

Effect of increased calcium intake on cardiac and vascular $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity in oral contraceptive-treated female *Sprague-Dawley* rats

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Received 5 January 2006; revised 8 August 2006

The present study aimed at investigating the influence of increased dietary calcium on $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity in heart and aorta of female *Sprague-Dawley* rats treated with oral contraceptive (OC) steroids. Rats were grouped as control (CR), OC-treated and OC+calcium-treated. OC-treated and OC+calcium-treated received a combination of OC steroids (ethinyloestradiol and norgestrel; ig). OC+calcium-treated rats were fed with 2.5% calcium diet, while OC-treated and CR groups were fed on 0.9% calcium diet. The activity of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ in heart and aorta was significantly lower in OC-treated rats than those in the other groups. OC treatment caused significant increase in plasma glucose and significant decrease in plasma K^+ as compared to control group. Decrease in $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity and plasma K^+ was abrogated by increased calcium intake, while increase in plasma glucose was not normalized by calcium supplementation. Plasma levels of Na^+ , lipid peroxidation index and ascorbic acid were comparable among the three groups. These results showed that OC treatment could lead to impaired activity of cardiac and vascular $\text{Na}^+ - \text{K}^+ - \text{ATPase}$, possibly due to reduced plasma K^+ level and these effects could be abolished by high calcium diet.

Keywords: Dietary calcium, Hypokalaemia, $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity, Oral contraceptive

Results from several studies have implicated depressed activity of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ in the development of cardiovascular disease^{1,2}. It has been shown that impaired activity of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ enhances myocardial and arterial contractility by increasing intracellular Ca^{2+} concentration^{3,4}. Oxidative stress has been suggested as a contributory factor to impaired $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity⁵. A number of prospective and cross-sectional studies have clearly established an association between increasing blood glucose and cardiovascular morbidity and mortality in both diabetic and non-diabetic subjects^{6,7}. One of the identified consequences of increased blood glucose is impairment in the activity of vascular $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ ⁸. Furthermore, studies have demonstrated that elevation of plasma K^+ concentration in the physiological range causes increase in vascular smooth muscle $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity⁹. Similarly, in heart and skeletal muscle, decreased activity of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ has been associated with hypokalaemia¹⁰.

An increased risk of venous thromboembolism, myocardial infarction and thrombotic stroke has been

demonstrated in women who used combined oral contraceptives (OCs). When compared with earlier studies, the cardiovascular morbidity tends to reduce, possibly due to an attempt directed at reducing the dosages of hormonal steroids and/or a tendency to refrain from OCs in smokers older than 35 years of age^{11,12}. However, a worldwide community-based study suggested that among current OC users, a history of hypertension increased the risk of ischaemic stroke by 11-fold in Western Europeans and even more in women from developing countries¹³. However, the pathophysiological factors contributing to the development of cardiovascular events in OC users are still poorly understood.

OC usage has been shown to increase the dietary requirements for calcium in order to prevent the risk of osteoporosis in later life¹⁴. Decreased serum Ca^{2+} concentration has been reported in women using OC^{15,16}. Serum levels of calcium have been shown to be inversely related to incidence of hypertension and cardiovascular disease in clinical and experimental studies^{17,18}. Calcium supplementation has been shown to elevate serum Ca^{2+} concentration and decrease blood pressure in hypertensive humans and rats^{17,19}. We, therefore, hypothesized that increase in cardiovascular risk in users of OC might be mediated

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by impaired cardiac and vascular $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. The present study also sought to determine whether the altered $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in OC-treated female rats is a result of hyperglycaemia, hypokalaemia or increased oxidative stress, and to examine the influence of high dietary calcium on $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in the rat model.

Materials and Methods

Animals—Female *Sprague-Dawley* rats weighing between 120 and 140g were housed in wire-bottomed, stainless steel cages to minimize coprophagy in a well ventilated room maintained at $25^\circ\pm 2^\circ\text{C}$, under 12 h light/dark cycle. Animals had free access to water and food (Pfizer livestock feeds, Ikeja, Lagos, Nigeria). The rats were divided into three groups (Control, OC-treated and OC+calcium-treated; $n= 9\text{-}10$ rats). Control rats received 0.2 ml of olive oil per 100g body weight by gavage per day and fed on rat chow containing calcium (0.9%). OC-treated rats received 0.2ml of olive oil per 100g body weight containing $1.0\mu\text{g}$ of ethinyloestradiol and $10.0\mu\text{g}$ of norgestrel (Wyeth-Ayerst, Inc., Canada) by gavage per day and fed on rat chow containing 0.9% calcium. OC+calcium-treated rats received 0.2 ml of olive oil per 100g body weight containing $1.0\mu\text{g}$ of ethinyloestradiol and $10.0\mu\text{g}$ norgestrel (Wyeth Ayerst, inc. Canada) by gavage per day and fed on rat chow containing 2.5% calcium and extra calcium was supplied as carbonate salt. Treatment with OC, with or without high calcium intake, lasted for 10 weeks. The animals were cared for in accordance with the "Guideline for the Care and Use of Laboratory Animals" of the National Institution of Health (NIH), USA.

