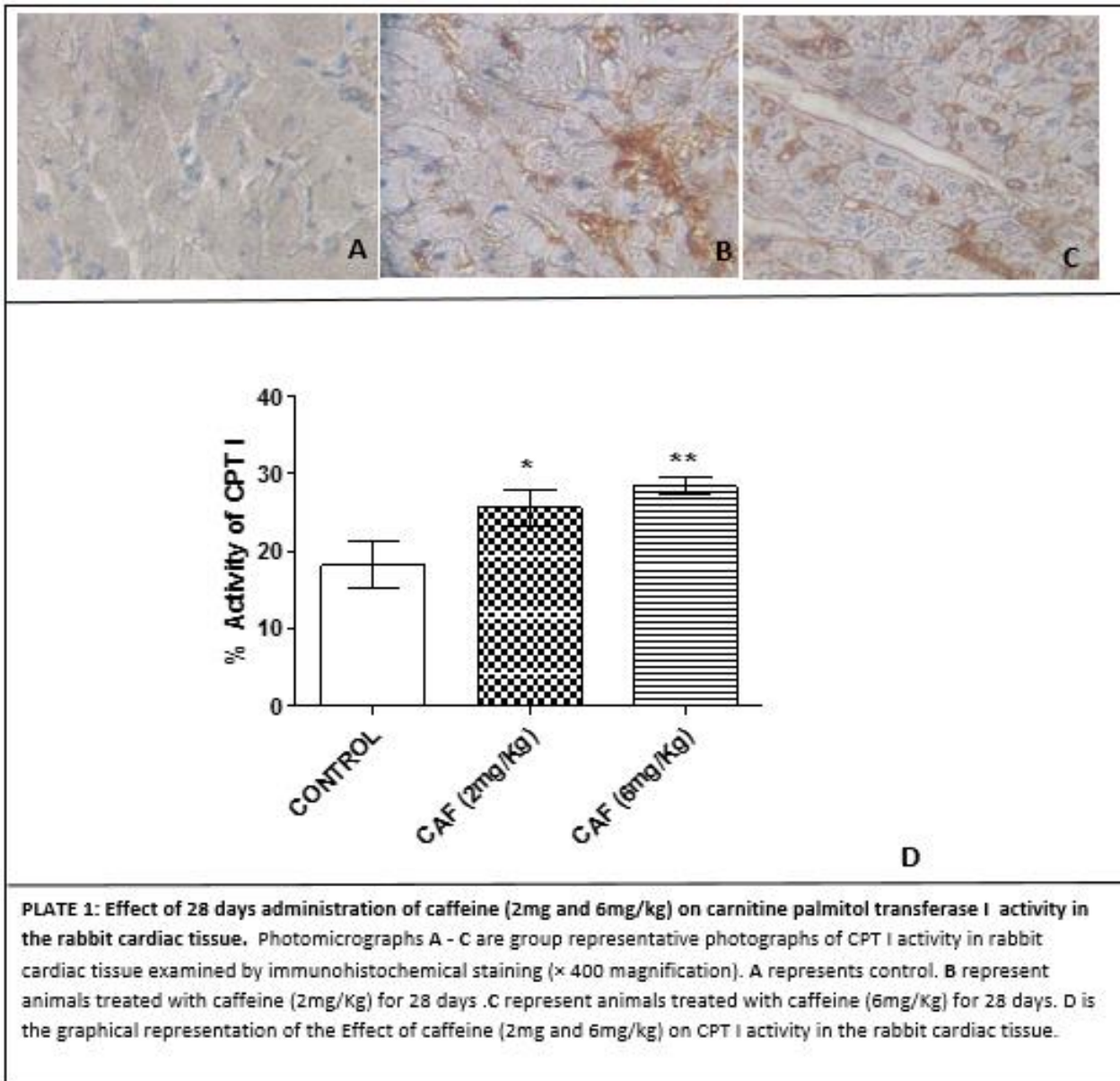
Effect of Chronic Administration of Caffeine on Cardiac CPT I Activity
The effect of chronic administration of caffeine (2 and 6 mg/kg) on cardiac CPT I percentage activity in the rabbit is shown in plate 1. Chronic administration of caffeine at the two doses of 2mg/kg and 6mg/kg significantly increased the percentage activity of CPT I enzyme in the rabbit cardiac muscle without any significant difference in the responses to the two doses of caffeine.

DISCUSSION

The cardiac muscle tissue has been described as a metabolic omnivore which can use many substrates such as lipids, glucose, lactate, ketones and amino acids as energy substrate to keep the heart functioning (Saddik *et al.,* 1991; Lopaschuk *et al.,* 1994). The purpose of the present study was therefore to determine if caffeine intake alters the choice of cardiac energy substrate and if the alteration is a possible contributory mechanism to the well-known ability of caffeine to boost performance and enhance endurance. Hence caffeine doses within the ranges commonly consumed by man were employed in the present study. The increase in blood glucose levels following caffeine administration has earlier been reported in man (Pizziol et al., 1998, Graham et al., 2001, Dekker et al., 2007), dog (Salahdeen and Alada 2009a), rats

PLATE 1: Effect of 28 days administration of caffeine (2mg and 6mg/kg) on carnitine palmitol transferase I activity in the rabbit cardiac tissue. Photomicrographs A - C are group representative photographs of CPT I activity in rabbit cardiac tissue examined by immunohistochemical staining (× 400 magnification). A represents control. B represent animals treated with caffeine (2mg/Kg) for 28 days .C represent animals treated with caffeine (6mg/Kg) for 28 days. D is the graphical representation of the Effect of caffeine (2mg and 6mg/kg) on CPT I activity in the rabbit cardiac tissue.

