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Biochemical Effectiveness of Cocoa Powder on Electrolytes Homeostasis, Liver and Cardiac Specific Enzymes and Renal Function

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Abstract: Cocoa beans are main ingredient of chocolate, cakes, cookies and coffee. Cocoa have health benefits effects, previously reported. This study was designed to evaluate the health effects of cocoa powder on different biochemical parameters. For this purpose 12 Male Albino Wistar rats were divided in to 2 groups (n = 6). Group I remain healthy control rats; Group II received cocoa powder at a dose of (1 g/kg b.w.) for 21 consecutive days orally. Biochemical analysis was evaluated by electrolytes homeostasis, liver specific enzymes, kidney markers and LDH cardiac specific enzyme. The cocoa-treated rats showed decreased intra-erythrocytes potassium, increased Na^+-K^+-ATPase, decreased direct bilirubin, increased plasma potassium, increased ALT level. Decreased mean body weight was observed. No mortality and sign of toxicity was observed at this dose. Considerable changes were observed in biochemical parameters after chronic administration of cocoa powder.

Key words: Cocoa powder, homeostasis, liver enzymes, LDH

INTRODUCTION

Cacao beans (Theobroma cacao) have been used worldwide as a major ingredient of cocoa and chocolate. Cacao beans are rich in polyphenols, such as EC (+)-catechin, quercetin (including its glucoside), clovamide, deoxyclovamide and procyanidin (Thompson *et al*., 1972; Sanbongi *et al*., 1998; Hammerstone *et al*., 1999). Chocolate and cocoa contain a high level of flavonoids, specifically epicatechin, which may have beneficial cardiovascular effects on health (Taubert *et al*., 2007). The ingestion of flavonol-rich cocoa is associated with acute elevation of circulating nitric oxide, enhanced flow-mediated vasodilation and augmented microcirculation (Bayard *et al*., 2007). It is believed that the improved flow after consumption of flavonol-rich cocoa may help to achieve health benefits in hearts and other organs. In particular, the benefit may extend to the brain and have important implications for learning and memory (Sorond *et al*., 2008).

According to research at Cornell University, cocoa powder has nearly twice the antioxidants of red wine, and up to three times the antioxidants found in green tea (Ottavani *et al*., 2002). Cacao also contains magnesium, iron, chromium, vitamin C, zinc and others (Lee *et al*., 2003).

Taubert and his co-workers published a research in 2007 according to which foods rich in cocoa appear to reduce blood pressure but drinking green tea may not (Taubert *et al*., 2007). 50 percent reduction in cardiovascular mortality and a 47% reduction in all-cause mortality for the men regularly consuming the most cocoa, compared to those consuming the least cocoa from all sources (Buijsse *et al*., 2006). It is suggested that the consumption of cocoa flavanols can have important beneficial effects on the function of the body's network of blood vessels. The body of research not only suggests that cocoa flavanols may provide a dietary approach to maintaining cardiovascular function and health (Hannum and Erdman, 2000), but also points at new possibilities for cocoa flavanol-based interventions for vascular complications associated with cognitive performance (Bisson *et al*., 2008), skin health (Gasser *et al*., 2008) and age-related blood vessel dysfunction (Hollenberg, 2006). A new study presented at the annual meeting of the International Society on Thrombosis and Haemostasis found that cocoa can prevent potential fatal blood clots from forming and causing strokes or heart attacks. The research showed that cocoa prevents platelets, the cell that help create clots, form causing blockages (Dietrich *et al*., 2000). Cocoa, cocoa extracts and purified cocoa flavanols and procyanidins exert strong antioxidant effects *in vitro*. Cocoa powder and cocoa extracts have been shown to exhibit greater antioxidant capacity than many other flavanol-rich foods and food extracts, such as green and black tea, red wine, blueberry, garlic and strawberry (Bearden *et al*., 2000; Lee *et al*., 2003).

On the basis of above mentioned health effects the present study was under taken to examine the health benefit effects of cocoa powder on different biochemical parameters in experimental rats model.