Tissue preparation—After 12h overnight fast, blood was withdrawn from animals under ether anaesthesia into heparinized specimen bottles by cardiac puncture. Plasma was separated by centrifugation for 10 min at 3000 rpm. All samples were refrigerated at 4°C until use. Plasma lipid peroxidation index²⁰, ascorbic acid²¹ and glucose²² were estimated immediately. The hearts and thoracic aorta were quickly removed, cleaned of connective tissues, stored in ice-cold 0.25mol/l sucrose solution (1:5 w/v) and homogenized. The homogenates were used for $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity assay after 24 hr.

Determination of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity— $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity was assayed spectrometrically by estimating the amount of inorganic phosphate

released following incubation of the tissue homogenates with disodium-ATP as reported previously²³. The tissues were rapidly thawed in ice-cold Tris-HCl buffer (20mmol/l; pH 7.4) containing sucrose (0.25mmol/l) and EDTA (0.5mmol/l). Subsequently, the tissues were blotted off by filter paper prior to weighing. Tissue homogenates were prepared in a pre-chilled mortar and the enzyme was assayed in a medium containing Tris buffer (0.05mol/l; pH 7.4); EDTA (1.0mmol/l); NaCl (1.5mmol/l); KCl (0.1mol/l); MgCl_2 (30mmol/l) and disodium-ATP (0.2mol/l) at 28°C . Reaction was started by addition of tissue homogenate and stopped after 30 min with trichloroacetic acid (10%). After centrifugation at 5000rpm for 15min, inorganic phosphate was determined in the supernatant using ammonium molybdate. Activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ was expressed as micromole of inorganic phosphate released per milligram protein per hour. Protein concentration was estimated by Biuret method using commercial kit supplied by Randox Laboratory Incorporated (UK).

Determinations of plasma electrolytes and glucose concentrations—The concentrations of plasma Na^+ and K^+ were estimated by flame photometry (Corning model 400) as described by Tietz *et al*²⁴. Fasting plasma glucose levels were estimated using glucose standard oxidative method (Glucose Autoanalyzer, Beckman Coulter Inc., Fullerton, CA, USA)²².

Determinations of plasma malondialdehyde (MDA) and ascorbic acid levels—The degree of lipid peroxidation (oxidative stress) in plasma was measured as the reaction product of MDA with thiobarbituric acid reactive-substances (TBARS)²⁰. Plasma ascorbic acid concentration was assayed using calorimetric method that involves formation of a complex of ferrous iron with α, α^1 dipyridyl after reduction of ferric to ferrous iron by ascorbic acid²¹.

Statistical analysis—Data analysis was carried out by One-way ANOVA supported by the Bonferroni's test when pairwise comparison was done between the groups. Results were presented as means \pm SEM, and the differences were considered significant at $P < 0.05$. Statistical analysis was performed with Statistical Package for Social Sciences (SPSS, Chicago, USA).

Results

Activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in thoracic aorta and heart were significantly reduced in OC-treated group,

but normalized by calcium supplementation (Figs 1, 2). Plasma concentration of glucose increased significantly, whereas plasma K⁺ concentration

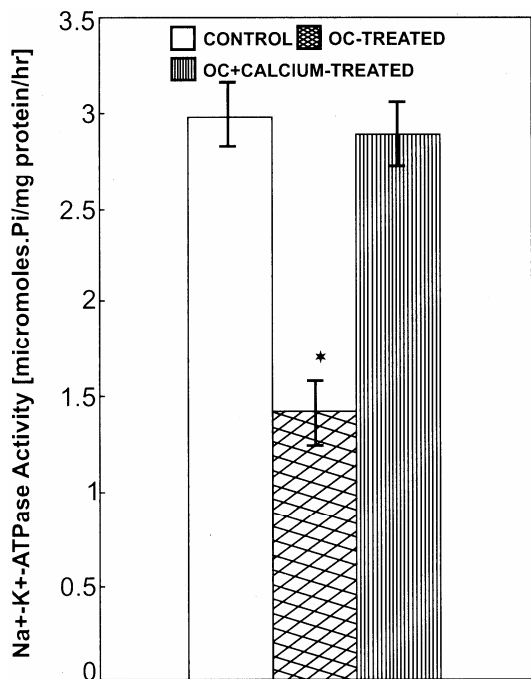


Fig. 1— Effect of high calcium diet on Na⁺-K⁺-ATPase activity in heart of OC-treated rats. *P<0.05 versus control and OC+calcium-treated groups.