(Leblanc et al., 1995) and mouse (Nagasawa et al., 2001). There are conflicting reports on the effect of caffeine administration on plasma lipid levels in rodents. Several studies in rodents have reported an increase (Yokogoshi et al., 1983, Kempf et al., 2010), or a decrease (Muroyama et al., 2003) plasma lipids levels following caffeine administration. However, the present study observed increases in both total cholesterol and triglycerides levels. The reason for the inconsistency observed in the effect of caffeine on plasma lipid levels is obscure. However, most of the previous studies worked with caffeine doses that are not relevant to human consumption. For example, 0.1% of caffeine in drinking water which is commonly reported in most rodent studies is about 100mg/kg which is totally off the range of caffeine consumption in humans (Milanez 2011). The observed increase in the heart carnitine palmitoltransferase-1 (CPT 1) activity and the high levels of cardiac glycogen in this study following caffeine administration suggest that the major substrate being used for energy metabolism

in the heart are fatty acids. While caffeine tends to promote the use of fatty acids in the cardiac tissue it stores up glucose which is evident in the observed increase in cardiac tissue glycogen in this study. The glycogen sparing effect of caffeine has earlier been reported in the skeletal muscle (Graham et al., 2001). CPT 1 is a mitochondrial enzyme that transports long-chain fatty acyl carnitine across the outer mitochondrial membrane. The carnitine palmitoyltransferase system is an essential step in the beta-oxidation of long chain fatty acids. This transfer system is necessary because, while fatty acids are activated (in the form of a thioester linkage to coenzyme A) on the outer mitochondrial membrane, the activated fatty acids must be oxidized within the mitochondrial matrix. Long chain fatty acids such as palmitoyl-CoA, unlike short- and medium-chain fatty acids, cannot freely diffuse through the mitochondrial inner membrane, and require a shuttle system to be transported to the mitochondrial matrix (Berg et al., 2007). Carnitine palmitoyltransferase I is the first

component and rate-limiting step of the carnitine palmitoyltransferase system, catalyzing the transfer of the acyl group from coenzyme A to carnitine to form palmitoylcarnitine. A translocase then shuttles the acyl carnitine across the inner mitochondrial membrane where it is converted back into palmitoyl-CoA. A sentinel paper in 1977 described the inhibition of the oxidation of fatty acids by isolated mitochondria by malonyl-CoA in vitro and its correlation with the inhibition of the ''outer'' CPT activity that could be measured in intact, well-coupled mitochondria (McGarry et al., 1977; McGarry et al., 1978). This explained the ability of some tissues to perform high rates of fatty acid synthesis or oxidation under separate physiological conditions. Malonyl-CoA, an intermediate of fatty acid synthesis, through inhibition of the ''rate limiting'' step of fatty acid oxidation, ensures that the two processes would not occur simultaneously. The inhibition of CPT 1 by malonyl-CoA is crucial because it is the point at which metabolism of fatty acids and glucose come into the most direct ''contact'' to influence each other's metabolism. Other interactions, such as the inhibition of phosphofructokinase by citrate, the provision of glucose-derived glycerol for acylglyceride synthesis, the regulation of pyruvate dehydrogenase activity by fatty acid-derived acetyl-CoA, do not have the same ability to affect the continuous substrate selection that tissues have to make primarily between glucose and fatty acid oxidation. The increase in CPT-1 activity due to caffeine administration in this study therefore corroborates the fact that caffeine spares the use of glucose in active muscle tissues thereby building up tissue glycogen. Thus, the increase in cardiac tissue glycogen due to caffeine administration in the present study shows that caffeine affects the cardiac choice of energy substrate and increases the ability of the heart to use fatty acids as energy substrate. Furthermore, the significant increase in circulating blood glucose and lipids following caffeine administrations in the present study suggest that the mechanism by which caffeine influences the cardiac choice of energy substrate is more dependent on metabolic effectors than on various substrate availability.

The observed increase in cardiac tissue cAMP following caffeine administration in the present study suggests the possibility of blockade of adenosine A1 receptors by caffeine and the role of cAMP in the signaling pathway leading to the chronic action of caffeine in the heart tissue. Stiles (1986) had earlier reported on the rise of tissue cAMP following adenosine A1 receptor blockade in animals chronically administered caffeine.

The reduction of MPO activity by caffeine in this study portends that caffeine could be cardioprotective. Since increase in MPO activity has earlier been reported to precipitate atherogenesis (Schindhelm et al; 2009). Possible mechanisms by which caffeine could reduce MPO activity may be due to its ability to decrease activation of resident macrophages and microglia (Lv et al. 2010; Chavez-Valdez et al. 2009).

Serum nitrite has been reported to be an indicator for NO production *in vivo* (Chaea et al.2004). Hence the increase in serum nitrite in this study indicates an increase in NO production due to caffeine administration. Increase in NO production promotes tissue perfusion which could be a contributory mechanism of caffeine's endurance enhancing ability (Christopherson and Bredt, 1997).

In conclusion, this study has demonstrated in the rabbit that caffeine consumption within dose ranges often consumed by man increased CPT1 activity suggesting increased utilization of free fatty acids in energy metabolism and sparing of cardiac tissue glycogen by mechanism(s) which probably involve blockade of A1 adenosine receptors and cAMP signaling pathway.

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