Materials and Methods

Animals and diet: 12 male Albino Wistar rats weighing 200-250 gm were purchased form the animal house of ICCBS (International center for Chemical and Biological sciences Karachi, Pakistan) for the study. Animals were acclimatized to the laboratory conditions before the start

of experiment and caged in a quiet temperature controlled animal room (23±4°C). Rats had free access to water and standard rat diet. The experiments were conducted in accordance with ethical guidelines for investigations in laboratory animals.

Study design: The rats were randomly divided into two groups (n = 6)
Group 1 was control and remain untreated.
Group 2 was cocoa-treated and received 1 g/kg body weight, orally for 21 consecutive days.

Drug preparation: 10 gm of cocoa powder was dissolved in 10 ml of deionized water, boiled for 10 min in order to avoid the lumps, cooled at room temperature and administered orally.

Sample collection: At the end of the experiment the animals were starved for 24 h, anesthetized, decapitated and the blood was sampled from the head wound in the lithium heparinized coated tubes. The samples were stored at -70°C. A portion of blood was taken in separate tube to collect the plasma.

Assessment of renal functions: Plasma samples were assayed for urea and creatinine. Urea was estimated spectrophotometry by the Oxime method (Butler *et al.*, 1981). Creatinine was estimated spectrophotometry by the Jeff's method (Spierto *et al.*, 1979).

Assessment of electrolytes homeostasis

Estimation of Plasma Electrolytes: Plasma was analyzed for the estimation of sodium and potassium by flame photometry (Corning 410).
Plasma Calcium is estimated by ion selective electrode using ion meter 3345 (Jenway).

Estimations of Intra erythrocyte sodium and potassium: Heparinized blood was centrifuged and plasma was separated. Buffy coat was aspirated and discarded. Erythrocytes were washed three times at room temperature by suspension in the magnesium chloride solution (112mmol/L), centrifugation at 450 X g at 4°C for 5 min and aspiration of the supernatant as described earlier (Fortes and Starkey, 1977). Final supernatant was retained for the estimation of intraerythrocyte sodium and potassium concentration. Neither electrolytes were detectable in the final wash. Washed erythrocytes were than used for the estimation of intraerythrocytes sodium and potassium (Tabssum *et al.*, 1996).

Erythrocyte membrane preparation: The packed red cells extracted by centrifugation at 4°C, 450 X g for 15 min were resuspended and diluted in 25 volumes of 0.011 mol/L Tris-HCl buffer at p H 7.4. The hemolyzed cells were then centrifuged for 30 min at 12,000 rpm at 4°C and the membrane pellet was resuspended in 30 ml of 0.0ll mol/L Tris-HCl buffer. This centrifugation step was repeated three times. The final concentration of the membrane suspension was ~4 mg protein/ml of Tris buffer. The membrane suspension was stored at -80°C until the assay was performed.

Erythrocyte Na-K-ATPase activity measurement: (Denis *et al.*, 1996): ATPase activity was measured in a final volume of 1 ml as follows: Membrane (400 µg) were preincubated for 10 min at 37°C in a mixture containing 92 mmol/L Tris-HCl (p H = 7.4), 100 mmol/L NaCl, 20 mmol/L KCl, 5 mmol/L $MgSO_4$. H_2O and 1 mmol/L EDTA. Assays were performed with or without 1 mmol/L Ouabain, a specific inhibitor of Na-K-ATPase activity was calculated as the difference between inorganic phosphate released during the 10 min incubation with and with out ouabain. Activity was corrected to a nanomolar concentration of inorganic phosphate released milligram protein/hour.
All assays were performed in duplicate and blanks for substrate, membrane and incubation time were included to compensate for endogenous phosphate and non-enzyme related breakdown of ATP. Under these experimental conditions, the coefficient of variation was 7.5%.

Assessment of liver enzymes: Plasma ALT (Reitman and Franhel, 1957), ALP (alkaline phosphatase) (Englehardt *et al.*, 1970) total and direct bilirubin (Sherlock, 1951) were analyzed using commercially prepared reagent kits from Randox.

Assessment of LDH: Plasma LDH was analyzed by (Anosike and Ejiofor, 1984) using commercially prepared kit from Randox.