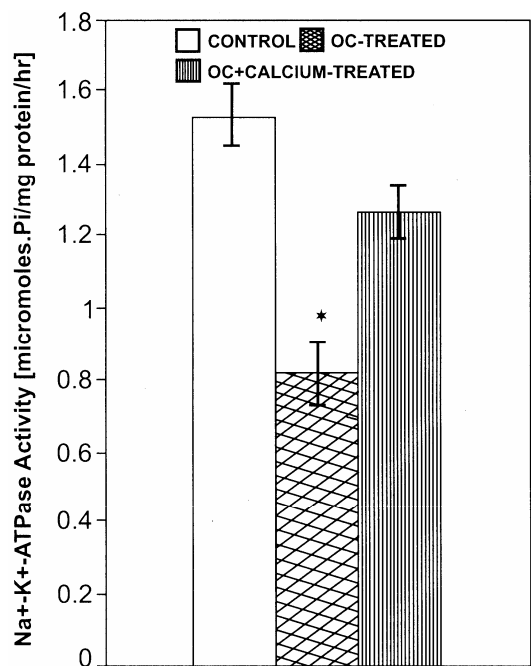


Fig. 2—Effect of high calcium diet on Na⁺-K⁺-ATPase activity in aorta of OC-treated rats. *P<0.05 versus control and OC+calcium-treated group.

decreased significantly in OC-treated group when compared with the control rats (Table 1). The decrease in K⁺ level induced by OC treatment was abolished by calcium supplementation while the increase in plasma glucose induced by OC treatment was not affected by calcium supplementation (Table 1). No change was observed in plasma Na⁺, lipid peroxidation index or ascorbic acid levels among the three groups (Table 1).

Discussion

Chronic administration of oral contraceptive (OC) steroids has been linked with increased incidence of arterial and heart diseases¹¹⁻¹³, all of which are known to be related to cellular Ca²⁺ overload^{3,4}. An increase in Ca²⁺ uptake has been associated with impaired activity of Na⁺-K⁺-ATPase^{1,3,4}. In the present study, we questioned whether impaired Na⁺-K⁺-ATPase activity might be the pathophysiological factor underlying the increase cardiovascular risk associated with OC usage and if calcium supplementation could ameliorate OC effects. We found that feeding rats with a high calcium diet abrogated impairment of cardiac and vascular Na⁺-K⁺-ATPase activity induced by OC treatment. We also observed that OC administration caused a decrease in plasma K⁺ levels and an increase in plasma glucose concentrations. The results further showed that the impaired activity of cardiac and vascular Na⁺-K⁺-ATPase accompanied with hypokalaemia seen in OC-treated rats was prevented by calcium supplementation, suggesting that involvement of an impaired Na⁺-K⁺-ATPase activity in the cardiovascular events seen in OC users might be secondary to OC-induced hypokalaemia.

This study seems to be the first to determine the effect of OC treatment on cardiac and vascular Na⁺-

Table 1—Plasma Na⁺, K⁺, fasting plasma glucose (FPG), malonyldialdehyde (MDA) and ascorbic acid levels in control, OC-treated and OC+calcium-treated rats [Values are mean ± SEM]

	Control	OC	OC+calcium
Na ⁺ (mmol/L)	137.7 ± 1.9	134.1 ± 1.5 ^{ns}	136.5 ± 1.8 ^{ns}
K ⁺ (mmol/L)	5.6 ± 0.1	5.0 ± 0.1*	5.4 ± 0.2 [#]
FPG (mmol/L)	3.1 ± 0.2	4.3 ± 0.2*	4.1 ± 0.2*
MDA (µmol/L)	12.9 ± 0.6	13.2 ± 0.5 ^{ns}	13.3 ± 0.3 ^{ns}
Ascorbic acid (mg/dl)	1.25 ± 0.03	1.28 ± 0.04 ^{ns}	1.23 ± 0.05 ^{ns}
n	10	10	9

^aValues are means ± SEM and significant at *P<0.05 compared with control group; [#]P<0.05 compared with OC-treated group [ns; not significantly different from control values].

K^+ -ATPase activity. However, the observed impairment in the Na^+ - K^+ -ATPase activity in the heart and aorta in these animals is consistent with the finding that OC treatment led to inhibition of Na^+ - K^+ -ATPase activity in rat liver²⁵ and erythrocyte²⁶. Azuma *et al*¹⁰ have found that hypokalaemia induces a decrease in Na^+ - K^+ -ATPase activity. Studies in vascular smooth muscle have indicated that the reduction in vascular tone due to elevation of K^+ concentration in physiological range has been associated with increased Na^+ - K^+ -ATPase activity²⁶. The fact that OC-induced impaired activity of Na^+ - K^+ -ATPase is accompanied with hypokalaemia and supplementation with calcium abolished these effects suggested that impaired Na^+ - K^+ -ATPase activity caused by OC treatment might be due to decreased plasma concentration of K^+ seen in these animals. The finding further implied that one of the mechanisms whereby calcium supplementation exerted its cardioprotective or antihypertensive effect could be attributed to its ability to correct hypokalaemia, which in turn enhanced the activity of Na^+ - K^+ -ATPase. This observation is consistent with finding of previous study that has reported that high calcium diet augment smooth muscle Na^+ - K^+ -ATPase in NaCl-induced hypertensive rats²⁷.

Previous study has reported that OC use can alter glucose metabolism⁷. This finding supports our results that OC-treated rats have significantly higher plasma glucose than control rats. Hyperglycaemia has been shown to inhibit Na^+ - K^+ -ATPase activity in rabbit aorta⁸. The increase in plasma glucose induced by OC treatment was not affected by high calcium diet in the present study, while impaired Na^+ - K^+ -ATPase activity was abolished by calcium supplementation. This observation suggested that impaired activity of Na^+ - K^+ -ATPase induced by OC treatment in these rats might not be due to increased plasma glucose levels. The fact that calcium supplementation did not have any effect on plasma glucose level could imply that high calcium diet might not improve glycaemic control in OC users. And hyperglycaemia induced by OC administration might have been due to the ability of OC steroids to reduce effectiveness of insulin action⁷.

The presence of lipid peroxides in plasma and even slight elevation of this oxidative product can cause undesirable cardiovascular effects²⁸. In the experimental animals used in the present study, there was no significant increase in plasma MDA levels. This finding implied that OC treatment and/or calcium supplement might not be associated with increased

oxidative stress. This finding is in confirmation of earlier study in humans²⁹, although contradictory reports exist³⁰. The discrepancy in these findings may be dose- and progestogen type-dependent because Ciavatti and co-workers³⁰ have fed female *Sprague-Dawley* rats on a combination of ethinyloestradiol (30.0 μ g) and lynestrenol (750.0 μ g), while we used ethinyl estradiol (1.0 μ g) and norgestrel (10.0 μ g). Vitamin C is a main aqueous phase anti-oxidant in the body³¹. As such, it plays a critical role in scavenging superoxide, hypochloride, the hydroxyl radical, peroxy radical, and singlet oxygen in plasma³¹. Peroxyl radicals mediate lipid peroxidation, hence, ascorbic acid has been said to be more efficient than other plasma antioxidants including α -tocopherol at preventing the initiation of lipid peroxidation by trapping the peroxy radical in aqueous phase before it can interact with lipids in plasma and cell membranes³¹. Therefore, the findings that OC treatment did not affect significantly, plasma levels of ascorbic acid in these animals could suggest that oxidative stress might not impact negatively. Hence, OC-related vasculopathy may neither be associated with increased lipid peroxidation nor decreased ascorbic acid.

In conclusion, the data of the present study showed that by correcting hypokalaemia induced by OC treatment, calcium supplementation enhanced markedly the OC-induced impaired activity of cardiac and vascular Na^+ - K^+ -ATPase. In addition, this study documented that impaired activity of Na^+ - K^+ -ATPase induced by OC steroids may not be attributed to oxidative stress or hyperglycaemia. Thus, some limitations of this animal model should be taken into account, especially the high dose of hormonal steroids used. However, it is conceivable that in addition to prevention of osteoporosis, high dietary calcium could exert cardioprotective effect in women taking OC.

Acknowledgement

The financial assistance from Stephen Oluwole Awokoya foundation for Science Education (Nigeria) is acknowledged and the authors are sincerely grateful to Dr J O Adebayo of Department of Physiology & Biochemistry, College of Medicine, University of Ilorin for his technical advice.

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