Statistical analysis: Results are presented as mean± SE. Statistical significance and difference from control and test values evaluated by Student's t-test. Statistical probability of $p<0.01$, <0.05 were considered to be significant.

RESULTS

Effect of cocoa powder on body weight in control and cocoa-treated rats: Decreased body weight was observed after chronic administration in cocoa-treated rats (Fig. 1).

Effect of cocoa powder on intra-erythrocytes and Na^+-K^+-ATPase in control and cocoa- treated rats: A decreased intra-erythrocytes potassium level was observed in cocoa-treated rats as compared to control (130.71±1.01, $p<0.01$). Similarly, increased Na^+-K^+-ATPase was ob served (81.05±2.08, $p<0.01$) and decreased sodium level was observed but results were not significant (Table 1).

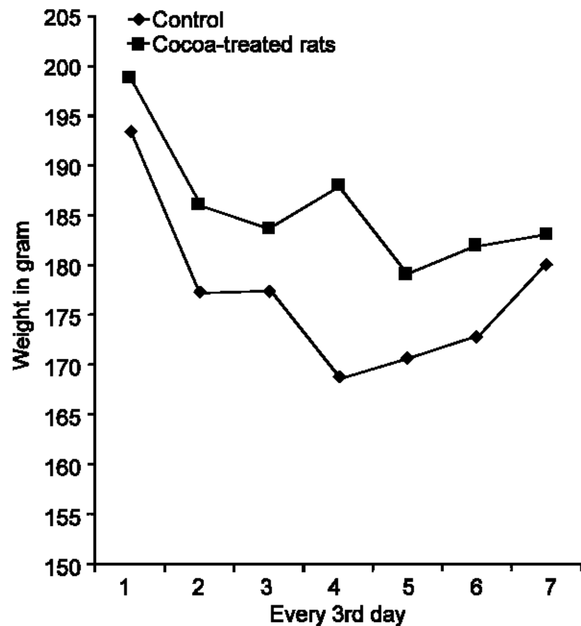

Fig. 1: Effect of cocoa powder on body weight in control and cocoa- treated rats

Table 1: Effect of cocoa powder on intra-erythrocytes and Na^+-K^+-ATPase in control and cocoa- treated rats

Parameters	Control group	Cocoa-treated group
Na+ (mmol/L)	12.02±2.40	9.67±1.43
K+ (mmol/L)	139.94±2.98	130.71±1.01*
Na+/K+ATPase (nm/mg of protein/hr)	73.93±6.86	81.05±20.08

Values are mean±SE Significant difference between control and cocoa-treated group by t-test *p<0.01

Table 2: Effect of cocoa powder on plasma erythrocytes in control and cocoa- treated rats

Parameters	Control group	Cocoa-treated group
Na^+ (mmol/L)	145.16±0.75	144.16±1.47
K^+ (mmol/L)	5.45±0.36	6.08±0.18**
Ca^+(mg/dl)	1.05±0.36	1.42±0.25

Values are mean±SE Significant difference between control and cocoa-treated group by t-test **p<0.05

Effect of cocoa powder on plasma erythrocytes in control and cocoa- treated rats: Table 2 showed increased level of potassium as compared to control (6.08±0.18, $p<0.05$). No change was ob served in plasma sodium level. Plasma calcium level was slightly increased but results were no significant.

Effect of cocoa powder on plasma liver enzymes in control and cocoa- treated rats: Table 3 showed decreased direct bilirubin level in cocoa-treated rats as compared to Control (10.23±5.08, $p<0.05$). Alanine transferase level was increased (9.97±1.42, $p<0.01$) in cocoa-treated rats as compared t o control. Decreased level of alkaline phosphatase and increased level of total bilirubin was observed but results were not significant.

Table 3: Effect of cocoa powder on plasma liver enzymes in control and cocoa-treated rats

Parameters	Control group	Cocoa-treated group
Total bilirubin (Unit/L)	0.53±0.22	0.64±0.32
Direct bilirubin (Unit/L)	19.02±2.46	10.23±5.08**
Alkaline phosphatase (Unit/L)	198.02±92.58	165.23±60.35
Alanine transferase (Unit/L)	6.59±0.77	9.97±1.42*

Values are mean±SE Significant difference between control and cocoa-treated group by t-test **p<0.05, *p<0.01

Effect of cocoa powder on plasma Urea and Creatinine level in control and cocoa- treated rats: Table 4 showed increased plasma creatinine level in cocoa-treated rats (5.45±1.19, $p<0.01$) as compared to control. No change was observed in plasma urea level.

Effect of cocoa powder on plasma lactate dehydrogenase level in control and cocoa- treated rats: No change was observed in plasma lactate dehydrogenase level as showed in Table 5.

DISCUSSION

In present study there is decreased levels of intra-erythrocytes K^+ and increased level of Na^+/K^+-ATPase, plasma K^+ were observed (Table 1, 2). Cocoa increased the Na^+-K^+-ATPase as it increased performance of this membrane bound enzyme due to the alterations of electrolytes. This type of high performance of Na^+-K^+-ATPase was observed in heart cells when digoxin was given to improve muscular contraction. (Carl *et al.*, 2005). The erythrocyte membrane is moreover characterized with respect to Na^+/ K^+-ATPase and Ca^{++} ATPase (Bookchin *et al.*, 2000). Previously it is reported that plasma membrane embedded -activated ATPase is usually supposed to have an essential role in counter balancing passive ionic leaks and oncotic forces from intracellular proteins and fixed phosphate groups i.e., in cell volume regulation (Dunham and Hoffman, 1980; Macknight and Leaf, 1980). It has been known for years that red blood cells in some mammalian species may be devoid of Na^+/K^+-ATPase and yet be able to maintain ionic balance and cell volume. Some carnivorous species, e.g. the cat and the dog, have low-potassium erythrocytes due to a lack of plasma membrane Na^+/K^+-ATPase (Bernstein, 1954; Chan *et al.*, 1964). Also red cells from ferrets (Mustela putorius furo), i.e. a Mustelidae species belonging to a collateral branch of the carnivorous phylogenetic tree have high sodium and low potassium content (Flatman and Andrews, 1983; Milanick, 1989). In other species, e.g. sheep and goat, the erythrocytes may be of a high-potassium or a low-potassium type (Evans and Phillipson, 1957). In the latter case the number of sodium pumps per red cell may be reduced or, more likely, the -ATPase activity is inhibited by a membrane-bound inhibitory factor closely related to the blood group L antigen (Tucker *et al.*, 1976). Potassium homeostasis is maintained predominantly

Table 4: Effect of cocoa powder on plasma Urea and Creatinine level in control and cocoa- treated rats

Parameters	Control group	Cocoa-treated group
Creatinine(mg/dl)	1.87±0.56	5.45±1.19*
Urea(mg/dl)	39.52±2.53	42.93±5.53

Values are mean±SE Significant difference between control and cocoa-treated group by t-test *p<0.01

Table 5: Effect of cocoa powder on plasma lactate dehydrogenase level in control and cocoa- treated rats

Parameters	Control group	Cocoa-treated group
LDH(Unit/L)	792.87±90.5	788.13±89.1

through the regulation of renal excretion. Cocoa increased plasma creatinine level (Table 4). It may be due to chronic administration of cocoa powder decrease the effective renal plasma flow precedes the decrease in GFR (Repta and Long, 1980). The another important finding of this study is the increased level of ALT and decreased level of direct bilirubin (Table 3). Previously reported studies showed that commonly elevated ALT following cellular damage as a result of enzymes leakage from cells to blood as a result of cell membrane permeability, has been seen in drug toxicities (Awadalla *et al*., 1975).

The study shows the pros and cons of chronic administration of cocoa powder using different biochemical parameters and suggests that it can be used alone as far as with conventional therapy in different biological impairments and illnesses, however, dose adjustment should be considered. Moreover, desirable changes in plasma ALT, bilirubin, K^+, intra-erythrocytes K^+ and Na^+-K^+-ATPase level could make up a defensive mechanism, which enables organism adaptation to various toxicities.

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